



UNIVERSITY  
OF TASMANIA

# **Genetic control of inflorescence development in pea**

**By**

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## Abstract

Angiosperm species exhibit incredible diversity in inflorescence architecture. Legumes comprise the third largest family of flowering plants, second only to the grasses in terms of agricultural importance. Several important crop legumes, including pea, lentil, common bean and chickpea, share a characteristic inflorescence form, the compound raceme, which has one extra level of inflorescence branching, the secondary inflorescence ( $I_2$ ), relative to the simple raceme of *Arabidopsis*. Historically, pea has been a popular model legume for studies of flowering, often through the characterisation of flowering and inflorescence mutants. In this study, pea genes with an apparent or putative role in inflorescence development were investigated, in order to improve understanding of the genetic control of inflorescence development in pea.

Unlike *Arabidopsis*, where no single gene mutant has a non-flowering phenotype, mutations at any of three pea loci can prevent flowering: *GIGAS/FTa1*, *VEG1/FULc* and *VEG2*. In this study, the roles of *VEG2* during inflorescence development were investigated using two mutant alleles: the non-flowering *veg2-1* mutant, and the late-flowering *veg2-2* mutant. The results indicate that *VEG2* is important for the correct timing of the inflorescence transition, initial specification and maintenance of  $I_2$  identity, and specification of floral meristems, under both LD and SD conditions.

Preliminary mapping results indicated a pea homolog of *FD* as a candidate for the *VEG2* locus. In this study, the legume *FD* gene family was characterised and the *VEG2* locus was shown to correspond to *FDa*. In the *veg2-1* mutant, the entire coding sequence was found to be deleted but putative flanking genes were unaffected. The *veg2-2* mutant was shown to contain a single nucleotide polymorphism (SNP), affecting a highly conserved amino acid within the DNA-binding, basic region of the bZIP domain.

The mechanisms of *FDa* action were further investigated through analysis of expression patterns of *FDa* and protein interactions with the pea FT and TFL1 homologs. *FDa* was found to be expressed in the wild-type apex throughout development. *FDa* protein was found to be capable of interacting with all five pea FT homologs, and DET (TFL1a), but not LF (TFL1c). Flowering genes regulated (either

directly or indirectly) by *FDa* were identified based on misregulation of expression in the *veg2* mutants. These included pea homologs of *FT*, *TFL1* and *LFY*, in addition to a range of MADS-box genes.

The *late5* mutant is a previously undescribed EMS-induced mutant that exhibits phenotypic similarity to *veg2-2*. To determine the role of *LATE5* during pea inflorescence development, the *late5* phenotype was characterised. The genetic interactions between *LATE5*, *DET* and *LF* were investigated through the phenotypes of double and triple mutants. The molecular roles of *LATE5* were also investigated by examining the effects of the *late5* mutation on expression of flowering genes. The map position of *LATE5* was refined to a region of less than 3.2cM towards the base of pea linkage group I, corresponding to a syntenic region of 0.6Mb containing 95 annotated genes in *Medicago*.

The legume family of *SVP*-like genes, which have important roles in flowering time, inflorescence branching and floral meristem identity in other species, was characterised and two new *SVP*-like genes (*SVPb* and *SVPc*) were isolated from pea. Investigation of expression patterns of pea *SVPa*, *SVPb* and *SVPc* genes, revealed developmental regulation of *SVPc* in wild-type pea, and misregulation of *SVPc* in the *veg2-2* mutant indicating regulation of *SVPc* (directly or indirectly) by *FDa/VEG2*.

Overall, the findings of this study make a significant contribution to knowledge of the genetic control of inflorescence development in pea.

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# Abbreviations

ABA	Abscisic acid
AF	<i>AFILA</i>
AG	<i>AGAMOUS</i>
AGL24	<i>AGAMOUS-LIKE24</i>
AGL79	<i>AGAMOUS-LIKE79</i>
ALOG	<i>Arabidopsis</i> LIGHT-SENSITIVE HYPOCOTYL 1 <i>Oryza</i> GI
AN	<i>ANANTHA</i>
AP1	<i>APETALA1</i>
AP2	<i>APETALA2</i>
AP3	<i>APETALA3</i>
AP01	<i>ABERRANT PANICLE ORGANIZATION1</i>
AREB3	ABA-Responsive Element Binding protein 3
ATC	<i>ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOG</i>
BiFC	Bimolecular fluorescence complementation
bp	Nucleotide base pairs
<i>BRC1</i>	<i>BRANCHED1</i>
bZIP	Basic region leucine zipper
CAL	<i>CAULIFLOWER</i>
CAPS	Cleaved amplified polymorphic sequence
cDNA	Complementary DNA
CDS	Coding sequence
<i>CEN</i>	<i>CENTRORADIALIS</i>
<i>CETS</i>	<i>CENTRORADIALIS/TERMINAL FLOWER 1/SELF-PRUNING</i>
<i>CO</i>	<i>CONSTANS</i>
cv.	Cultivar
<i>DAM</i>	Dormancy-associated MADS-box
dbEST	GenBank Database of Expressed Sequence Tags
dCAPS	Derived cleaved amplified polymorphic sequence
<i>DET</i>	<i>DETERMINATE</i>
DSCI TGI	Dana Farber Cancer Institute Gene Indices
<i>DJC23</i>	<i>DNA J PROTEIN C23</i>
<i>DJC24</i>	<i>DNA J PROTEIN C24</i>
<i>DLF1</i>	<i>DELAYED FLOWERING1</i>
DNA	Deoxyribonucleic acid
<i>DNE</i>	<i>DIE NEUTRALIS</i>
<i>DPBF4</i>	<i>Dc3 promoter-binding factor 4</i>
DTF	Plant age at first open flower
EMS	Ethyl methanesulfonate
<i>FA</i>	<i>FALSIFLORA</i>
<i>FDP</i>	<i>FD PARALOG</i>
<i>FLC</i>	<i>FLOWERING LOCUS C</i>

<i>FT</i>	<i>FLOWERING LOCUS T</i>
<i>FUL</i>	<i>FRUITFUL</i>
gDNA	Genomic DNA
<i>gi</i>	<i>gigas</i>
<i>Hd3a</i>	<i>Heading date 3a</i>
I <sub>1</sub>	Primary inflorescence
I <sub>2</sub>	Secondary inflorescence
<i>INCO</i>	<i>INCOMPOSITA</i>
<i>J</i>	<i>JOINTLESS</i>
KAL	Kaliski
kb	1000 nucleotide base pairs
<i>LARP1C</i>	<i>LA RELATED PROTEIN 1C</i>
<i>LATE1</i>	<i>LATE BLOOMER 1</i>
<i>LATE5</i>	<i>LATE BLOOMER 5</i>
LB	Lurio-Bertoni media
LD	Long day photoperiod
<i>LF</i>	<i>LATE FLOWERING</i>
<i>LFY</i>	<i>LEAFY</i>
LG	Linkage group
<i>LSH3</i>	<i>LIGHT-DEPENDENT SHORT HYPOCOTYLS 3</i>
<i>LSH4</i>	<i>LIGHT-DEPENDENT SHORT HYPOCOTYLS 4</i>
<i>LUX</i>	<i>LUX ARRHYTHMO</i>
Mb	1,000,000 nucleotide base pairs
<i>MC</i>	<i>MACROCALYX</i>
miR156	microRNA156
NFI	Node of flower initiation
PCR	Polymerase chain reaction
<i>phyA</i>	<i>phytochrome A</i>
<i>PI</i>	<i>PISTILLATA</i>
qRT-PCR	Quantitative reverse transcription PCR
QTL	Quantitative trait loci
RACE	Rapid amplification of cDNA ends
RAPD	Random amplified polymorphic DNA
RN	Reproductive node
RNA	Ribonucleic acid
RNAi	RNA interference
SAM	Shoot apical meristem
SD	Short day photoperiod
SDW	Autoclaved Milli-Q water
SE	Standard error
SEM	Scanning electron microscopy
<i>SEP1</i>	<i>SEPALATA1</i>
<i>SEP2</i>	<i>SEPALATA2</i>
<i>SEP3</i>	<i>SEPALATA3</i>
<i>SEP4</i>	<i>SEPALATA4</i>

<i>SFT</i>	<i>SINGLE FLOWER TRUSS</i>
<i>SN</i>	<i>STERILE NODES</i>
SNP	Single nucleotide polymorphism
<i>SOC1</i>	<i>SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1</i>
<i>SP</i>	<i>SELF PRUNING</i>
<i>SPGB</i>	<i>SELF-PRUNING G-BOX</i>
<i>SPL</i>	<i>SQUAMOSA PROMOTER BINDING PROTEIN-LIKE</i>
<i>SQUA</i>	<i>SQUAMOSA</i>
<i>SVP</i>	<i>SHORT VEGETATIVE PHASE</i>
TAE	Tris acetate ethylenediamine tetra-acetic acid
TAIR	The Arabidopsis Information Resource
TER	Térèse
<i>TFL1</i>	<i>TERMINAL FLOWER1</i>
T <sub>m</sub>	Optimal annealing temperature
TN	Total nodes
TSA	Transcriptome Shotgun Assembly
<i>UFO</i>	<i>UNUSUAL FLORAL ORGANS</i>
<i>UNI</i>	<i>UNIFOLIATA</i>
UTR	Untranslated region
<i>VEG1</i>	<i>VEGETATIVE 1</i>
<i>VEG2</i>	<i>VEGETATIVE 2</i>
V/I <sub>1</sub> transition	Transition of the SAM from vegetative to I <sub>1</sub> meristem identity
<i>VRN1</i>	<i>VERNALIZATION1</i>
WT	Wild-type
<i>WUS</i>	<i>WUSCHEL</i>
YFC	C-terminal half of yellow fluorescent protein
YFN	N-terminal half of yellow fluorescent protein
YFP	Yellow fluorescent protein
<i>ZCN8</i>	<i>Zea mays CENTRORADIALIS8</i>
<i>ZFL1</i>	<i>Zea mays FLO/LFY 1</i>



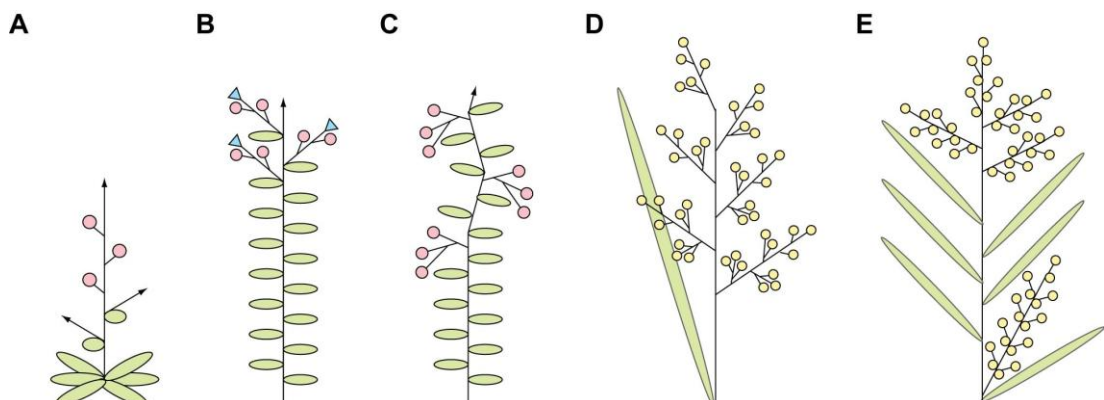


# CHAPTER 1: General introduction

## 1.1 Introduction

### 1.1.1 Variation in inflorescence architecture

The arrangement of flowers and flower-bearing structures on the plant stem, known as inflorescence architecture or form, has important implications for reproductive success and ease of harvest in agricultural systems (e.g. Wyatt, 1982; Pnueli et al., 1998). Angiosperm species exhibit incredible diversity in inflorescence architecture, most commonly in terms of complexity and pattern of branching, number and position of flowers and the capacity of the inflorescence for continued growth (Eames, 1961; Weberling, 1989; see Figure 1.1). Inflorescences are classed as indeterminate or determinate, depending on the fate of the shoot apical meristem (SAM). In indeterminate inflorescence forms, a population of pluripotent cells is maintained in the SAM, enabling potentially indefinite shoot growth, as occurs in the racemes of *Arabidopsis* and pea (Figure 1.1A-B). In determinate inflorescence forms, the SAM eventually adopts floral identity and is consumed in floral organ production, as seen in (i) the cyme of tomato and (ii) the panicle, which is an inflorescence form typical of most monocots (Figure 1.1C-E). Inflorescences are further classified as simple, when flowers are borne directly on the main stem, as seen in *Arabidopsis* (Figure 1.1A), or compound when flowers are borne on secondary or higher order inflorescence branches, which occurs in numerous legume species (Figure 1.1B).



**Figure 1.1.** Examples of different inflorescence forms.

(A) The simple raceme of *Arabidopsis*. (B) The compound raceme of pea. (C) The modified cyme of tomato. Panicles of (D) rice and (E) maize. Arrows indicate indeterminate growth, pink circles are flowers, yellow circles are spikelets (each spikelet contains a single floret in rice and two florets in maize), triangles are terminal stubs, and ovals are leaves or bracts. Some vegetative growth is not shown.

The exact nature of the most primitive inflorescence form has been the subject of historic debate. Popular theories have included (i) a solitary flower terminating the shoot, (ii) an inflorescence with two axillary flowers and a terminal flower, known as a dichasial cyme, and (iii) a leafy cyme with terminal flowers borne on shoots that alternate vegetative and floral growth (Parkin, 1914; Rickett, 1944; Stebbins, 1973). The common thread is that each of these theories describes the ancestral inflorescence form as determinate, and indeterminate inflorescence forms would need to have arisen through the evolution of mechanisms to repress formation of terminal flowers. The alternative possibility of an indeterminate ancestral inflorescence form has also been raised (Coen and Nugent, 1994), but this has received less attention. Regardless of this uncertainty, it is likely that ancestral inflorescences were simple rather than compound (Parkin, 1914). Compound inflorescence forms could have arisen through the evolution of mechanisms to repress or delay formation of flowers on axillary structures, restricting flowers to second or higher order inflorescence branches (Coen and Nugent, 1994; Koes, 2008).

### ***1.1.2 The importance of meristem identity***

At the tissue level, variations in inflorescence architecture reflect differences in the identity and activity of shoot meristems, as these meristems generate the tissues that comprise the inflorescence. Meristems are dynamic, organised structures that comprise undifferentiated, dividing cells and are responsible for self-renewal and production of new shoot cells, tissues, and ultimately organs (see Sablowski, 2007). Each component within the inflorescence is generated by a specific meristem with distinct identity characterised by what it forms (meristem product), where it forms (location) and whether or not it is determinate. Vegetative meristems are generally indeterminate and produce the vegetative shoot tissues of the plant, including the vegetative stem and leaves. As the plant undergoes the transition to reproductive development, the vegetative SAM becomes an inflorescence meristem, which generates the inflorescence stem and may bear flowers or higher-order inflorescence branches, depending on inflorescence form. The development of compound inflorescences involves an additional type of inflorescence meristem for each level of inflorescence branching. Floral meristems are determinate, consumed in the production of a set number of floral organs. Normal inflorescence development

depends on correct specification and maintenance of identity for each meristem involved.

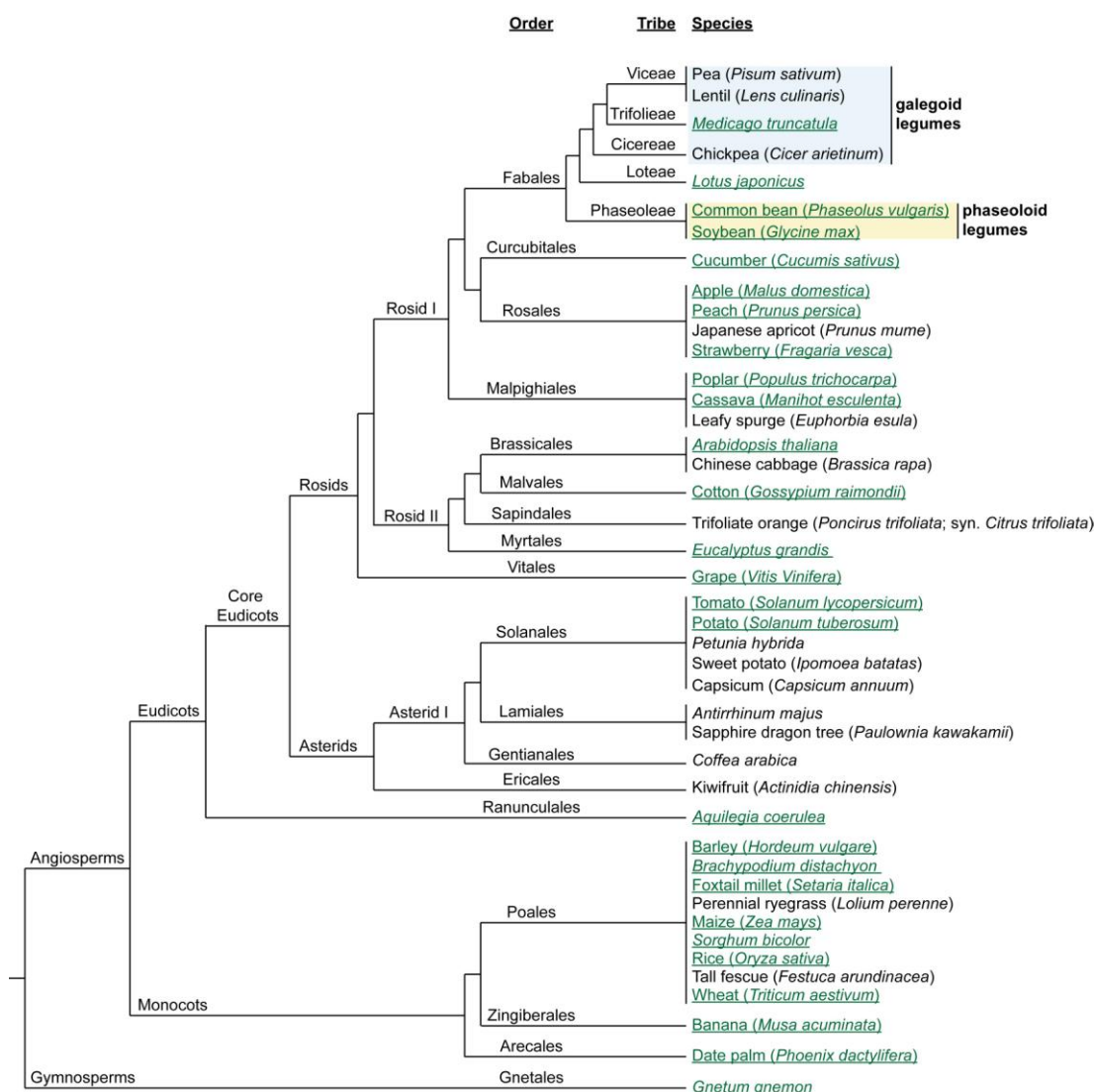
Inflorescence development can be considered as the central stage of the flowering process, which occurs after the plant receives and integrates signals that determine flowering time and before floral development, with some overlap between the three stages. Before a plant commits to flowering, cues are received about the plant environment (e.g. photoperiod, vernalisation, ambient temperature, light quality) and internal state of the plant (e.g. age, stress, hormonal state). These cues provide information about the suitability of conditions for flowering and are transmitted via a series of signal-specific pathways (see Srikanth and Schmid, 2011). Genes known as floral integrators integrate signals from multiple pathways to ensure flowering occurs at an optimal time for reproductive success. As described above, the transition to reproductive development involves changes in meristem identity, and some key floral integrators can also have important roles in inflorescence or floral meristem identity, in addition to affecting flowering time. These genes can be grouped with other genes that have important roles in specifying and/or maintaining meristem identity, as meristem identity genes. Floral promoters are genes that promote floral identity and ultimately meristem determinacy and floral repressors are genes that repress floral identity and maintain meristem indeterminacy by restricting expression of floral meristem identity genes to axillary meristems. Once floral meristem identity is established, floral development begins, and homeotic genes specify floral organ identity according to the ABCE model of floral patterning (Coen and Meyerowitz, 1991; Theissen, 2001). To control meristem determinacy, these genes regulate the gene system that maintains a population of undifferentiated stem cells within indeterminate meristems (e.g. Ferrario et al., 2006; Sablowski, 2007).

### ***1.1.3 Model species***

The vast majority of current knowledge of the genetic control of inflorescence development is based on studies in the well-known model species *Arabidopsis*. In recent years, this knowledge has formed a foundation for studies in a range of crop species, which show greater complexity in inflorescence form. Rapid development of genome and transcript resources for many diverse species (examples in Figure 1.2), has been a significant advantage for both forward and reverse genetic approaches.

Although sequences are now available for many angiosperm species, examination of gene function is still a slow process, and in general it is still the model species with a long history of usage that offer the greatest insight into the gene systems controlling specific aspects of plant development.

This thesis will investigate compound inflorescence development in pea, using knowledge of the systems operating in *Arabidopsis* and other model species. This chapter will review current knowledge of key genes involved in *Arabidopsis* inflorescence development and corresponding genes that are important for this process in tomato, and the grasses rice and maize. The known components in pea will then be introduced in this context. Figure 1.2 shows the taxonomic relationships between all species mentioned in this thesis, for ease of reference.

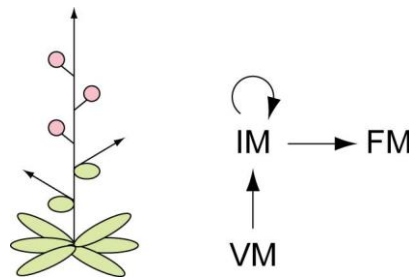


**Figure 1.2.** Taxonomic relationships between the species mentioned in this thesis, shown to the order level. Tribe is indicated for each legume species. For species shown in green and underlined, genome sequence was available at the time of this study.

## 1.2 *Arabidopsis thaliana*

### 1.2.1 Inflorescence form

*Arabidopsis* is a small annual plant from the mustard family (Brassicaceae), with an inflorescence in the form of a simple raceme (Figure 1.3). During vegetative development, the *Arabidopsis* SAM has vegetative meristem identity, remaining indeterminate and producing rosette leaves with minimal internode elongation. Prior to the initiation of flowers, the main stem bolts with dramatic elongation of internode length. During the floral transition, the SAM is converted to an inflorescence meristem, which remains indeterminate for the life of the plant (Figure 1.3). At early inflorescence nodes, the inflorescence meristem yields axillary, indeterminate inflorescence shoots, known as co-florescences, subtended by cauline leaves, and bears axillary, bractless flowers at later nodes (Figure 1.3). Key genes important for correct specification of meristem identity and consequent inflorescence development in *Arabidopsis* are summarised in Table 1.1 and discussed further in this section.



**Figure 1.3.** The simple raceme of *Arabidopsis*.

Diagram of the *Arabidopsis* inflorescence (left) and schematic of the meristem transitions involved in its development (right). In *Arabidopsis*, inflorescence development involves conversion of the vegetative shoot apical meristem (VM) into an inflorescence meristem (IM) which remains indeterminate and produces axillary floral meristems (FM) directly on the main stem axis.

In the diagram, arrows indicate indeterminate growth, circles are flowers and ovals are leaves or bracts. In the schematic, straight arrows indicate meristem transitions and products, and circular arrows indicate meristem indeterminacy.

**Table 1.1.** Summary of key genes important for determining meristem identity during inflorescence development in *Arabidopsis*. Abbreviations for meristems are as follows: vegetative meristem (VM), inflorescence meristem (IM), and floral meristem (FM).

Gene family	Gene	Expression domain	Overexpression	Mutant phenotype	Inferred function	References
<i>CENTRORADIALIS</i> / <i>TERMINAL FLOWER1</i> / <i>SELF-PRUNING</i> ( <i>CETS</i> )	<i>FLOWERING LOCUS T</i> ( <i>FT</i> )	Leaves, cotyledons	IM → FM	Late-flowering	Promotes the floral transition and FM identity	Koornneef et al. (1983); Koornneef et al. (1991); Kardailsky et al. (1999); Kobayashi et al. (1999)
	<i>TERMINAL FLOWER1</i> ( <i>TFL1</i> )	VM, IM	Late-flowering, FM → IM	Early-flowering, IM → FM	Represses the floral transition and FM identity, maintains IM identity	Shannon and Meeks-Wagner (1991); Alvarez et al. (1992); Schultz and Haughn (1993); Ratcliffe et al. (1998)
Basic region leucine zipper (bZIP)	<i>FD</i>	VM, IM, early FM, leaf primordia	Early-flowering	Late-flowering	Promotes/represses the floral transition with various <i>CETS</i> proteins	Koornneef et al. (1991); Abe et al. (2005); Wigge et al. (2005)
	<i>FD PARALOG</i> ( <i>FDP</i> )	VM, IM	-	Late-flowering (minor)	As above	Abe et al. (2005); Wigge et al. (2005); Jaeger et al. (2013)
<i>LEAFY</i> ( <i>LFY</i> )	<i>LFY</i>	Leaves (weak), FM	Early-flowering, IM → FM	Late-flowering (minor), FM → IM, floral defects	Promotes the floral transition and FM identity	Schultz and Haughn (1991); Huala and Sussex (1992); Weigel et al. (1992); Weigel and Nilsson (1995)
F-box	<i>UNUSUAL FLORAL ORGANS</i> ( <i>UFO</i> )	VM, FM	Floral defects	FM → IM, floral defects	<i>LFY</i> co-factor	Ingram et al. (1995); Lee et al. (1997); Hepworth et al. (2006)
MADS-box	<i>AGAMOUS-LIKE24</i> ( <i>AGL24</i> )	IM, FM	Early-flowering, FM → IM, floral defects	Late-flowering	Promotes the floral transition and controls FM identity	Yu et al. (2002); Michaels et al. (2003); Yu et al. (2004); Liu et al. (2007)
	<i>SHORT VEGETATIVE PHASE</i> ( <i>SVP</i> ; prev. <i>AGL22</i> )	VM, FM	Late-flowering, FM → IM, floral defects	Early-flowering	Represses the floral transition and controls FM identity	Hartmann et al. (2000); Liu et al. (2007)
	<i>SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1</i> ( <i>SOC1</i> ; prev. <i>AGL20</i> )	Leaves, VM, IM, late FM	Early-flowering, floral morphology defects	Late-flowering	Promotes the floral transition and FM identity	Borner et al. (2000); Lee et al. (2000); Onouchi et al. (2000); Samach et al. (2000)
	<i>APETALA1</i> ( <i>AP1</i> )	FM, sepal and petal whorls	Early-flowering, IM → FM	FM → IM, floral defects	Specifies FM identity	Irish and Sussex (1990); Bowman et al. (1993); Mandel and Yanofsky (1995)

### 1.2.2 The *FT/FD* pathway

*FLOWERING LOCUS T* (*FT*) encodes a small protein with similarity to mammalian phosphatidylethanolamine binding protein (PEBP; Kardailsky et al., 1999; Kobayashi et al., 1999), which serves as a major component of the long-sought florigen signal connecting the detection of photoperiod in the leaf with floral initiation in the apex (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). *FT* expression in leaves is under the control of *CONSTANS* (*CO*), which integrates cues from the internal circadian clock with light signals, to upregulate expression of *FT* under favourable long day (LD) photoperiods. *FT* is a key floral integrator and expression of *FT* is controlled by the gibberellin, vernalisation and ambient temperature pathways in addition to the photoperiod pathway (Blazquez et al., 2003; Searle et al., 2006; Porri et al., 2012). *FT* protein is transported into phloem sieve elements, a process mediated by FT-INTERACTING PROTEIN 1 (FTIP1), and *FT* then travels via the phloem from the leaves to the apex (Corbesier et al., 2007; Liu et al., 2012). In the apex, *FT* interacts as a protein complex with the basic region leucine zipper (bZIP) transcription factor *FD*, or the closely related *FD PARALOG* (*FDP*; Abe et al., 2005; Wigge et al., 2005). Together, *FT/FD* florigenic complexes upregulate expression of the floral integrator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), and ultimately floral meristem identity genes including *FRUITFUL* (*FUL*), *APETALA1* (*API*), *SEPALLATA1* (*SEP1*) and *SEP3* (Abe et al., 2005; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005; Searle et al., 2006). The floral specification activities of *FT/FD* are dependent on two redundant *BELL1*-like homeobox proteins *PENNYWISE* (*PNY*) and *POUND-FOOLISH* (*PNF*), which act in conjunction with the *KNOTTED1*-like homeobox protein *SHOOT MERISTEMLESS* (*STM*) to regulate inflorescence patterning events (Kanrar et al., 2008; Smith et al., 2011). *TWIN SISTER OF FT* (*TSF*), the closest homolog of *FT* in *Arabidopsis*, acts redundantly with *FT*, to promote flowering via protein interaction with *FD* and *FDP* (Yamaguchi et al., 2005; Jang et al., 2009). Overexpression of either *FT* or *TSF* results in early flowering and altered inflorescence architecture with termination of the SAM in an ectopic flower (Kardailsky et al., 1999; Kobayashi et al., 1999; Yamaguchi et al., 2005; Jang et al., 2009).

### 1.2.3 Repression by *TERMINAL FLOWER1 (TFL1)*

*TFL1* is a founding member of the *CENTRORADIALIS/TERMINAL FLOWER1/SELF-PRUNING (CETS)* family, which also includes *FT* and *TSF*, but *TFL1* has an opposite role in flowering (Kardailsky et al., 1999; Kobayashi et al., 1999; Hanzawa et al., 2005). Within the SAM, *TFL1* competes with *FT* and *TSF* through protein interaction with *FD* and *FDP* to repress expression of floral meristem identity genes, particularly *API* (Wigge et al., 2005; Ahn et al., 2006; Hanano and Goto, 2011; Jaeger et al., 2013). In this way, *TFL1* performs a dual role, delaying the floral transition until an appropriate plant age and maintaining the indeterminacy of the inflorescence meristem by restricting expression of floral meristem identity genes to axillary meristems (Shannon and Meeks-Wagner, 1991; Ratcliffe et al., 1998). Expression of *TFL1* is limited to the central cells of the SAM, and *TFL1* protein remains absent from cells developing from the flanks of the inflorescence meristem, despite its capacity to move beyond its expression domain (Conti and Bradley, 2007). In the wild-type SAM, *TFL1* is expressed in proportion to *FT*, with expression rising to match the increase in *FT* associated with the transition to flowering (Jaeger et al., 2013). In the *tfl1* mutant, flowering occurs earlier than in wild-type, axillary flowers with subtending cauline leaves replace the wild-type co-florescences, and an ectopic flower terminates the main stem (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). Conversely, constitutive overexpression of *TFL1* results in a delay in the floral transition and a highly branched inflorescence, with axillary shoots that lack subtending cauline leaves gradually acquiring floral identity acropetally up the inflorescence stem until normal axillary flowers are eventually produced (Ratcliffe et al., 1998).

*BROTHER OF FT (BFT)* is a close homolog that functions redundantly with *TFL1*, and has recently been found to have a specific role in delaying flowering under high salinity through competition with *FT* to bind with *FD* (Ryu et al., 2013). Similar to *TFL1*, overexpression of *BFT* results in delayed flowering and severe floral defects characterised by the replacement of flowers with compact axillary inflorescences that gradually acquire floral identity, or the production of ectopic flowers (Yoo et al., 2010).

*ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOG (ATC)* is the most closely related *Arabidopsis* gene to the *CETS* founding member *Antirrhinum*



*CENTRORADIALIS* (*CEN*; Mimida et al., 2001). Constitutive expression of *ATC* also results in a similar phenotype to transgenic plants overexpressing *TFL1*, suggesting that *ATC* may act redundantly with *TFL1* (Mimida et al., 2001). *ATC* is not normally expressed in the inflorescence meristem of wild-type plants (Mimida et al., 2001), but results of a recent study suggest that *ATC* may move from vasculature to the apex to act as an antiflorigenic signal (Huang et al., 2012).

#### ***1.2.4 LEAFY (LFY) and UNUSUAL FLORAL ORGANS (UFO) - an alternative pathway***

*LFY* encodes a plant-specific transcription factor that specifies and maintains floral meristem identity, acting independently of the *FT/FD* pathway to activate floral meristem and/or organ identity genes including *API*, *APETALA3* (*AP3*), *PISTILLATA* (*PI*), *AGAMOUS* (*AG*), *SEP3* and *SEP4* (Weigel and Meyerowitz, 1993; Parcy et al., 1998; Winter et al., 2011). In the *lfy* mutant, shoot structures bearing bracts are produced in place of flowers at axillary positions on the inflorescence stem (Schultz and Haughn, 1991; Huala and Sussex, 1992). These shoots progressively acquire floral identity acropetally up the inflorescence stem, with later shoots bearing sepal-like or carpel-like organs in place of bracts (Schultz and Haughn, 1991). The lack of normal flowers in the *lfy* mutant indicates *LFY* has a major role in correct specification of floral meristems. Single *lfy* mutants never produce petals or stamens, which is consistent with a critical role for *LFY* in the activation of B-class genes within the ABCE model for floral patterning (Schultz and Haughn, 1991). *LFY* integrates signals from the photoperiod and gibberellin pathways, but only has a minor role in promoting the floral transition, resulting in a slight delay in flowering in the *lfy* mutant (Blazquez et al., 1997; Blazquez and Weigel, 2000). Constitutive expression of *LFY* results in a similar phenotype to *FT/TSF* overexpression, with early flowering, replacement of all co-florescences with flowers and termination of the shoot apex in a flower (Weigel and Nilsson, 1995).

*LFY* also provides a link between the meristem identity genes and genes that form the machinery that maintain meristem pluripotency. *LFY* acts together with the homeodomain protein WUSCHEL (*WUS*) to activate *AG* expression by concomitant binding (Busch et al., 1999; Lohmann et al., 2001; Hong et al., 2003). Subsequent

AG-mediated repression of the *CLAVATA* (*CLV*) and *WUS* pathway ultimately leads to termination of the floral meristem in a carpel (Lenhard et al., 2001; Lohmann et al., 2001).

*UFO* encodes a co-factor that binds directly to *LFY* as part of a transcriptional complex and promotes floral meristem identity and correct floral patterning, accordingly (Lee et al., 1997; Hepworth et al., 2006; Chae et al., 2008). In the *ufo* mutant, some flowers are occasionally replaced with co-florescences whereas others exhibit floral defects including replacement of petals and stamens with chimeric floral tissues (Ingram et al., 1995; Hepworth et al., 2006). In addition, co-florescences and the main stem may terminate in carpeloid or sepaloid structures (Ingram et al., 1995). *UFO* belongs to the F-box protein family, which are known for binding targets for ubiquitin-mediated proteolysis (Samach et al., 1999). It is possible that *UFO* may stimulate the transcriptional activity of *LFY* through mediating polyubiquitylation at promoter regions to stimulate rapid turnover and associated activation of target gene expression (Chae et al., 2008).

### ***1.2.5 Dual roles for AGAMOUS-LIKE24 (AGL24) and SHORT VEGETATIVE PHASE (SVP)***

*AGL24* and *SVP* are two closely related members of a large group of genes known as the MADS-box transcription factors (Hartmann et al., 2000). A number of MADS-box genes play important roles during the transition to flowering, and in the specification of identity for floral meristems and floral organs (see Gramzow and Theissen, 2010). These genes are further subdivided into subfamilies named after the first isolated member, and *AGL24* and *SVP* fall within the *StMADS11* subfamily (Hartmann et al., 2000). *AGL24* and *SVP* have opposite roles in the control of flowering time but act redundantly to control floral meristem identity (Hartmann et al., 2000; Yu et al., 2002; Gregis et al., 2006).

*AGL24* is a dosage-dependent promoter of the floral transition, regulated by the photoperiod, vernalisation, autonomous and gibberellin pathways, and acts together with the floral integrator *SOC1* to directly upregulate *LFY* expression (Yu et al., 2002; Michaels et al., 2003; Lee et al., 2008a). A positive feedback loop conferred by direct transcriptional regulation exists between *AGL24* and *SOC1* (Liu et al., 2008). In contrast, *SVP* is a dosage-dependent floral repressor that is controlled by the

photoperiod, autonomous, thermo-sensory and gibberellin pathways, and interacts via protein binding with FLOWERING LOCUS C (FLC) to repress targets in the absence of vernalisation (Lee et al., 2007b; Fujiwara et al., 2008; Li et al., 2008). Direct targets of *SVP* include floral integrators (*FT*, *TSF* and *SOC1*), *AP2*-like genes and microRNAs (Li et al., 2008; Jang et al., 2009; Tao et al., 2012).

After the floral transition, *SVP* and *AGL24* act with *AP1* during early stages of floral development, to ensure correct floral patterning by repressing B-class (*PI* and *AP3*), C-class (*AG*), and E-class (*SEP3*) genes, within the ABCE model for floral patterning (Gregis et al., 2006; Gregis et al., 2009). To do this, *AGL24* and *SVP* proteins each dimerise with *AP1* and interact with the LEUNIG-SEUSS (LUG-SEU) co-repressor complex to recruit it to the promoter regions of target genes (Gregis et al., 2006; Gregis et al., 2009).

Both the *svp* and *agl24* single mutants have altered flowering time, but no morphological defects (Hartmann et al., 2000; Michaels et al., 2003). In contrast, *35S:SVP* plants exhibit abnormal floral morphology in addition to delayed flowering, with flowers exhibiting chimeric vegetative shoot and floral characteristics with elongated internodes and leaves that sometimes have axillary stamens or exhibit fusion to carpel structures (Liu et al., 2007). Similarly, in addition to reducing flowering time, overexpression of *AGL24* results in floral defects with each floral meristem yielding a central flower that has axillary flowers borne in the axils of sepals (Yu et al., 2002; Yu et al., 2004).

### 1.2.6 Regulation by *SOC1*

*SOC1*, a member of the *TOMATO MADS 3 (TM3)* subclade of MADS-box transcription factors, is a key floral integrator that integrates signals from the photoperiod, autonomous, vernalisation and gibberellin pathways (Borner et al., 2000; Lee et al., 2000; Hepworth et al., 2002). *SOC1* is expressed in leaves and in the SAM, where it is up-regulated close to the time of the floral transition, within 24 hours of transfer from short day (SD) to inductive LD photoperiods (Borner et al., 2000; Samach et al., 2000). *SOC1* is also expressed in floral meristems during stage 3 of floral development in the region that will become stamen and carpel whorls, where it acts to ensure correct floral patterning (Samach et al., 2000; Gregis et al., 2009; Liu et al., 2009). *soc1* mutant plants are late-flowering, and can exhibit

aerial rosettes which are a sign of delayed flowering in the axillary meristems that form co-florescences (Onouchi et al., 2000; Dorca-Fornell et al., 2011). *SOC1* has recently been found to play a redundant role with *AGL24*, *SVP* and *SEP4* in specifically suppressing *TFL1* in emerging lateral meristems, to allow acquisition of floral identity (Liu et al., 2013). Studies of the double mutant phenotypes suggest that *SOC1* may act with *FT* and *FUL* to prevent secondary growth and regulate meristem longevity, which are both traits that distinguish annual and perennial growth habits (Melzer et al., 2008). *SOC1* overexpression can affect flower morphology in addition to altering flowering time, and overexpression of *SOC1* enhances the ectopic flower phenotype of *35S:AGL24* transgenic plants (Borner et al., 2000; Liu et al., 2007). Closely related members of the same subfamily of MADS-box genes, including *AGAMOUS-LIKE 42* (*AGL42*), *AGL71* and *AGL72*, are regulated by *SOC1* and function redundantly to regulate flowering time and axillary meristem identity (Dorca-Fornell et al., 2011).

### ***1.2.7 AP1 - a key floral meristem identity gene***

*AP1*, a member of the *SQUAMOSA* (*SQUA*) subfamily of MADS-box transcription factors, is a key regulator of floral development in *Arabidopsis*, with important roles in specification of floral meristem identity and correct specification of sepal and petal whorls (Mandel et al., 1992; Kaufmann et al., 2010). *AP1* is expressed throughout floral meristems as they arise from the flanks of the SAM and remains expressed until later stages of floral development (Mandel et al., 1992). *AP1* directly represses *TFL1* from floral meristems by binding to two sites within the 3' untranslated region (UTR) of *TFL1* (Kaufmann et al., 2010). *AP1* also interacts with SEP proteins to specify floral organ identity (Sridhar et al., 2006). Before stage 3 of floral development, *AP1* represses *AGL24*, *SVP* and *SOC1* to maintain floral meristem determinacy (Liu et al., 2007). In the absence of *AP1*, these genes are expressed ectopically and transform flowers into shoots (Yu et al., 2004; Liu et al., 2007). *AP1* represses expression of *FD* in young floral meristems in a similar manner (Wigge et al., 2005; Kaufmann et al., 2010). During later stages of floral development, *AP1* is itself repressed by *AG* from the cells that will become stamens and carpels, and becomes confined to sepals and petals (Mandel et al., 1992). *ap1* mutants produce inflorescences with extra cauline leaves before forming abnormal

flowers that completely lack petals and have leaf-like sepals that subtend ectopic flowers (Irish and Sussex, 1990; Bowman et al., 1993). The ectopic flowers can proliferate in a complex, branched floral structure, but decrease in frequency acropetally and with increased temperature (Bowman et al., 1993). Overexpression of *API* results in early flowering, replacement of co-florescences with flowers, and termination of the SAM in a terminal flower (Mandel and Yanofsky, 1995).

*FUL* and *CAULIFLOWER* (*CAL*) are related members of the same subfamily of MADS-box genes, which also function in meristem identity (Kempin et al., 1995; Gu et al., 1998). *FUL* acts to promote flowering and to control carpel and fruit development and *ful* single mutants flower late with abnormal carpel and fruit morphology (Gu et al., 1998; Ferrandiz et al., 2000a). *CAL* is a brassica-specific *API* paralog that acts redundantly with *API* (Kempin et al., 1995; Purugganan, 1997). *ap1 cal* double mutants exhibit a ‘cauliflower’ appearance wherein flowers behave as inflorescences, producing additional meristems in a spiraled phyllotaxy that each act in the same manner, resulting in a proliferation of meristems borne in place of each flower (Bowman et al., 1993). Additional mutation of *FUL* results in a completely non-flowering triple mutant that bears proliferating leafy shoots in place of flowers (Ferrandiz et al., 2000b).

### ***1.2.8 Local balance between promoters and repressors of floral identity***

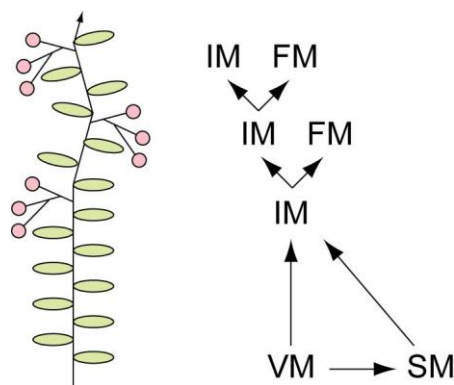
A recurring theme apparent in the null and overexpression phenotypes for meristem identity genes is that the local balance between promoters and repressors of floral identity is important for correct development of the indeterminate raceme of *Arabidopsis*. To produce this inflorescence form, the floral repressor *TFL1* prevents floral meristem identity genes from being expressed within the SAM, maintaining shoot indeterminacy, and genes that promote floral fate upregulate floral meristem identity genes within axillary meristems. When the system is unbalanced by excess of a floral promoter, then inflorescence meristems acquire floral meristem identity and the SAM terminates in a flower. Conversely, when a floral repressor is expressed ectopically in flanking cells, indeterminacy of axillary structures is increased and floral meristems are replaced with inflorescence meristems. In a wild-type plant, correct balance is maintained by complex positive and negative feedback loops between genes to ensure that the plant becomes committed to reproductive

development once the decision to flower is made and to restrict gene expression patterns to appropriate domains.

## 1.3 Tomato

### 1.3.1 Inflorescence form

In contrast to the monopodial growth pattern of *Arabidopsis*, in which the main shoot axis maintains a single line of growth, tomato exhibits a sympodial growth pattern. In tomato, growth starts with an initial segment that ends in an inflorescence, and growth continues by sympodial segments which each produce vegetative nodes, end in an inflorescence and bear the next sympodial segment (Figure 1.4). This pattern of growth involves an additional type of meristem, the sympodial meristem, which forms in the axil of the node prior to the inflorescence in each segment (Figure 1.4). To produce the modified cyme of tomato, the vegetative SAM or sympodial meristem becomes an inflorescence meristem, which remains indeterminate and bifurcates a number of times, each bifurcation producing a floral meristem and another inflorescence meristem (Figure 1.4; Allen and Sussex, 1996; Welty et al., 2007). A number of important meristem identity genes have been characterised in tomato, and the majority of these are homologs of *Arabidopsis* genes described above. This subset will be the focus of this introduction (Table 1.2).



**Figure 1.4.** The modified cyme of tomato.

Diagram of the tomato inflorescence (left) and schematic of the meristem transitions involved in its development (right). In tomato, inflorescence development involves conversion of the vegetative shoot apical meristem (VM) into an inflorescence meristem (IM) which bifurcates to produce a number of floral meristems (FM). Stem growth continues via a sympodial meristem (SM) borne on the stem node beneath the IM, which in turn becomes an IM after three nodes. In the diagram, arrows indicate indeterminate growth, circles are flowers and ovals are leaves. In the schematic, arrows indicate meristem transitions and products.

**Table 1.2.** Summary of key genes important for determining meristem identity during inflorescence development in tomato, focussing on homologs of the *Arabidopsis* genes shown in Table 1.1. Abbreviations for meristems are as follows: vegetative meristem (VM; includes vegetative primary shoot meristem and sympodial meristem), inflorescence meristem (IM; on primary shoots and sympodial segments), and floral meristem (FM). Question marks indicate uncertainty in the literature.

Gene family	Gene	<i>Arabidopsis</i> homolog(s)	Expression domain	Overexpression in tomato	Mutant/knockdown phenotype	Inferred function	References
CETS	<i>SINGLE FLOWER TRUSS</i> (SFT; prev. <i>SP3D</i> )	<i>FT/TSF</i>	Leaves, stem, apex, floral organs	Early-flowering, termination of IM in single FM	Late-flowering, IM identity lost	Promotes the floral transition and IM identity	Carmel-Goren et al. (2003); Molinero-Rosales et al. (2004); Lifschitz et al. (2006)
	<i>SELF PRUNING</i> (SP)	<i>ATC/TFL1</i>	Leaves, VM, IM, FM	Late-flowering, partial loss of IM identity	Progressive reduction of sympodial segments until two consecutive inflorescences produced	Regulates sympodial growth by delaying SAM transition to IM identity	Pnueli et al. (1998); Thouet et al. (2008)
bZIP	<i>SELF-PRUNING G-BOX</i> (SPGB)	<i>FD/FDP</i>	Leaves, VM	-	-	Promotion of IM identity? Control of leaf development?	Pnueli et al. (2001); Lifschitz et al. (2006)
<i>LFY</i>	<i>FALSIFLORA</i> (FA)	<i>LFY</i>	Leaves, shoot, VM, FM, floral organs	-	Late-flowering, all FM → IM/VM, no FM	Promotes the floral transition and specifies FM identity	Molinero-Rosales et al. (1999)
F-box	<i>ANANTHA</i> (AN)	<i>UFO</i>	IM, FM	-	All FM → IM, no FM	Specifies FM identity	Allen and Sussex (1996); Lippman et al. (2008)
MADS-box	<i>JOINTLESS</i> (J)	<i>SVP</i>	VM, IM, FM, floral organs during early development	-	Late-flowering (minor), IM identity lost	Promoting floral transition (minor) and IM identity	Szymkowiak and Irish (1999); Mao et al. (2000); Szymkowiak and Irish (2006); Quinet et al. (2006)
	<i>MACROCALYX</i> (MC; <i>LeMADS-MC</i> )	<i>AP1</i>	Floral organs (sepals, petals, carpels)	-	IM identity lost, leafy sepals	Maintains IM identity, specifies sepals	Vrebalov et al. (2002)
<i>Arabidopsis</i> LIGHT-SENSITIVE HYPOCOTYL 1 <i>Oryza</i> Gl (ALOG)	<i>TERMINATING FLOWER</i> (TMF)	<i>LIGHT-DEPENDENT SHORT HYPOCOTYLS 3/4</i> (LSH3/LSH4)	VM, IM (weak), FM (weak)	IM → VM	Early-flowering, IM → FM for primary shoot	Maintains VM or specifies IM identity	MacAlister et al. (2012)

### 1.3.2 Antagonism between CETS proteins

Similar to *Arabidopsis FT*, the tomato ortholog *SINGLE FLOWER TRUSS* (*SFT*) encodes a florigenic, graft-transmissible signal that travels from leaf to apex to trigger flowering (Lifschitz et al., 2006). The *sft* mutant is late-flowering and the first inflorescence produces only one or two flowers, before reverting to vegetative development for several nodes and again producing flowers in a repeated pattern (Molinero-Rosales et al., 2004). In this manner, the sympodial growth pattern is lost and one continuous inflorescence shoot alternates leaves and flowers (Molinero-Rosales et al., 2004). This phenotype indicates that *SFT* acts to promote the floral transition and has important roles in maintaining inflorescence meristem identity and co-ordinating sympodial development. *SFT* has additional pleiotropic roles in compound leaf development, stem growth and the formation of abscission zones (Shalit et al., 2009).

The functional homolog of *TFL1* in tomato, *SELF PRUNING* (*SP*), acts to delay the transition of each sympodial meristem to inflorescence identity (Pnueli et al., 1998). *sp* mutant plants exhibit a progressive decrease in the number of leaves in each sympodial segment, until the last segment is reduced to an inflorescence and the tomato shoot ends in two consecutive inflorescences (Pnueli et al., 1998). Introduction of the *sp* mutation into tomato cultivars to create 'determinate' varieties is credited with revolutionising the tomato industry, due to desirable traits including a bushy, compact form with nearly homogeneous fruit setting, enabling mechanical harvest (Pnueli et al., 1998).

The antagonism between *FT* and *TFL1* homologs is also apparent in tomato, and the *SFT/SP* ratio is thought to control the balance of determinate and indeterminate growth to establish the sympodial growth pattern seen in wild-type plants (Lifschitz and Eshed, 2006; McGarry and Ayre, 2012; Jiang et al., 2013). *SP* and *SFT* proteins both interact with the FD homolog SELF-PRUNING G-BOX (*SPGB*; Pnueli et al., 2001; Lifschitz et al., 2006), indicating that these proteins may share a similar mechanism of action with *Arabidopsis* homologs.



### ***1.3.3 FALSIFLORA (FA) and ANANTHA (AN) - conserved roles in promoting floral identity***

Mutation to *FA* or *AN*, tomato homologs of *LFY* and *UFO*, respectively, results in a highly similar phenotype, whereby inflorescence meristems fail to yield floral meristems, instead remaining indeterminate and proliferating more inflorescence meristems (Allen and Sussex, 1996; Lippman et al., 2008). This results in highly branched, non-flowering structures, which bear leaves in *fa* mutants and some sepal- or bract-like structures in *an* mutants (Allen and Sussex, 1996; Molinero-Rosales et al., 1999; Lippman et al., 2008). These phenotypes indicate that both genes have a critical role in specification of floral meristem identity, and suggest that *FA* and *AN* proteins may act together in tomato in a similar manner as homologous *Arabidopsis* proteins (Allen and Sussex, 1996; Molinero-Rosales et al., 1999; Chae et al., 2008; Lippman et al., 2008). Inflorescence defects are notably more severe in *an* than *ufo*, indicating *UFO* may share greater redundancy with other F-box genes in *Arabidopsis* (Ingram et al., 1995; Allen and Sussex, 1996; Hepworth et al., 2006). This is consistent with findings that *LFY* is not exclusively dependent on *UFO* for function in *Arabidopsis* (Chae et al., 2008). The *sft fa* double mutant is non-flowering, indicating that *SFT* and *FA* act in two alternative pathways, similar to homologous genes in *Arabidopsis* (Molinero-Rosales et al., 2004). Although unrelated to inflorescence development, *fa* mutants also exhibit a reduction in the number of small leaflets relative to wild-type indicating that *FA* has an additional role in tomato compound leaf development, which is not seen for *LFY* in *Arabidopsis* (Molinero-Rosales et al., 1999).

### ***1.3.4 A role for JOINTLESS (J) - an StMADS11 subfamily member***

*J* belongs to the same subclade of MADS-box genes as *SVP* (Mao et al., 2000; Leseberg et al., 2008), but appears to share some functional similarity with *AGL24* (Thouet et al., 2012). In *j* mutants, there is a minor delay in flowering time and inflorescences revert to leaf production after formation of a few flowers, suggesting that *J* promotes inflorescence identity, possibly through suppression of sympodial identity in inflorescence meristems, with an additional minor role in promoting the floral transition (Szymkowiak and Irish, 1999; Quinet et al., 2006; Szymkowiak and Irish, 2006). This phenotype is similar to the *sft* mutant, except a sympodial growth

pattern is retained (Szymkowiak and Irish, 1999). As *SP* has a role in promoting sympodial identity, and the inflorescences of *j* mutants share characteristics with sympodial segments, it has been suggested that *J* may act by antagonising *SP* activity within the inflorescence meristem (Szymkowiak and Irish, 2006). An alternative theory is that *J* may act to maintain inflorescence identity through repression of floral identity, by restricting expression of *FA* within the inflorescence meristem (Thouet et al., 2012). In support of this, *FA* expression is higher in the inflorescence meristem of *j* mutants than in wild-type (Thouet et al., 2012). Although not relevant for inflorescence development, *J* has an additional role in formation of the pedicel abscission zone and *j* mutants lack this feature (Szymkowiak and Irish, 1999). The absence of pedicel abscission zones is preferable for mechanical harvest of ‘stemless’ fruit without the sepals and portion of the pedicel that are normally abscised with the fruit in wild-type plants (Szymkowiak and Irish, 1999). These findings suggest that there is some divergence of function between *JOINTLESS* and its *Arabidopsis* homologs.

### **1.3.5 MACROCALYX (MC) - a role in inflorescence meristem identity**

*MC* is the tomato homolog of *API* (Vrebalov et al., 2002). Knockdown of *MC* function in transgenic tomato plants by RNA interference (RNAi) results in inflorescences that produce several flowers before reverting to vegetative growth (Vrebalov et al., 2002). Flowers are normal except for leaf-like sepals, which is in sharp contrast to the severe floral defects, including floral indeterminacy and complete absence of petals, which is observed in *Arabidopsis ap1* mutants, suggesting that *MC* is less important than *API* for floral meristem specification (Irish and Sussex, 1990; Bowman et al., 1993; Vrebalov et al., 2002). In contrast, *MC* has an important role in maintenance of inflorescence identity, which is not apparent for *API* in *Arabidopsis*, and may reflect a divergence of how these genes act at the molecular level. The mechanism for *MC* action during tomato inflorescence development has not been investigated in any great detail, instead recent literature has focussed on a separate role for *MC* in controlling development of abscission zones in conjunction with *J* (Nakano et al., 2012).

### ***1.3.6 TERMINATING FLOWER (TMF) - an ALOG gene with a role in tomato inflorescence development***

*TMF* is a member of the *Arabidopsis* LIGHT-SENSITIVE HYPOCOTYL 1 *Oryza* GI (ALOG) gene family, which has not yet been mentioned in this chapter, as homologous *Arabidopsis* genes *LIGHT-DEPENDENT SHORT HYPOCOTYLS* 3 and 4 (*LSH3* and *LSH4*; discussed below) have not been recognised as important meristem identity genes in *Arabidopsis* (MacAlister et al., 2012). In the tomato *tmf* mutant the first inflorescence comprises a solitary flower, but subsequent sympodial shoots are normal (Hareven et al., 1994). Constitutive expression of *TMF* imparts vegetative characteristics to inflorescence meristems, which suggests that *TMF* may act to maintain vegetative identity of the SAM rather than specifying inflorescence identity (MacAlister et al., 2012; Périlleux et al., 2014). *TMF* functions by delaying expression of floral meristem genes including *AN* and members of the *SEP* family within the SAM (MacAlister et al., 2012).

In *Arabidopsis*, the homologous genes *LSH3* and *LSH4* are thought to suppress differentiation within the boundary regions which separate organ primordia from the SAM (Takeda et al., 2011). Knockdown of *LSH3* and *LSH4* gene function in transgenic *Arabidopsis* plants does not result in any noticeable phenotype, but overexpression of *LSH4* causes leaf and floral defects, including extra floral organs and production of ectopic shoots within flowers, and overexpression of *LSH3* causes milder defects of a similar nature (Takeda et al., 2011). Although *LSH3* and *LSH4* have not been highlighted as key genes involved in inflorescence development in *Arabidopsis*, overexpression phenotypes are similar to *TMF* in tomato, with all three genes appearing to promote vegetative identity in the SAM (Takeda et al., 2011; MacAlister et al., 2012). It is possible that genetic redundancy, possibly with other ALOG genes, underlies an absence of inflorescence phenotype when *LSH3* or *LSH4* function is compromised in *Arabidopsis*.

### **1.3.7 *A matter of balance***

Similar to *Arabidopsis*, the balance between the genes that promote an inflorescence or floral fate, and those that repress these fates, is thought to be a key factor in controlling tomato inflorescence development (see McGarry and Ayre, 2012). Unlike *Arabidopsis*, the SAM in tomato acquires a semi-determinate fate through direct production of floral meristems while axillary sympodial meristems adopt an indeterminate fate for continued shoot growth. This difference can be explained by dominant action of inflorescence and floral identity genes in the SAM, and dominance of genes repressing inflorescence and floral identity in axillary (sympodial) meristems (McGarry and Ayre, 2012).

## **1.4 The grasses - rice and maize**

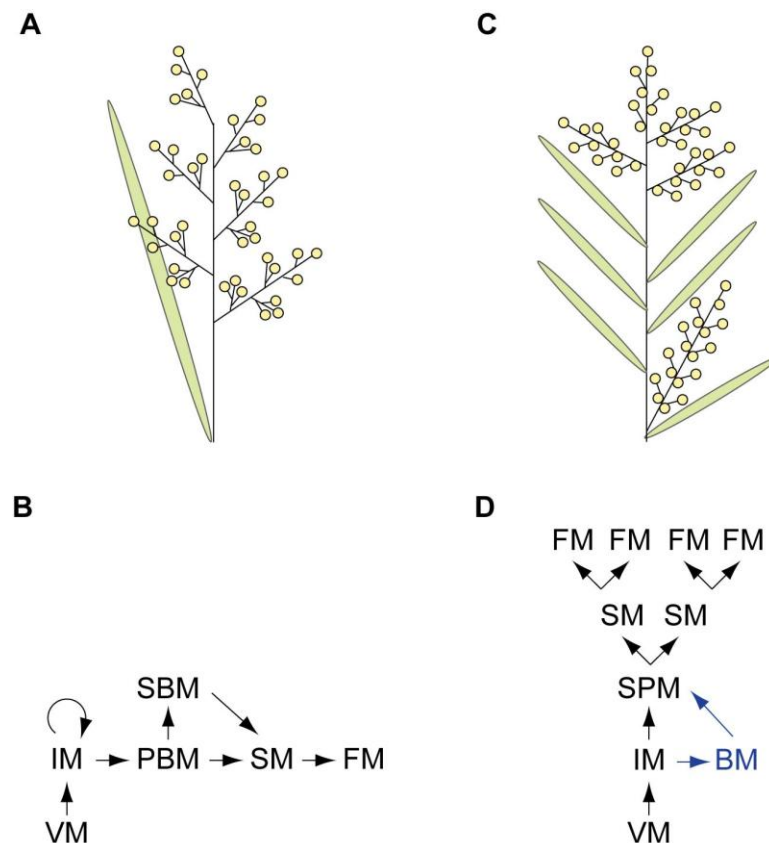
There is considerable evolutionary distance between the monocot grasses (Poaceae) and previously discussed eudicot models, as lineage divergence occurred approximately 150 million years ago (Chaw et al., 2004). The panicles seen in grass species are complex, involving multiple specialised inflorescence branches that are produced by specific types of meristems, including branch meristems that arise directly from the main stem or other branches, and spikelet meristems that bear florets (grass flowers). Here, rice and maize have been selected as models for grass inflorescence development, as these two grass species have been prominent in recent literature.

### **1.4.1 *Inflorescence form***

In rice, the SAM undergoes the transition to inflorescence identity and produces the main inflorescence stem, bearing ten or more primary branch meristems, before degenerating and remaining as a vestige (Figure 1.5A-B). Each primary branch bears several secondary branch meristems, then both primary and secondary branch meristems produce lateral spikelet meristems and terminate in spikelets (Figure 1.5A-B). Each spikelet, in turn, bears a single floral meristem (Figure 1.5B), subtended by two pairs of bract-like structures known as glumes.

In contrast, maize has two distinct inflorescence types - the male tassel and female ear, and also differs from the rice panicle in terms of branching pattern and SAM fate (Figure 1.5C-D). After floral induction, the maize SAM and an axillary

meristem that is five or six nodes beneath the SAM are each converted into inflorescence meristems. The apical inflorescence meristem gives rise to the tassel; the axillary inflorescence meristem gives rise to the ear. The tassel meristem produces an indeterminate number of branch meristems (Figure 1.5C-D). Inflorescence and branch meristem yield a number of spikelet pair meristems that each produce two spikelets (Figure 1.5C-D). In maize, each spikelet produces a pair of glumes and two floral meristems, but only the upper floret develops in the spikelets present in the ear (Figure 1.5D).



**Figure 1.5.** The panicles of rice and maize.

(A) Diagram of the rice inflorescence form and (B) schematic of meristem transitions involved in its development. In rice, inflorescence development involves conversion of the vegetative shoot apical meristem (VM) into an inflorescence meristem (IM) which bears an axillary primary branch meristem (PBM) at each node, before aborting. Primary branches bear secondary branch meristems (SBM), and both primary and secondary branches bear spikelet meristems (SM) then terminate in an SM. Each SM produces a single floral meristem (FM).

(C) Diagram of the maize inflorescence form and (D) schematic of meristem transitions involved in its development. In maize, a female inflorescence (ear) is borne in an axillary position several nodes before the male inflorescence (tassel) terminates the main stem. IMs bears branch meristems (BM) in the tassel only, and spikelet pair meristems (SPM) in both ear and tassel. Each SPM bears two SMs, which each bear two FMs. In the ear the lower FM aborts during early development.

In diagrams, arrows indicate indeterminate growth, circles are spikelets and ovals are leaves. In schematics, straight arrows indicate meristem transitions and products, and circular arrows indicate meristem indeterminacy.

**Table 1.3.** Summary of key genes important for determining meristem identity during inflorescence development in rice and maize, focussing on homologs of the *Arabidopsis* genes shown in Table 1.1. Rows for rice genes are shown in yellow and rows for maize genes are shown in blue. Abbreviations for meristems are as follows: vegetative meristem (VM), inflorescence meristem (IM), branch meristem (BM), primary branch meristem (PBM), secondary branch meristem (SBM), spikelet pair meristem (SPM), spikelet meristem (SM) and floral meristem (FM). Overexpression phenotypes are restricted to transgenic rice/maize plants. Question marks indicate uncertainty in the literature.

Gene family	Gene	<i>Arabidopsis</i> homolog(s)	Expression domain	Overexpression or gain-of-function phenotype	Mutant/knockdown phenotype	Inferred function	References
CETS	<i>Heading date 3a</i> ( <i>Hd3a</i> )	<i>FT/TSF</i>	Leaves	Early-flowering	Late-flowering under SD conditions	Promoting IM identity	Kojima et al. (2002); Tamaki et al. (2007); Komiya et al. (2008)
	<i>RICE FLOWERING LOCUS T 1</i> ( <i>RFT1</i> )	<i>FT/TSF</i>	Leaves	Early-flowering	Late-flowering under LD conditions	Promoting IM identity	Izawa et al. (2002); Komiya et al. (2008)
	<i>Zea mays CENTRORADIALIS8</i> ( <i>ZCN8</i> )	<i>FT/TSF</i>	Leaves	Early-flowering	Late-flowering	Promoting IM identity	Danilevskaya et al. (2008a); Meng et al. (2011)
	<i>RICE CENTRORADIALIS1</i> ( <i>RCN1</i> )	<i>TFL1/ATC</i>	IM	Late-flowering, increased panicle branching (delayed BM → SM)	-	Repressing IM identity, promoting meristem indeterminacy	Nakagawa et al. (2002)
	<i>RCN2</i>	<i>TFL1/ATC</i>	VM, IM	As above	-	As above	
	<i>ZCN2</i>	<i>TFL1/ATC</i>	VM, tassel base. Ear: IM, SPM, SM, FM, floral organs	Late-flowering, delayed IM → SPM	-	Repressing IM identity, promoting meristem indeterminacy	Danilevskaya et al. (2008a); Danilevskaya et al. (2010)
	<i>ZCN4</i>	<i>TFL1/ATC</i>	Tassel primordia, ear vasculature	As above	-	As above	
	<i>ZCN5</i>	<i>TFL1/ATC</i>	Tassel primordia. Ear: SPM, SM	As above	-	As above	
bZIP	<i>OsFD1</i>	<i>FD/FDP</i>	Leaf sheath, tiller bud, stem, apex	Early-flowering	Late-flowering, minor floral defects	Promoting IM identity	Taoka et al. (2011); Tsuji et al. (2013b)
	<i>OsFD2</i>	<i>FD/FDP</i>	Leaf blade, leaf sheath, tiller bud, stem, apex	Increased panicle branching (delayed BM → SM), leaf defects	-	Promoting meristem indeterminacy, leaf development	Tsuji et al. (2013b)

Gene family	Gene	<i>Arabidopsis</i> homolog(s)	Expression domain	Overexpression or gain-of-function phenotype	Mutant/knockdown phenotype	Inferred function	References
bZIP	<i>DELAYED FLOWERING 1 (DLF1)</i>	<i>FD/FDP</i>	VM, IM	-	Late-flowering, increased branching in tassel and ear, multiple ear IMs	Pleiotropic roles in inflorescence development	Muszynski et al. (2006)
MADS-box	<i>OsMADS22</i>	<i>SVP/AGL24</i>	VM, IM, PBM, FM, leaves, roots	Decreased panicle branching? Increased panicle branching?	No flowering phenotype	Promotes panicle branching? Inhibits panicle branching?	Pelucchi et al. (2002); Sentoku et al. (2005); Fornara et al. (2008); Lee et al. (2008b); Yoshida et al. (2013)
	<i>OsMADS47</i> (prev. <i>OsMDP1</i> )	<i>SVP/AGL24</i>	VM, leaves, roots	-	No flowering phenotype	Acts redundantly with other MADS-box genes?	Duan et al. (2006); Arora et al. (2007); Fornara et al. (2008)
	<i>OsMADS55</i>	<i>SVP/AGL24</i>	VM, young panicles, roots	Decreased panicle branching? Increased panicle branching?	No flowering phenotype	Promotes panicle branching? Inhibits panicle branching?	Lee et al. (2003); Arora et al. (2007); Lee et al. (2008b); Yoshida et al. (2013)
	<i>Zea mays MADS19 (ZMM19; Tunicate/Tu)</i>	<i>SVP/AGL24</i>	Leaf blades, leaf husks	Large glumes, partial loss of sex determination in ear and tassel	-	Wild-type function unknown	Han et al. (2012); Wingen et al. (2012)
	<i>OsMADS14</i> (prev. <i>RAP1b</i> )	<i>AP1/FUL</i>	IM, FM, floral organs	Early-flowering	Triple knockdown: Late-flowering	Promotes IM identity, and floral meristem identity?	Moon et al. (1999); Jeon et al. (2000b); Kyoizuka et al. (2000); Pelucchi et al. (2002); Masiero et al. (2002); Fornara et al. (2004); Komiya et al. (2009); Kobayashi et al. (2012); Lu et al. (2012)
	<i>OsMADS15</i> (prev. <i>RAP1a</i> )	<i>AP1/FUL</i>	Leaves, stem, roots, FM, floral organs	Early-flowering, reduced number of PBMs, no SBMs			
	<i>OsMADS18</i>	<i>AP1/FUL</i>	Shoot, leaves, roots, SBM, SM, FM	Early-flowering			
	<i>ZMM4</i>	<i>AP1/FUL</i>	IM, BM, SPM, SM	Early-flowering	-	Promotes IM identity, later roles in inflorescence development?	Danilevskaya et al. (2008b)

Gene family	Gene	<i>Arabidopsis</i> homolog(s)	Expression domain	Overexpression or gain-of-function phenotype	Mutant/knockdown phenotype	Inferred function	References
LFY	<i>ABERRANT PANICLE ORGANIZATION2</i> ( <i>APO2</i> ; <i>RFL</i> )	<i>LFY</i>	VM, leaf primordia, early PBM, early SBM, FM, floral organs	Early-flowering, no inflorescence defects reported	Late-flowering, IM → SM, precocious BM → SM, floral defects	Promotes transition to IM identity, promotes panicle branching by repressing SM identity, maintains FM identity	Kyozuka et al. (1998); Rao et al. (2008); Ikeda-Kawakatsu et al. (2012)
	<i>Zea mays FLO/LFY1</i> ( <i>ZFL1</i> )	<i>LFY</i>	VM, SM, SPM, FM, floral organs	-	Double mutants: late-flowering, loss of sex determination in ear and tassel, floral defects	Promotes transition to IM identity, panicle branching, and FM identity	Bomblies et al. (2003)
	<i>ZFL2</i>	<i>LFY</i>	As above	-			
F-box	<i>ABERRANT PANICLE ORGANIZATION1</i> ( <i>APO1</i> )	<i>UFO</i>	IM, BM	Increased panicle branching (delayed BM → SM)	Late-flowering (minor), IM → SM, precocious BM → SM, floral defects	Promotes transition to IM identity, promotes panicle branching by repressing SM identity, maintains FM identity	Ikeda et al. (2005); Ikeda et al. (2007); Ikeda-Kawakatsu et al. (2009)
ALOG	<i>TAWAWA1</i> ( <i>TAW1</i> )	<i>LSH3/LSH4</i>	VM, IM, BM	SM → BM	IM → SM, precocious BM → SM	Promotes meristem indeterminacy and panicle branching	Yoshida et al. (2013)



### 1.4.2 The *FT/FD* pathway in grasses

*Heading date 3a* (*Hd3a*), a rice homolog of *FT*, encodes a florigenic signal that moves from leaves to the apex to promote flowering under favourable SD conditions (Tamaki et al., 2007). Within the apex, *Hd3a* acts within a ‘florigen activation complex’ which also contains *OsFD1*, a homolog of *Arabidopsis* *FD/FDP*, and 14-3-3 proteins that act as a molecular bridge between *Hd3a* and *OsFD1* molecules (Taoka et al., 2011). This complex is able to bind to a C-box element present within the promoter region of the *API/FUL* homolog *OsMADS15*, and upregulate its expression (Taoka et al., 2011). Overexpression of *Hd3a* or *OsFD1* in transgenic rice plants results in early flowering, whereas knockdown of expression by RNAi delays flowering with additional stamen defects seen in *OsFD1* RNAi plants (Kojima et al., 2002; Komiya et al., 2008; Taoka et al., 2011). A second rice FD homolog, *OsFD2*, has also been found to act in a florigen activation complex with *Hd3a*, and overexpression of *OsFD2* delays the transition from branch meristem (primary or secondary) to terminal spikelet meristem resulting in increased production of branches and spikelets and a dense panicle phenotype (Tsuji et al., 2013b). Additional defects in leaf morphology for plants overexpressing *OsFD2*, in conjunction with the finding that co-expression of *Hd3a* and *OsFD2* cannot induce *OsMADS15* expression in transient assays, has led to the conclusion that *OsFD2* has a divergent role in leaf development, particularly that of the flag leaf, the last leaf before the panicle (Tsuji et al., 2013b). However, the inflorescence phenotype of *OsFD2* transgenic overexpression lines suggests that this gene may still have a role during inflorescence development, regulating different target genes, which has not yet been investigated. No interaction has been detected between *Hd3a* and a third FD homolog, *OsFD3*, and no flowering or inflorescence phenotype is apparent in *OsFD3* RNAi plants, suggesting that *OsFD3* does not have an important role during inflorescence development in rice (Tsuji et al., 2013b).

The role of a second *FT* homolog, *RICE FLOWERING LOCUS T 1* (*RFT1*), has also been investigated in rice. *RFT1* RNAi plants exhibit delayed flowering under LD conditions only, but double knockdown of *RFT1* and *Hd3a* blocks flowering completely under SD conditions (Komiya et al., 2008). This indicates that *RFT1* has an important role in promoting the floral transition from vegetative to inflorescence identity, particularly under non-favourable LD conditions, but also

under SD conditions, where *RFT1* appears to act partially redundantly with *Hd3a* (Komiya et al., 2008). Interactions between RFT1 and the rice FD homologs have not yet been reported, but it seems likely that *RFT1* may act through a similar mechanism to *Hd3a*.

In maize, the strongest candidate for the florigen signal is the *FT* homolog *Zea mays CENTRORADIALIS8* (*ZCN8*), which is expressed in the leaf vasculature in a photoperiod-dependent manner (Danilevskaya et al., 2008a). Increased expression of *ZCN8* in transgenic maize plants causes early flowering whereas transgenic downregulation of *ZCN8* expression delays flowering (Meng et al., 2011). *ZCN8* protein interacts with DELAYED FLOWERING 1 (*DLF1*), a maize FD homolog (Danilevskaya et al., 2008a; Meng et al., 2011). *DLF1* is expressed in the apex during vegetative growth, with expression level peaking at around the time of the transition to flowering and decreasing to undetectable levels during early reproductive growth (Muszynski et al., 2006). *dlf1* mutant plants are late-flowering with inflorescence defects including (i) increased branching in both tassel and ear, (ii) multiple stem nodes bearing axillary ears and (iii) abnormal floret morphology in tassels (Muszynski et al., 2006). This phenotype indicates that *DLF1* is important for multiple stages of inflorescence development in maize (Muszynski et al., 2006). *ZCN26*, another *FT* homolog in maize, is also expressed in leaves and shows strong interaction with *DLF1*, but is not a candidate for a role in promoting inflorescence identity, as it is not regulated by photoperiod (Meng et al., 2011). It is possible that *ZCN26* may act with *DLF1* during later stages of maize inflorescence development, but this remains to be investigated.

Overall, these findings indicate that the transition to inflorescence development in both rice and maize, involves *FT* and *FD* homologs, in a mechanism that is remarkably well conserved with *Arabidopsis*. Subfunctionalisation between *FT* and *FD* homologs in the grasses is an interesting aspect of this process that is still being investigated.

### ***1.4.3 Repressive functions of TFL1 homologs***

The repressive influence of *TFL1* homologs is apparent in both rice and maize, similar to *Arabidopsis*. Overexpression of either of two *TFL1* homologs, *RICE CENTRORADIALIS1* or 2 (*RCN1* or *RCN2*), in transgenic rice plants results in either a severe non-flowering phenotype where reproductive structures are never produced, or a late-flowering phenotype with increased panicle branching due to delayed termination of branch meristems in a spikelet meristem (Nakagawa et al., 2002). These inflorescence defects are similar to those described for overexpression of *OsFD2* (Tsuji et al., 2013b). Although interactions between the rice RCN and FD proteins have not been reported, the residues on the 14-3-3 binding interface of Hd3a are well conserved in the RCN proteins (Taoka et al., 2011), indicating that RCN proteins may act in repressor complexes with FD proteins (Tsuji et al., 2013a). Three of the six *TFL1* homologs that have been identified in maize, *ZCN2*, *ZCN4*, and *ZCN5*, are expressed in wild-type meristems during inflorescence development (Danilevskaya et al., 2008a; Danilevskaya et al., 2010). Overexpression of each gene individually in transgenic maize plants can delay flowering by up to two weeks and result in increased tassel branching and high spikelet density in the tassel and ear. These phenotypes suggest that *ZCN2*, *ZCN4*, and *ZCN5* may be involved in repressing the transition to inflorescence meristem identity and maintaining the indeterminacy of the inflorescence and branch meristems. Of the three proteins, only *ZCN2* can interact with DLF1, but two other maize FD homologs have recently been identified (Tsuji et al., 2013b), and may be candidates for interaction with *ZCN4* and *ZCN5*. Overall, these results indicate that *TFL1* homologs in grasses repress the transition to flowering and promote meristem indeterminacy, similar to the role of *Arabidopsis TFL1*, and likely act through a similar mechanism.

### ***1.4.4 A possible role for SVP-like genes in grass inflorescence development***

The maize *Tunicate* (*Tu*) mutant, also known as pod corn, was first grown by native tribes of American Indians for supposed magical and curative properties and was later thought by scientists to represent an ancestral form of maize (e.g. Mangelsdorf and Galinat, 1964). *Tu* mutant plants exhibit a characteristic elongation of outer glumes in addition to seed development in tassels and branching and development of both florets within each spikelet pair in the ear (Mangelsdorf, 1948).

With the exception of increased glume size, this phenotype represents partial loss of sex determination in male and female inflorescences. Recently the *Tu* locus has been identified as a co-dominant gain-of-function allele of *Zea mays MADS19* (*ZMM19*), a gene from the same clade of MADS-box genes as *SVP*, and the *Tu* mutation has been found to have originated after the domestication of maize (Han et al., 2012; Wingen et al., 2012). The *Tu* mutant phenotype appears to have resulted from duplication of *ZMM19* and chromosomal rearrangement within the 5' regulatory region, causing ectopic expression of this gene in the maize ear (Han et al., 2012; Wingen et al., 2012). *ZMM19* expression is normally restricted to vegetative organs (leaf blades and husk leaves) in wild-type plants, as is expression of the other *StMADS11* subfamily genes in maize, suggesting that this family of genes may not normally have a role in wild-type maize inflorescence development (Wingen et al., 2012). The function of these genes in wild-type plants is not yet clear.

Three *SVP*-like genes are present in rice: *OsMADS22*, *OsMADS47*, and *OsMADS55* (Arora et al., 2007). These genes exhibit different expression patterns in wild-type plants, with *OsMADS22* present in the vegetative meristem, inflorescence meristem, primary branch meristems and young floral buds, *OsMADS55* expressed in vegetative shoots and developing panicles, and *OsMADS47* predominantly expressed in leaves (Pelucchi et al., 2002; Sentoku et al., 2005; Duan et al., 2006; Fornara et al., 2008). Overexpression of *OsMADS22* or *OsMADS55* in transgenic rice plants results in shortened panicles and abnormal floret morphology (Sentoku et al., 2005; Lee et al., 2008b). This decrease in panicle length is particularly interesting, as both of these genes also have roles in brassinosteroid signalling, and overexpression of either gene results in increased internode length for vegetative portions of the stem (Lee et al., 2008b). Although panicle length in lines overexpressing these genes has not been directly measured in terms of number of branches or spikelets, published photos suggest that this reduction in length may be due to decreased branching within the panicle (Lee et al., 2008b), indicating a possible role for these genes in promoting meristem determinacy. Consistent with this, recent results indicate that these three *SVP*-like genes may act redundantly with rice *SOC1* and *SEP* homologs to downregulate *RCN* genes for decreased panicle branching (Liu et al., 2013). This is similar to the redundant roles played by *SOC1*, *SVP*, *AGL24* and *SEP4* in regulation of *TFL1* in *Arabidopsis* (Liu et al., 2013). Confusingly, a separate study has recently

found that overexpression of *OsMADS22* or *OsMADS55* in transgenic rice plants increases panicle branching (Yoshida et al., 2013), in sharp contrast to earlier findings which showed the opposite (Lee et al., 2008b). It is clear that the function of these rice genes requires further investigation.

#### **1.4.5 A role for *LFY* and *UFO* homologs in panicle branching**

The function of *LFY* and *UFO* homologs has been characterised in the most detail in rice. Mutation to *ABERRANT PANICLE ORGANIZATION2* (*APO2*; *LFY* homolog), or *ABERRANT PANICLE ORGANIZATION1* (*APO1*; *UFO* homolog), results in (i) a delay in flowering time, (ii) reduced panicle branching due to termination of the inflorescence meristem (which normally remains as a vestige) in a spikelet and early transition of branch meristems to spikelet meristems, and (iii) defects in floret morphology including indeterminacy (Ikeda et al., 2005; Ikeda-Kawakatsu et al., 2012). This indicates that both *APO2* and *APO1* function to promote the transition to inflorescence development in a manner similar to *LFY* and *UFO* in *Arabidopsis*, but also have a distinct additional role in inflorescence development - the repression of spikelet meristem identity to promote panicle branching (Ikeda-Kawakatsu et al., 2012). Similar to the mechanism in *Arabidopsis*, *APO1* and *APO2* proteins physically interact in rice, and *APO1* is dependent on *APO2* for function (Ikeda-Kawakatsu et al., 2012).

In maize, two *LFY* homologs, *Zea mays FLO/LFY1* (*ZFL1*) and *ZFL2*, appear to promote the transition to inflorescence development, panicle branching and floral identity (Bomblies et al., 2003) in a manner similar to *APO2* in rice (Ikeda-Kawakatsu et al., 2012), but their mechanism of action is less clear. In rice, *APO2* is thought to repress spikelet meristem identity, and *APO2* is downregulated in wild-type branch meristems before transition to spikelet meristem identity, accordingly (Ikeda-Kawakatsu et al., 2012). However in maize, *ZFL1* and *ZFL2* are expressed in wild-type spikelet meristems (Bomblies et al., 2003), which seems inconsistent with a role in repression of spikelet meristem identity. The mechanism by which maize *ZFL* genes control panicle branching is yet to be explored. Two maize homologs of *UFO* have been identified –*ZmFIMa* and *ZmFIMb* (Ikeda et al., 2007), but details of expression pattern or function have not yet been reported for these genes.

#### 1.4.6 A divergent role for grass homologs of *API*/*FUL*

Rice members of the same *SQUA* clade of MADS-box genes as *Arabidopsis* *API* and *FUL*, include *OsMADS14*, *OsMADS15*, and *OsMADS18*, which fall into two subclades of monocot ‘*FUL*-like’ genes (Moon et al., 1999; Litt and Irish, 2003; Malcomber et al., 2006). Expression of these three genes in rice floral meristems has led to suggestions that these genes may function during early stages of floral development, similar to *API* in *Arabidopsis* (Kyoizuka et al., 2000; Pelucchi et al., 2002). *OsMADS14* is expressed in floral meristems in wild-type but not in *RFT1* RNAi plants and transcription of *OsMADS15* can be induced in the presence of both *OsFD1* and *Hd3a*, which suggests that these genes may be downstream targets of florigen activation complexes in the SAM (Komiya et al., 2009; Taoka et al., 2011). However, no floral defects have been reported for these genes as a result of compromised gene function or overexpression, so the putative roles of *OsMADS14*, *OsMADS15* and *OsMADS18* in floral development require further confirmation. Nonetheless, there is evidence that these genes do have a role in inflorescence development. Individual RNAi knockdown of *OsMADS14*, *OsMADS15* or *OsMADS18* has no apparent influence on flowering, but knockdown of all three results in a delay in flowering time, indicating that these three genes act redundantly to promote the inflorescence transition in rice (Kobayashi et al., 2012). In support of this conclusion, overexpression of any of these three genes reduces flowering time in transgenic rice plants (Jeon et al., 2000a; Fornara et al., 2004; Lu et al., 2012). In triple RNAi lines, additional mutation to the *SEP*-like MADS-box gene *PANICLE PHYTOMER2* (*PAP2*) can prevent the transition from vegetative SAM to inflorescence meristem (Kobayashi et al., 2012). This suggests that *OsMADS14*, *OsMADS15* and *OsMADS18* act together with *PAP2* to promote the inflorescence transition in rice (Kobayashi et al., 2012). Curiously, expression of *Hd3a* and *RFT1* is reduced in quadruple knockdown lines, leading to the suggestion that *OsMADS14*, *OsMADS15*, *OsMADS18* and *PAP2* may have a role in amplifying florigenic signals through positive feedback loops with *Hd3a* and *RFT1* in leaves (Kobayashi et al., 2012).

Of the four *API/FUL*-like genes present in maize (Malcomber et al., 2006), *ZMM4* appears the most interesting in terms of inflorescence development. *ZMM4* is strongly up-regulated in the shoot apex close to the time of the floral transition, and induction is delayed in the *dlf1* mutant, suggesting that *ZMM4* may be an early target of a maize florigen activation complex (Danilevskaya et al., 2008b). Over-expression of *ZMM4* results in early flowering, suggesting a role in promoting IM identity, and later expression in branch, spikelet pair and spikelet meristems suggests that *ZMM4* may have additional roles during inflorescence development that remain to be determined (Danilevskaya et al., 2008b).

Overall, these findings indicate that *API/FUL*-like genes in rice and maize may have a role beyond floral meristem specification, which is divergent from that of *Arabidopsis API*. It is particularly interesting that homologs of MADS-box genes that are confined to roles in floral development in *Arabidopsis* (e.g. *SEP* genes, *API*) appear to be important during early stages of inflorescence development in the grasses. As MADS-box genes are known to act in hetero-dimers or higher order complexes (see Kaufmann et al., 2005), it seems likely that patterns of overlapping expression between specific MADS-box genes may be important for specification of meristem identity in grasses, but this will become clearer as these genes are investigated further.

#### ***1.4.7 A conserved role in inflorescence branching for an ALOG gene***

Similar to *TMF* in tomato, a homologous gene in rice, *TAWAWAI* (*TAWI*), appears to have an important role in promoting inflorescence meristem indeterminacy and panicle branching (MacAlister et al., 2012; Yoshida et al., 2013). Loss of *TAWI* function results in decreased panicle branching, with early abortion of the inflorescence meristem and precocious termination of branch meristems with spikelets, relative to wild-type plants (Yoshida et al., 2013). In gain-of-function alleles, the termination of inflorescence meristem activity and of branch meristems in spikelets is delayed, leading to increased panicle branching (Yoshida et al., 2013). Expression of *SVP*-like genes *OsMADS22*, *OsMADS47*, and *OsMADS55*, were found to be significantly upregulated in these plants, and *TAWI* was confirmed as an upstream regulator of these three genes using a two-component induction system (Yoshida et al., 2013). These findings have led to the theory that *TAWI* promotes

panicle branching through upregulation of *SVP*-like genes (Yoshida et al., 2013), but this is a confusing result in light of other results that indicate *OsMADS22* and *OsMADS55* decrease panicle branching (Lee et al., 2008b; Liu et al., 2013). Overall, similarity in the roles of *TMF* and *TAWI* suggest that homologous ALOG family genes in other angiosperm species may share important roles in inflorescence branching.

#### **1.4.8 Summary**

In summary, a core set of genes that control inflorescence and floral meristem identity in *Arabidopsis*, also appear to be important for meristem identity during inflorescence development in the taxonomically distant grass species rice and maize. Although the precise nature of gene function has diverged between lineages and the exact details are still being investigated, it seems likely that this group of genes could be important for inflorescence development across angiosperm species.

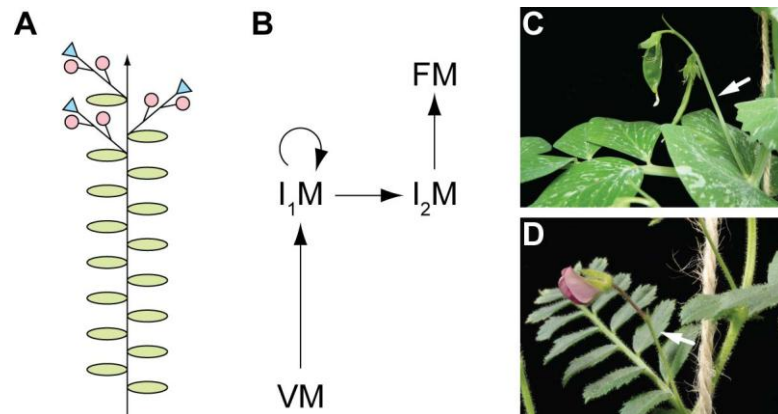
### **1.5 Pea**

#### **1.5.1 A model legume species**

Legumes are the third largest family of flowering plants (approx. 18,000 species), behind only orchids and daisies (Polhill and Raven, 1981). In terms of agricultural importance, legumes are second only to the grasses (FAO, 2013). The majority of cultivated legumes fall into two groups within the Papilionoideae: the phaseoloid legumes (including *Glycine* and *Phaseolus*) and the galegoid legumes (including *Cicer*, *Lens* and *Pisum*; Figure 1.2). Pea is a crop plant in itself, and is also representative of other agronomically significant legume species, which share a similar inflorescence form, including lentil, chickpea, common bean and soybean (Figure 1.6). Compared to other legumes, pea offers useful attributes for a model species that include ease of self- or cross-pollination, short generation time, ease of grafting, the availability of diverse lines and a long history of use in studies of the genetic control of flowering. Mendel first used pea as a model in experiments investigating the fundamental laws of genetics, which included a brief examination of the inheritance of flowering time (Mendel, 1866). In the last four decades, a number of mutants that exhibit altered inflorescence architecture have been isolated in pea, and many of these have subsequently been characterised at a molecular level



(Table 1.4). So far, the genes involved in pea inflorescence development appear to be largely conserved with *Arabidopsis*, rather than involving novel genes, but pea homologs often exhibit increased copy number and/or minor functional divergence (Table 1.4). Here, a brief overview will be given to introduce the genes, gene families and genetic loci thought to have significant roles in pea inflorescence development.



**Figure 1.6.** The compound raceme of pea.

(A) Diagram of the inflorescence form of pea, which is shared by other model legume species including chickpea, lentil and common bean, and (B) schematic of meristem transitions involved in its development. After floral induction the shoot apical meristem converts from a vegetative meristem (VM) to a primary inflorescence meristem ( $I_1M$ ), which remains indeterminate and generates multiple secondary inflorescence meristems ( $I_2M$ ) and eventually senesces. Each  $I_2M$  yields one or more floral meristems (FM). In (A) arrows indicate indeterminate growth, circles are flowers, triangles are stubs and ovals are leaves. In (B) straight arrows indicate meristem transitions and products, and circular arrows indicate meristem indeterminacy.

(C-D) Photos of an axillary secondary inflorescence (arrow) and subtending leaf at a node on the main stem of (C) pea and (D) chickpea.

**Table 1.4.** Summary of genes with known or putative roles in meristem identity during inflorescence development in pea. Abbreviations for meristems are as follows: vegetative SAM (VM); vegetative axillary meristem (AM), primary inflorescence meristem (I<sub>1</sub>M), secondary inflorescence meristem (I<sub>2</sub>M), and floral meristem (FM). Question marks indicate uncertainty in the literature.

Gene family	Gene	<i>Arabidopsis</i> homolog(s)	Expression domain	Mutant name	Mutant phenotype	Inferred function	References
CETS	<i>FTa1</i>	<i>FT/TSF</i>	Leaves Apex	<i>gigas</i>	Non-flowering LD, late-flowering SD	Graft transmissible stimulus. Promotes I <sub>1</sub> M identity (SD)? Specification of I <sub>2</sub> M and FM (LD)?	Murfet (1992); Taylor and Murfet (1994); Beveridge and Murfet (1996); Hecht et al. (2011)
	<i>FTb2</i>	<i>FT/TSF</i>	Leaves (LD)	-	-	Promotes I <sub>1</sub> M identity?	Hecht et al. (2011)
	<i>FTc</i>	<i>FT/TSF</i>	Apex	-	-	Promotes I <sub>2</sub> M identity?	Hecht et al. (2011)
	<i>DETERMINATE</i> ( <i>DET</i> ; <i>TFL1a</i> )	<i>TFL1</i>	I <sub>1</sub> M	<i>det</i>	I <sub>1</sub> M → I <sub>2</sub> M	Maintains I <sub>1</sub> M identity	Singer et al. (1990); Foucher et al. (2003)
	<i>LATE FLOWERING</i> ( <i>LF</i> ; <i>TFL1c</i> )	<i>TFL1</i>	AM	<i>lf</i>	Early flowering	Represses flowering	Murfet (1975); Foucher et al. (2003)
MADS-box	<i>VEGETATIVE1</i> ( <i>VEG1</i> ; <i>FULc</i> )	<i>AGAMOUS-LIKE79</i> ( <i>AGL79</i> )	I <sub>2</sub> M	<i>veg1</i>	Non-flowering under all conditions	Specification of I <sub>2</sub> M identity	Reid and Murfet (1984); Berbel et al. (2012)
	<i>PROLIFERATING INFLORESCENCE MERISTEM</i> ( <i>PIM</i> ; prev. <i>PEAM4</i> )	<i>AP1</i>	FM	<i>pim</i>	Floral meristem identity defects	Specification of floral meristems	Berbel et al. (2001); Taylor et al. (2002)
<i>LFY</i>	<i>UNIFOLIATA</i> ( <i>UNI</i> )	<i>LFY</i>	Leaves, FM, IM, I <sub>2</sub> M	<i>uni</i>	Reduced leaf complexity, floral defects	Compound leaf development, specification of FM identity	Hofer et al. (1997); Gourlay et al. (2000)
F-box	<i>STAMINA PISTILLOIDA</i> ( <i>STP</i> )	<i>UFO</i>	VM, FM, leaf primordia	<i>stp</i>	Reduced leaf complexity, floral defects, occasional IM → FM	Compound leaf development, specification of FM identity	Taylor et al. (2001)
-	-	-	-	<i>vegetative2-1</i> ( <i>veg2-1</i> )	Non-flowering under all conditions	Promotes I <sub>1</sub> M identity? Specification of I <sub>2</sub> M and FM identity?	Murfet (1992); Murfet and Reid (1993); Reid et al. (1996); Sussmilch (2008)
				<i>veg2-2</i> (prev. <i>veg2<sup>inc</sup></i> )	Late-flowering with I <sub>2</sub> and floral defects		
-	-	-	-	<i>late bloomer5</i> ( <i>late5</i> )	Late-flowering with I <sub>2</sub> and floral defects	Promotes I <sub>1</sub> M identity? Specification of I <sub>2</sub> M and FM identity?	Weller (2007); Sussmilch (2008)

### 1.5.2 *Pea inflorescence architecture*

The pea inflorescence is in the form of a compound raceme, which has one extra level of branching relative to the simple raceme of *Arabidopsis* (Figures 1.3 and 1.6A). Accordingly, pea inflorescence development involves specification of one extra meristem type, the secondary inflorescence ( $I_2$ ) meristem (Figure 1.6B). When a pea seedling commits to flowering, the vegetative SAM undergoes a transition to a primary inflorescence ( $I_1$ ) meristem, which will be referred to in this study as the V/ $I_1$  transition. The  $I_1$  meristem remains indeterminate and produces the shoot tissues of the main stem, including the stem and leaves, similar to the vegetative SAM, except that the  $I_1$  bears axillary  $I_2$  structures (Figure 1.6A-B). Each  $I_2$  is leafless and terminates in a stub after bearing several axillary flowers (Figure 1.6).

### 1.5.3 *The FT family in pea*

In pea, the *FT* family comprises three subclades, containing a total of five members: *FTa1*, *FTa2*, *FTb1*, *FTb2* and *FTc* (Hecht et al., 2011). Three mutant alleles (*gigas-1*, *gigas-2* and *gigas-3*) have been described for *FTa1* (Hecht et al., 2011), but mutants have not yet been reported for any of the other four *FT* genes. The most severe *gigas* mutant, *gigas-2* (previously *fsd* or *gi<sup>fsd</sup>*), is non-flowering under LD photoperiods, but merely late-flowering, with normal  $I_2$  and floral morphology under SD conditions (Murfet, 1992; Taylor and Murfet, 1994; Reid et al., 1996). Early grafting studies revealed that *gigas* scions can flower normally under LD conditions when grafted to a wild-type graft stock bearing leaves, leading to the conclusion that *FTa1/GIGAS* encodes a floral stimulus or precursor (Taylor and Murfet, 1994; Beveridge and Murfet, 1996). Later, *FTa1* was found to be expressed in both the leaf and the apex, under both LD and SD conditions, with expression induced in conjunction with or immediately prior to expression of inflorescence and floral identity genes (Hecht et al., 2011). These results indicate that *FTa1* may function in the timing of the floral transition, and the subsequent specification of  $I_2$  and floral meristems under LD conditions only.

The remaining four *FT* genes exhibit distinct patterns of expression in terms of tissue specificity, timing and regulation by photoperiod (Hecht et al., 2011). *FTb2* is expressed specifically in the leaf under favourable LD conditions within the time-frame that the plant becomes physiologically committed to flowering, indicating that

*FTb2* might have a role in the production of a florigen signal in response to LD photoperiods (Hecht et al., 2011). *FTc* is expressed only in the apex and is induced immediately prior to apical induction of inflorescence and floral meristem identity genes (Hecht et al., 2011). Expression of *FTc* is delayed in the *gigas* mutant, suggesting that *FTc* may act as an integrator of long-range signals transmitted by other *FT* genes (Hecht et al., 2011). The roles of *FTa2* and *FTb1* are not currently clear without knowledge of their mutant phenotypes. The five *FT* genes differ in their activity when over-expressed in the *Arabidopsis ft-1* mutant under LD; *FTc* results in an extreme early-flowering phenotype, *35S::FTa1* plants flower earlier than wild-type, and *35S::FTa2*, *35S::FTb1*, and *35S::FTb2* plants flower earlier than *ft-1* but similar to or later than wild-type (Hecht et al., 2011). Overall, these findings indicate that *FT* genes have retained a key role in promoting flowering in pea, but some divergence in function has occurred within the *FT* family.

#### 1.5.4 Subfunctionalisation of *TFL1* homologs in pea

Three homologs of *TFL1* have been identified in pea: *DETERMINATE* (*DET*; *TFL1a*), *TFL1b* and *LATE FLOWERING* (*LF*; *TFL1c*; Foucher et al., 2003). Mutants have been described for *DET* and *LF* and the functions of these genes have been characterised in detail, but no putative role has yet been reported for *TFL1b*. *DET* expression is restricted to the I<sub>1</sub> meristem, but *DET* itself is not critical for the V/I<sub>1</sub> transition to occur (Singer et al., 1990; Singer et al., 1999; Berbel et al., 2012). The *det* mutant flowers at the same time as wild-type, but after a few reproductive nodes, the SAM exhibits ectopic expression of the I<sub>2</sub> meristem identity gene *VEGETATIVE1* (*VEG1*) and terminates in an ectopic I<sub>2</sub> (Singer et al., 1990; Berbel et al., 2012). This phenotype indicates that *DET* maintains I<sub>1</sub> indeterminacy by excluding *VEG1* from the SAM (Berbel et al., 2012). Additional mutation to *DET* on a non-flowering *veg1* mutant background allows expression of the floral meristem identity gene *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*), suggesting that *DET* also acts to repress *PIM* from the SAM (Berbel et al., 2012). The *lf* mutant flowers earlier than wild-type but exhibits normal I<sub>1</sub> morphology, indicating that *LF* acts to delay flowering time in wild-type pea (Murfet, 1975). In this manner, the two roles of *Arabidopsis TFL1* (delaying flowering time and maintaining SAM indeterminacy)

are carried out by two different pea homologs, and pea thus provides a system where these separate roles can be studied in isolation.

### ***1.5.5 VEG1 - a gene with a novel role in I<sub>2</sub> specification***

The *veg1* mutant is non-flowering under all known conditions (Gottschalk, 1979; Reid and Murfet, 1984). Instead of I<sub>2</sub> structures, branches that exhibit expression of *DET* are borne at aerial nodes (Berbel et al., 2012). These observations led to the conclusion that *VEG1* has a critical role in I<sub>2</sub> meristem specification and the development of a model in which the expression domains of *VEG1* and *DET* are restricted by mutually repressive interactions between these two genes (Berbel et al., 2012). This antagonism allows floral development on lateral structures and indeterminate stem growth, thus having a significant influence on pea inflorescence architecture. *VEG1* has been identified as *PsFULc*, a pea MADS-box gene from the *AGAMOUS-LIKE79* (*AGL79*) clade that is deleted in the *veg1-1* mutant (Berbel et al., 2012). In *Arabidopsis*, *AGL79* is expressed predominantly in the roots and does not appear to be important for inflorescence architecture (Parenicova et al., 2003), indicating that the pea ortholog, *VEG1*, has acquired a novel role for compound inflorescence development (Berbel et al., 2012).

### ***1.5.6 The uncharacterised VEGETATIVE2 (VEG2) locus***

Knowledge of the *VEG2* locus is based on two recessive fast neutron mutant alleles: *veg2-1* and *veg2-2* (Murfet, 1992; Murfet and Reid, 1993; Reid et al., 1996). At the beginning of this study, this locus had not been described in a primary research paper and knowledge of the *veg2* mutants was limited to preliminary observations. Plants carrying the more severe *veg2-1* mutation fail to produce any I<sub>2</sub> or floral structures under any conditions (Murfet and Reid, 1993). Instead, lateral branches grow out to yield a non-flowering, aerial branching phenotype that is remarkably similar to that of non-flowering *veg1* and *gigas-2* mutants (Murfet, 1992). The *veg2-2* mutant flowers late, with I<sub>2</sub> and floral defects (Murfet, 1992). These mutant phenotypes indicate that *VEG2* may have roles in flowering time and specification of I<sub>2</sub> and floral meristems. Prior to commencement of this study, the *VEG2* locus had not been fully characterised at a molecular level, but a pea *FD*-like gene was considered a likely candidate (Weller, 2007; Sussmilch, 2008).

### 1.5.7 The uncharacterised *LATE BLOOMER 5 (LATE5)* locus

The *LATE5* locus is known from a single recessive late-flowering mutant with  $I_2$  and floral defects that was identified in EMS mutant screens at the University of Tasmania (Weller, 2007; Sussmilch, 2008). This phenotype indicates that *LATE5* may have a role similar to *VEG2*, in flowering time and specification of  $I_2$  and floral meristems. Preliminary mapping results indicated that *LATE5* was located towards the base of pea linkage group I, but no candidate genes for this locus were apparent (Sussmilch, 2008).

### 1.5.8 Pea homologs of *LFY* and *UFO*

Mutation to *UNIFOLIATA (UNI)*, the pea ortholog of *LFY*, causes severe floral defects, with *uni* flowers lacking petals and stamens and instead comprising a proliferation of sepalloid and carpeloid organs, and containing ectopic flowers of a similar nature (Hofer et al., 1997). This indicates that *UNI* has a critical role in floral meristem specification in pea, comparable to that of *LFY* in *Arabidopsis*. The *uni* mutant exhibits additional defects in leaf morphology, with decreased complexity of the pea compound leaf, and *UNI* is thought to fulfil pleiotropic functions in floral meristem identity and leaf development by regulating indeterminacy during morphogenesis of these organs (Hofer et al., 1997).

The pea ortholog of *UFO* has also been identified in pea. *stamina pistilloida (stp)* mutant plants have floral defects similar to the *uni* mutant, but sometimes exhibit additional termination of the SAM in an aberrant flower (Ferrandiz et al., 1999; Taylor et al., 2001). *uni* is epistatic to *stp*, suggesting the functional dependence of *STP* on *UNI*, and synergistic effects of weak *stp* and *uni* alleles in double mutant plants indicates that *STP* and *UNI* may act together in pea, similar to other systems (Taylor et al., 2001).

### ***1.5.9 PIM - a conserved role in floral meristem specification***

Expression of the pea *API* homolog, *PIM*, is limited to floral meristems, indicating a conserved role as a floral meristem identity gene (Berbel et al., 2001). Accordingly, *pim* mutant plants fail to correctly specify floral meristems, instead exhibiting replacement of single flowers with structures ranging from leafy branches to triads of abnormal flowers with bract-like sepals and petals and stamens that are absent or mosaic (Singer et al., 1999; Taylor et al., 2002). Mutation to *PIM* specifically affects floral meristems, and *pim* mutant plants exhibit normal flowering time, and morphology of I<sub>1</sub> and I<sub>2</sub> structures (Singer et al., 1999; Taylor et al., 2002). Overexpression of *PIM* in *Arabidopsis* causes early flowering and conversions of inflorescence to floral identity (Berbel et al., 2001).

## **1.6 Aims for this study**

At the onset of this study, a number of genes with important roles in pea inflorescence development had been identified, but little was known about the pathways controlling transitions in meristem identity. Mutants were available for two loci *VEG2* and *LATE5*, which showed clear defects in multiple stages of inflorescence development, suggesting that these loci may have important regulatory roles during this process. However, these mutants had not been characterised in any detail, and the corresponding genes had not yet been identified. In addition, the *StMADS11* subfamily genes, which have important roles in inflorescence development in other species, had not been characterised in pea. The aim for this study was to improve knowledge of the genetic system controlling development of the compound raceme in pea, focussing on the roles, functions and nature of the *VEG2* and *LATE5* loci, and including preliminary investigation of *SVP/AGL24* homologs in pea. Towards this aim, Chapters 3, 4 and 5 characterise the roles, molecular identity, and mechanisms of action of *VEG2*, respectively, Chapter 6 investigates the *LATE5* locus and Chapter 7 describes isolation and preliminary investigation of novel *SVP*-like genes in pea.





## CHAPTER 2: General Materials and Methods

This chapter describes the general materials and methods used for all research presented in this thesis. For detailed materials and methods for specific experiments see also the materials and methods sections of individual chapters.

### 2.1 Plant materials and growth conditions

Details of progenitor *Pisum sativum* lines and pea flowering mutants used for research presented in this thesis are given in Tables 2.1 and 2.2, respectively. For ease of growth, mutant lines were created or introgressed (with multiple backcrosses) into line NGB5839, a gibberellin deficient dwarf mutant (*le-3*) of cultivar Torsdag (Lester et al., 1999). Seed for single mutants partially or fully introgressed into NGB5839 background and populations segregating single, double and triple mutants were provided by J. Weller and J. Vander Schoor. For sowing, all seeds were coated in thiram (fungicidal powder) and sown in 14cm slim-line pots containing a 1:1 mixture of dolerite chips and vermiculite, topped with 3cm of native nursery grade potting mix with controlled release fertilizer (CRF) added (Horticultural and Landscape Supplies, Brighton, TAS, Australia). Plants were watered regularly and supplied with nutrient solution on a weekly basis. All plants described in this thesis were grown in controlled-environment growth cabinets or phytotrons at the University of Tasmania. Growth cabinets were used for all experiments in which highly accurate temperature or photoperiod controls were required. Growth cabinets were maintained at a temperature of 20°C and white light provided by cool-white fluorescent tubes (L40 W/20S cool white; Osram Germany) at an irradiance of 120-140  $\mu\text{mol m}^{-2} \text{s}^{-1}$  unless otherwise specified. Temperature in the phytotron was maintained at approximately 24°C during the day and 16°C at night and plants were exposed to a base photoperiod of 8 hours of natural light in conjunction with darkness or extended lighting to create different photoperiod conditions.

**Table 2.1.** Details of pea progenitor lines used in this study as wild-type lines and/or parental lines for mapping crosses.

Line	Purpose/Description	Reference(s)
Jl1794	Parental line for distant mapping crosses with mutants in NGB5839 background. Accession of the northern race of <i>P. sativum</i> var. <i>humile</i> .	Weller et al. (2012)
Jl399 (Cennia)	Parental line for intermediate mapping crosses with mutants in NGB5839 background. Early-flowering ( <i>sn</i> mutant) line.	Ellis et al. (1992)
Kaliski (KAL;Wt4042)	Wild-type line originally used in mutagenesis programmes.	Swiecicki (1987)
NGB5839	Wild-type line originally used in mutagenesis programmes. Gibberellin deficient ( <i>le-3</i> ) dwarf of cv. Torsdag.	Lester et al. (1999); Weller et al. (2003)
Solara (SOL)	Parental line for mapping crosses with mutants in Kaliski background.	Samec et al. (1998)
T�r�se (TER)	Parental line for narrow mapping crosses with mutants in NGB5839 background.	Laucou et al. (1998)

**Table 2.2.** Details of pea flowering mutants grown in this study.

Line	Details	Wild-type line	Reference(s)
<i>det-2</i> (J11358)	Spontaneous mutant crossed to NGB5839 (3 backcrosses).	NGB5839	Marx (1986); Singer et al. (1990); Foucher et al. (2003)
<i>gigas-2</i>	Created by fast-neutron mutagenesis of cultivar Porta and crossed to NGB5839 (5 backcrosses).	NGB5839	Murfet (1992); Taylor and Murfet (1994); Reid et al. (1996); Hecht et al. (2011)
<i>late5</i>	Created by EMS mutagenesis of NGB5839.	NGB5839	Weller (2007)
<i>lf-22</i>	Created by EMS mutagenesis of NGB5839.	NGB5839	Weller (2007)
<i>pim-2</i>	Spontaneous mutation in cv. Torsdag crossed to NGB5839.	NGB5839	Taylor et al. (2002)
<i>veg1</i>	Created by X-irradiation of cv. 'Dippes gelbe Viktoria' and partially introgressed into NGB5839 background.	Wild-type siblings	Gottschalk (1979); Reid and Murfet (1984); Berbel et al.(2012)
<i>veg2-1</i>	Isolated from fast-neutron mutagenesis of cv. Kaliski. Plants in Kaliski background and plants partially introgressed into NGB5839 background both grown in this study.	Kaliski/ wild-type siblings	Murfet and Reid (1993)
<i>veg2-2</i>	Isolated from fast-neutron with N-nitroso-N-ethylurea (NEU) mutagenesis of cv. Kaliski and crossed to NGB5839 (2 backcrosses).	NGB5839	Murfet and Reid (1993); Reid et al. (1996)

## 2.2 Plant measurements

Details of traits measured on plants grown in this study are shown in Table 2.3. All traits were measured on the main stem, unless otherwise stated. All lengths were measured to the nearest millimetre. Data from any plants which exhibited stunted or abnormal growth were excluded. For all traits involving numbering of nodes, the lowest scale leaf was counted as node 1. For measurement of internodes, internodes were numbered with internode 1 between the first and second scale leaf. Where reproductive nodes were numbered, the node of floral initiation (NFI) was counted as reproductive node 1. For branching measurements, aerial nodes were defined as those from node 8 and above, based on preliminary observation of pattern of branch outgrowth (see Chapter 3, Section 3.3.2). A 5mm minimum length was chosen for measurements of timing of axillary bud outgrowth in order to focus on axillary buds that were being released from dormancy, as the majority of nodes bear dormant axillary buds 1-2mm in length (personal observation; see Chapter 3, Section 3.3.2).

**Table 2.3.** Details of plant traits measured during this study.

Trait	Details
Days to first open flower (DTF)	Number of days between sowing and first fully developed/open flower with standard and wing petals fully unfurled.
Internode length	Length between the base of a node and the base of the next higher node (mm)
Length of main lateral	Length from axil junction to tip of apex for the main lateral structure at a node, not including determinate $I_2$ structures (mm).
No. nodes of main lateral	Number of nodes bearing a fully expanded leaf on each lateral branch.
Node of floral initiation (NFI)	Node of first $I_2$ on main stem, regardless of whether a fully developed flower is borne on this $I_2$ .
Reproductive nodes	Number of nodes on the main stem bearing an axillary $I_2$ .
Secondary inflorescence ( $I_2$ ) length	Length of $I_2$ from axil junction to stub tip (mm).
Stem length	Length of stem from node 1 to top of apex (mm).
Timing of first 'enlarged aerial bud' development	(a) The number of days between sowing and development of an axillary bud 5mm or longer in length, at node 8 or above; or (b) The first node on the plant stem (spatially and temporally) to develop an axillary bud 5mm or longer in length, at node 8 or above.
Timing of first 'aerial branch' development	(a) The number of days between sowing and development of a branch with one expanded leaf at node 8 or above; or (b) The first node (spatially and temporally) to develop a branch with at least one expanded leaf, at node 8 or above.
Total lateral length	Length of all laterals greater than 5mm in length including any extra primary laterals (arising directly from the main stem; additional to the main lateral per node), laterals on higher order branch levels on compound branches, and the branch/primary inflorescence ( $I_1$ ) portion of <i>veg2-2</i> ' $I_2$ s' after reversion to $I_1$ s (see Section 3.3.4).
Total nodes	Total number of nodes with fully expanded leaves on the main stem.

## 2.3 Online sequence resources

Online sequence resources shown in Table 2.4 were used in this study for identification, analysis of gene homologs and some primer design. Versions listed here were used unless stated differently in text. Where genes were not annotated, or found to be annotated incorrectly based on expressed sequences or alignments between species, coding sequence and protein sequence was predicted or corrected accordingly for use in phylogenetic analyses. Transcriptome Shotgun Assembly (TSA) sequences for pea became available partway through this study (Franssen et al., 2011; Kaur et al., 2012). Prior to this, the majority of primers for new genes (not previously isolated from pea) were designed directly from *Medicago* sequence (see Appendix 1).

**Table 2.4.** Online resources used for sequence and expression information.

Resource type	Species	Resource	Website	Version	Reference(s)
Genomic sequence	Apple ( <i>Malus domestica</i> )	Phytozome	<a href="http://www.phytozome.net/apple">http://www.phytozome.net/apple</a>	v1.0	Velasco et al. (2010)
	<i>Aquilegia coerulea</i>	Phytozome	<a href="http://www.phytozome.net/aquilegia">http://www.phytozome.net/aquilegia</a>	v1.1	US Department of Energy Joint Genome Institute, unpublished
	<i>Arabidopsis thaliana</i>	The Arabidopsis Information Resource (TAIR)	<a href="http://www.arabidopsis.org">http://www.arabidopsis.org</a>	TAIR10	Lamesch et al. (2012)
	Banana ( <i>Musa acuminata</i> )	The Banana Genome Hub	<a href="http://banana-genome.cirad.fr">http://banana-genome.cirad.fr</a>	-	D'Hont et al. (2012)
	Cassava ( <i>Manihot esculenta</i> )	Phytozome	<a href="http://www.phytozome.net/cassava">http://www.phytozome.net/cassava</a>	v.4.1	Prochnik et al. (2012)
	Common bean ( <i>Phaseolus vulgaris</i> )	Phytozome	<a href="http://www.phytozome.net/commonbean">http://www.phytozome.net/commonbean</a>	v1.0	US Department of Energy Joint Genome Institute, unpublished
	Cotton ( <i>Gossypium raimondii</i> )	Phytozome	<a href="http://www.phytozome.net/cotton">http://www.phytozome.net/cotton</a>	v2.1	US Department of Energy Joint Genome Institute, unpublished
	Cucumber ( <i>Cucumis sativus</i> )	Phytozome	<a href="http://www.phytozome.net/cucumber">http://www.phytozome.net/cucumber</a>	v1	US Department of Energy Joint Genome Institute, unpublished
	Date palm ( <i>Phoenix dactylifera</i> )	Date palm draft sequence	<a href="http://qatar-weill.cornell.edu/research/datepalmGenome/download.html">http://qatar-weill.cornell.edu/research/datepalmGenome/download.html</a>	v3	Al-Dous et al. (2011)
	<i>Eucalyptus grandis</i>	Phytozome	<a href="http://www.phytozome.net/eucalyptus">http://www.phytozome.net/eucalyptus</a>	v1.1	US Department of Energy Joint Genome Institute, unpublished
	Foxtail millet ( <i>Setaria italica</i> )	Phytozome	<a href="http://www.phytozome.net/foxtailmillet">http://www.phytozome.net/foxtailmillet</a>	v2.1	US Department of Energy Joint Genome Institute, unpublished
	<i>Gnetum gnemon</i>	ConGenIE	<a href="http://congenie.org/">http://congenie.org/</a>	-	Nystedt et al. (2013)
	Grape ( <i>Vitis vinifera</i> )	Phytozome	<a href="http://www.phytozome.net/grape">http://www.phytozome.net/grape</a>	12X March 2010	The French–Italian Public Consortium for Grapevine Genome Characterization (2007)
	<i>Lotus japonicus</i>	Kazusa DNA Research Institute	<a href="http://www.kazusa.or.jp/lotus">http://www.kazusa.or.jp/lotus</a>	build 2.5	Sato et al. (2008)
	<i>Medicago truncatula</i>	<i>Medicago truncatula</i> Hapmap Project	<a href="http://medicagohapmap.org">http://medicagohapmap.org</a>	Mt3.5v4	Young et al. (2011)

Resource type	Species	Resource	Website	Version	Reference(s)
Genomic sequence (continued)	Peach ( <i>Prunus persica</i> )	Phytozome	<a href="http://www.phytozome.net/peach">http://www.phytozome.net/peach</a>	v1.0	The International Peach Genome Initiative (2013)
	Poplar ( <i>Populus trichocarpa</i> )	Phytozome	<a href="http://www.phytozome.net/poplar">http://www.phytozome.net/poplar</a>	v3.0	Tuskan et al. (2006)
	Potato ( <i>Solanum tuberosum</i> )	Phytozome	<a href="http://www.phytozome.net/potato">http://www.phytozome.net/potato</a>	v3.4	Potato Genome Sequencing Consortium (2011)
	Rice ( <i>Oryza sativa</i> )	Rice Genome Annotation Project	<a href="http://rice.plantbiology.msu.edu">http://rice.plantbiology.msu.edu</a>	MSU Release 7.0	Kawahara et al. (2013)
	Soybean ( <i>Glycine max</i> )	Phytozome	<a href="http://www.phytozome.net/soybean">http://www.phytozome.net/soybean</a>	v1.1	Schmutz et al. (2010)
	Strawberry ( <i>Fragaria vesca</i> )	Phytozome	<a href="http://www.phytozome.net/strawberry">http://www.phytozome.net/strawberry</a>	v1.1	Shulaev et al. (2011)
	Tomato ( <i>Solanum lycopersicum</i> )	Phytozome	<a href="http://www.phytozome.net/tomato">www.phytozome.net/tomato</a>	ITAG2.3	Tomato Genome Consortium (2012)
	Various	GenBank	<a href="http://www.ncbi.nlm.nih.gov">http://www.ncbi.nlm.nih.gov</a>	-	-
Expressed sequences	Pea ( <i>Pisum sativum</i> )	GenBank TSA Sequence Database	<a href="http://www.ncbi.nlm.nih.gov">http://www.ncbi.nlm.nih.gov</a>	-	Franssen et al. (2011); Kaur et al. (2012)
	Various	Dana Farber Cancer Institute Gene Indices (DFCI TGI)	<a href="http://compbio.dfci.harvard.edu/tgi/plant.html">http://compbio.dfci.harvard.edu/tgi/plant.html</a>	-	Quackenbush et al. (2001)
	Various	GenBank Database of Expressed Sequence Tags (dbEST)	<a href="http://www.ncbi.nlm.nih.gov/nucest">http://www.ncbi.nlm.nih.gov/nucest</a>	-	Boguski et al. (1993)
Gene expression	<i>Lotus japonicus</i>	<i>Lotus japonicus</i> Gene Expression Atlas	<a href="http://ljgea.noble.org/v2">http://ljgea.noble.org/v2</a>	v2	Verdier et al. (2013)
	<i>Medicago truncatula</i>	<i>Medicago truncatula</i> Gene Expression Atlas	<a href="http://mtgea.noble.org/v3">http://mtgea.noble.org/v3</a>	v3	Benedito et al. (2008); He et al. (2009)
	Soybean ( <i>Glycine max</i> )	RNA-Seq Atlas of Glycine max	<a href="http://soybase.org/soyseq">http://soybase.org/soyseq</a>	-	Severin et al. (2010)

## 2.4 Primer design

Primers were designed from pea sequence where available, or regions of *Medicago* sequence found through nucleotide alignments to be well conserved with other legumes (see Table 2.4 for details of online legume sequence resources) where pea sequence was not available. Primers were designed using the web-based application Primer3 (<http://primer3.wi.mit.edu/>; Rozen and Skaletsky, 2000; Koressaar and Remm, 2007; Untergasser et al., 2012), optimised for primer length (18-25bp), product length, G/C content, annealing temperature, minimal self- and cross-compatibility and presence of a GC clamp at 3' end. Details for all primers used for research presented in this thesis are given in Appendix 1.

## 2.5 DNA and RNA extractions and processing

### 2.5.1 Standard genomic DNA (gDNA) extraction

For extraction of gDNA for mapping populations or genotyping of presence/absence PCR-based markers, tissue samples were collected in liquid nitrogen and stored at -70°C until processing. Frozen tissue samples were ground using either mortar and pestle, or carbide beads and a mechanical homogeniser (Retsch MM30 or Qiagen TissueLyserII), depending on sample size. Samples were stabilised with 500 µL of 2x Extraction Buffer (100mM Tris-HCl, 1.4M NaCl, 20mM EDTA, 2% w/v CTAB, 20mM 2-β-mercaptoethanol, pH 8 with HCl) and incubated for 10-15 minutes at 60°C with gentle agitation. Solvent extraction was performed twice using chloroform-isoamyl alcohol (24:1) solution. DNA was precipitated with 1mL of Precipitation Buffer (50mM Tris-HCl, 10mM EDTA, 1% w/v CTAB, pH 8 with HCl), pelleted by centrifugation for 10 minutes at 10,000g and resuspended in 300µL 1.5M NaCl containing 1µL RNase A (25mg/mL) and incubated for 10-15 minutes at 50°C. DNA was precipitated in chilled 95% ethanol, pelleted by centrifugation at 20,000g for 15 minutes, washed in 70% ethanol, air dried and dissolved in autoclaved Milli-Q water (Milli-Q Plus, Merck Millipore, Billerica, MA, USA). Dilutions of 50ng/µL gDNA were used for PCR and HRM analysis.

### **2.5.2 Fast gDNA extraction for simple genotyping**

For fast gDNA extraction for simple genotyping with PCR-based size or CAPS markers, QuickExtract<sup>™</sup> Plant DNA Extraction Solution (Epicentre) was used according to manufacturer's instructions on a small circle of leaf tissue (0.5mm radius).

### **2.5.3 RNA extraction and cDNA synthesis**

Frozen tissue samples were ground using either mortar and pestle or carbide beads and mechanical homogeniser (Retsch MM30 or Qiagen TissueLyserII), depending on sample size. Total RNA was extracted using the Promega SV Total RNA Isolation System (Promega, Madison, WI) in accordance with the manufacturer's instructions. First strand cDNA was synthesised from 1µg RNA using the ImProm-II<sup>™</sup> Reverse Transcription System (Promega, Madison, WI, USA), Tetro Reverse Transcriptase (Bioline, London, UK), or MMLV High Performance Reverse Transcriptase (Epicentre, Madison, WI, USA), each in a total volume of 20µL, in accordance with the manufacturers' instructions. To check for contamination, a negative control without reverse transcriptase (RT-) was included for each sample. The cDNA product was diluted one in five, and used for PCR or qRT-PCR.

## **2.6 PCR**

### **2.6.1 Standard PCR**

Standard PCR was performed in a 50µL volume, comprising 5µL of template DNA, 10µL of 5x reaction buffer, 1µL of dNTPs (10mM), 1µL of forward primer (10µM), 1µL of reverse primer (10µM), 1.5µL MgCl<sub>2</sub> (50mM), and 0.2µL of MangoTaq<sup>™</sup> DNA polymerase (Bioline, Alexandria, NSW, Australia), with autoclaved Milli-Q water to final volume. Reactions were conducted in a thermal cycler with heated lid as follows: 94°C for 5 minutes, 35-40x [94°C for 45 seconds, annealing temperature for 45 seconds, 72°C for 1 minute per kb according to expected product size], 72°C for 5 minutes.



### 2.6.2 Low stringency PCR

Low stringency PCR was performed when using primers with expected mismatches (e.g. primers designed from *Medicago* or dCAPS primers) with reaction set up and conditions as for standard PCR except optimised to decrease stringency by decreasing annealing temperature (generally to 50-55°C) and increasing concentration of MgCl<sub>2</sub>.

### 2.6.3 High Fidelity PCR

High fidelity PCR was performed using the same reaction mix as for standard PCR except using 0.2µL of Phusion<sup>®</sup> DNA polymerase (Finnzymes, Espoo, Finland), with 5x Phusion HF Buffer and without addition of MgCl<sub>2</sub>, in accordance with the manufacturer's instructions. Reactions were conducted in a thermal cycler with heated lid as follows: 98°C for 30 seconds, 25-35x [98°C for 10 seconds, primer T<sub>m</sub> + 3°C for 30 seconds, 72°C for 30 seconds per kb of PCR product], 72°C for 5 minutes.

### 2.6.4 Colony PCR

For colony PCR, bacterial colonies were picked and suspended in 5µL of autoclaved Milli-Q water. Bacteria were lysed by incubation at 95°C for 5 minutes. 20µL of reaction mix containing 0.5µL of forward primer (10µM), 0.5µL of reverse primer (10µM), 0.5µL of dNTPs (10mM), 5µL 5x reaction buffer, 0.8µL MgCl<sub>2</sub> and 0.1µL MangoTaq<sup>™</sup> DNA polymerase (Bioline, Alexandria, NSW, Australia), made up to 20µL with autoclaved Milli-Q water, was added to each tube. PCR conditions were as follows: 94°C for 5 minutes, 30x [94°C for 1 minute, annealing temperature for 1 minute, 72°C for 1 minute per kb of PCR product], 72°C for 5 minutes.

### 2.6.5 Quantitative reverse transcription PCR (qRT-PCR)

For analysis of relative gene expression, qRT-PCR was conducted using a Rotor-Gene 3000 Real-time Thermal Cycler with Rotor-Gene 6 Version 6.1 (Corbett Research, Australia). A Corbett Robotics CAS-1200<sup>™</sup> pipetting robot (Corbett Research, Australia) with CAS Robotics 4 Version 4.9.8 (1.6.61) software was used to prepare reactions. Each 10µL reaction comprised 2µL cDNA template, 5µL 2x Quantace SensiMixPlus SYBR reagent (Alexandria, NSW, Australia), 0.3µL each of forward and reverse primer (10µM) and 2.4µL autoclaved Milli-Q water. A no-

template control (containing water instead of cDNA) was included for each run to check for contamination, and each sample was run in duplicate for increased accuracy. For each cDNA sample, *ACTIN* was run on the reverse transcriptase negative control (RT-) to check for contamination. Reactions were run for 50 cycles.

A standard curve for the target gene was included in every run. Standard curves were generated from a 10-fold dilution series from  $1 \times 10^{-2}$  to  $10^{-6}$  ng/ $\mu$ L. Standard curve regression was considered acceptable if the  $R^2$  value was equal to or higher than 0.99. *ACTIN* was chosen as the reference gene for evaluating transcript levels of flowering genes as previously described (Foo et al., 2005; Hecht et al., 2011; see Appendix 1 for primer details). Calculations of gene expression relative to *ACTIN* were based on non-equal amplification efficiencies and deviation in threshold cycle using the means for two technical replicates (Pfaffl, 2001).

#### **2.6.6 Rapid amplification of cDNA ends (RACE)**

RACE PCR was conducted using the Clontech SMART RACE cDNA Amplification Kit (Clontech, CA, USA) in accordance with the manufacturer's instructions. Gene-specific primers and nested gene-specific primers were designed according to the manufacturer's recommended guidelines. RACE was performed on cDNA synthesised from total RNA from shoot apex and leaf samples. RACE products were visualised and selected products were purified, cloned and sequenced.

#### **2.6.7 Genome walking**

Genome walking was conducted using the Clontech Universal GenomeWalker™ kit (Clontech, CA, USA) in accordance with the manufacturer's instructions. Gene-specific primers and nested gene-specific primers were designed according to recommended guidelines. Genome walking was performed on separate libraries of wild-type (NGB5839 or Kaliski) or *veg2-1* mutant gDNA digested with restriction enzymes as outlined in specific chapters. Genome walking products were visualised and selected products were purified, cloned and sequenced.

### **2.6.8 Visualisation of DNA**

To visualise PCR and digest products, DNA was separated on agarose gel in TAE buffer (40mM Tris Acetate and 1mM EDTA), containing GoldView<sup>™</sup> Nucleic Acid Stain (Acridine orange; SBS Genetech Co., Ltd, Beijing, China), alongside an appropriate DNA ladder and visualised under UV light.

### **2.6.9 Purification of PCR products**

For cloning and/or sequencing, PCR products were purified using Promega Wizard<sup>®</sup> SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and eluted in sterile, nuclease free water in accordance with the manufacturer's instructions.

## **2.7 Cloning**

### **2.7.1 Routine cloning**

For routine cloning, purified PCR products were ligated into pGEM<sup>®</sup>-T Easy vectors (Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. Electroporation at 1200V was used to insert vectors into competent *E. coli* cells (DH5 $\alpha$  prepared on site or Promega JM109). Transformed bacteria were allowed to recover in 400 $\mu$ L of Luria Broth (LB, 10g/L Bacto-tryptone, 5g/L Bacto-yeast extract, 10g/L NaCl, pH 7.5) with incubation at 37°C for 1 hour with shaking. Transformation reactions were spread across LB agar plates (ingredients as for LB broth with 15g/L agar and 100 $\mu$ g/mL ampicillin and 1 $\mu$ L/mL X-gal added) and incubated overnight at 37°C. White colonies were screened for an insert of desired length by colony PCR using a combination of gene-specific primers and vector-specific primers (pGEMT-F + pGEMT-R; see Appendix 1).

### **2.7.2 Gateway cloning**

The Gateway<sup>®</sup> cloning system (Invitrogen Corporation, Carlsbad, CA, USA) was used for creation of constructs for yeast two- and three-hybrid analyses and bimolecular fluorescence complementation. Full-length coding sequence was amplified by high fidelity PCR from wild-type cDNA. Successful PCR products were purified and a poly-A tail was added to PCR products using MangoTaq<sup>™</sup> DNA polymerase (Bioline, Alexandria, NSW, Australia) in a reaction mix containing 40 $\mu$ L

PCR product, 10µL of 5x reaction buffer, 1µL of dNTPs (10mM) and 0.1µL of MangoTaq™ DNA polymerase, incubated at 72°C for 10 minutes. A-tailed PCR products were purified again, cloned into an entry vector for the Gateway® system using the pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen Corporation, Carlsbad, CA, USA) and transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen Corporation, Carlsbad, CA, USA) with heat shock and selection of transformants on LB agar plates containing 100mg/L spectinomycin (for selection of TOPO vector) and 50mg/L streptomycin (for selection of TOP10 *E. coli*), in accordance with the manufacturer's instructions.

LR recombination reactions were performed using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen Corporation, Carlsbad, CA, USA) to shuttle gene inserts from entry vectors into destination vectors and used to transform One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen Corporation, Carlsbad, CA, USA) with heat shock and selection of transformants on LB agar plates containing 50 mg/L streptomycin (for selection of TOP10 *E. coli*) and the additional appropriate antibiotics required for selection of destination vector (Table 2.5), in accordance with the manufacturer's instructions.

**Table 2.5.** Antibiotics used for selection of desired LR recombinants in destination vectors.

Destination vector	Vector source	Purpose	Antibiotics	Concentration (mg/L)
pDEST32	Invitrogen ProQuest™ Two-Hybrid System	Yeast two- and three- hybrid bait	Gentamicin	10
pDEST22		Yeast two- and three- hybrid prey	Ampicillin	100
pARC351	Derived from pRED-NLSa (P. Ouwerkerk) with gateway modifications (R. Immink)	Yeast three-hybrid analysis	Kanamycin	50
pYFN43	A. Ferrando (Belda-Palazón et al., 2012), derived from pMDC43 (Curtis and Grossniklaus, 2003)	Bimolecular fluorescence complementation (BiFC) analysis	Kanamycin	50
pYFC43			Kanamycin	50

Colony PCR using gene specific PCR primers in combination with vector-specific primers, purification of DNA from selected colonies and sequencing using vector specific primers were performed to confirm correct coding sequence, reading frame, and orientation of inserts for each entry and destination construct.

### ***2.7.3 Purification of plasmid DNA from bacterial clones***

For sequencing of inserts in vectors, plasmid DNA was purified from selected colonies using the centrifugation protocol of the Promega Wizard Plus SV Mini-preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

## **2.8 Quantification of DNA, RNA and PCR products**

Concentration of DNA, RNA and PCR products was measured with a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) in accordance with the manufacturer's instructions.

## **2.9 Sequencing and sequence analysis**

Purified DNA was sent for sequencing at either the Australian Genome Research Facility Ltd (AGRF, Brisbane, Australia) or Macrogen Inc. (Seoul, Korea). Sequences were edited manually using Sequencher 4.8 (Gene Codes, Ann Arbor, MI) to correct falsely identified bases, remove unreadable sequence at the 3' and 5' ends and group sequences in contigs. Sequence identity was confirmed by BLAST search or alignment with existing sequence. Sequences were annotated using DNASTAR® Lasergene SeqBuilder (Version 8.1.4 (7); DNASTAR, Inc, Madison, WI, USA).

## **2.10 Design and use of molecular markers for mapping and genotyping**

Whole genes or gene sections containing introns were isolated from parental lines, or mutant and wild-type, by standard PCR. PCR products were visualised and any visible size differences between the pea lines of interest were identified and used in design of size markers. If no immediate size marker was identified, PCR products were purified and sequenced and any polymorphisms found between parental lines during sequence analysis were used for molecular marker design as follows. Specific marker details are given in Appendix 1 (Table A1.4).

### 2.10.1 Selection of marker genes for mapping

Where possible, existing molecular markers (size or CAPS) or marker genes published in pea were utilised (e.g. Aubert et al., 2006; Bordat et al., 2011). To further refine map position of mutant loci and candidate genes, close synteny between pea and *Medicago* (Choi et al., 2004; Kaló et al., 2004), was used to select genes as potential marker genes based on position in *Medicago* corresponding to a region of interest in pea. Other criteria for marker gene selection included presence of multiple introns, single gene copy in *Medicago* as identified by BLAST search of the *Medicago* genome, and availability of pea sequence as determined by BLASTn search with *Medicago* sequence against available *Pisum* expressed sequence resources (generally GenBank TSAs; see Section 2.3).

### 2.10.2 Size markers

Size differences between pea lines that were 15bp or larger and visible through visualisation of PCR products ( $\geq 10\%$  of PCR product size) were used for design of PCR-based size markers, with new primers designed closer to the deletion site if necessary to maximise relative size differences. PCR-based size markers were scored in segregating populations simply by standard PCR and visualisation of PCR products. Size differences identified through sequencing that were too small for PCR-based size markers were used in the design of HRM markers.

### 2.10.3 Cleaved Amplified Polymorphic Sequence (CAPS) markers

Single nucleotide polymorphisms (SNPs) between pea lines that altered a restriction enzyme recognition site, and would result in visible differences in length of digest product(s) for each parental line, were identified in sequenced PCR products with DNASTAR<sup>®</sup> Lasergene SeqBuilder (Version 8.1.4 (7); DNASTAR, Inc, Madison, WI, USA) and used for design of CAPS markers. If necessary, new primers were designed closer to restriction sites. Enzyme digests were conducted according to the manufacturer's instructions (New England Biolabs, Inc., Ipswich, MA). Prospective CAPS markers were tested on PCR products from parental lines and successful markers were used to genotype samples from the appropriate plant population(s) by standard PCR, restriction enzyme digest and visualisation of restriction enzyme products.

#### ***2.10.4 Derived Cleaved Amplified Polymorphic Sequence (dCAPS) markers***

For SNPs which did not alter a restriction enzyme recognition site, dCAPS markers were designed using the dCAPS Finder 2.0 with a minimal number of mismatches (<http://helix.wustl.edu/dcaps/dcaps.html>; Neff et al., 2002). dCAPS markers were tested and used in the same manner as CAPS markers.

#### ***2.10.5 High Resolution Melt (HRM) markers***

HRM markers were designed to target indels and C/T, G/A, C/A and G/T SNPs with primers designed to amplify small fragments (<200bp). HRM markers were tested and scored in segregating populations using a Rotorgene Q HRM machine (Qiagen). A Corbett Robotics CAS-1200<sup>TM</sup> pipetting robot (Corbett Research, Australia) with CAS Robotics 4 Version 4.9.8 (1.6.61) software was used to prepare reactions containing 2µL template, 1.05µL forward primer, 1.05µL reverse primer, 7.5µL HRM PCR Master Mix from Type-it HRM PCR Kit (Qiagen), and 3.4µL sterile milli-Q water. Conditions were as follows: 95°C for 5 minutes, 50x [95°C for 10 seconds, annealing temperature (T<sub>m</sub>; 50-60°C) for 30 seconds], 95°C for 5 minutes, 50°C for 5 minutes, HRM (temperature increasing with 0.1°C increments from 60-90°C, or from product melt temperature -5°C to +5°C). HRM results were analysed with Rotor-Gene<sup>®</sup> ScreenClust HRM<sup>®</sup> Software (Qiagen).

### **2.11 Linkage analysis**

Classical and molecular markers were scored in plants from F<sub>2</sub> generation mapping populations generated from crosses between mapping parents with the F<sub>1</sub> generation allowed to self-fertilise. For dominant classical markers, the F<sub>3</sub> generation was grown to distinguish F<sub>2</sub> plants that were heterozygous from homozygous dominant plants, where possible. Linkage maps were constructed from estimations of genetic distance between molecular and morphological markers based on segregation data using JoinMap<sup>®</sup> 4 (Kyazma B.V., Wageningen, Netherlands).

## 2.12 Construction of alignments and phylogenetic trees

For phylogenetic analyses, amino acid sequences of proteins were aligned using ClustalX (Thompson et al., 1997) and adjusted manually, where necessary, using GeneDoc (Version 2.7.000; Nicholas and Nicholas, 1997; <http://www.psc.edu/biomed/genedoc>). Using these alignments, distance-based methods were used for phylogenetic analyses in PAUP\* 4.0b10 (<http://paup.csit.fsu.edu/>). For comparison of homologous proteins, percentage identity at the amino acid level was calculated in GeneDoc from full-length protein alignments constructed using ClustalX.

## 2.13 Statistical analysis

All statistical analyses were conducted using IBM® SPSS® Statistics Version 21, using a significance level of 0.05. Where sample size differed between groups, Levene's test for homogeneity of variance was used to test the assumption of equal variance. For comparisons between only two groups, two-tailed t-tests were conducted with equal variance either assumed or not assumed, depending on whether the assumption of equal variance had previously been rejected. For comparisons between three or more groups, one-way ANOVA was conducted. Where the assumption of equal variance was retained, a standard ANOVA was conducted to test for any significant differences between groups, and where significant differences were found, Tukey's HSD post-hoc test was conducted to further examine the differences. Where the assumption of equal variance was rejected, Welch's test was conducted to test for any significant differences between groups, coupled with the Games-Howell post hoc test if significant differences were found. *p*-values are reported in text for each t-test, standard ANOVA/Welch's test where no significance was found between groups, and post-hoc test (Tukey's HSD/Games-Howell) for specific comparisons where a standard ANOVA/Welch's test identified a significant difference between groups.

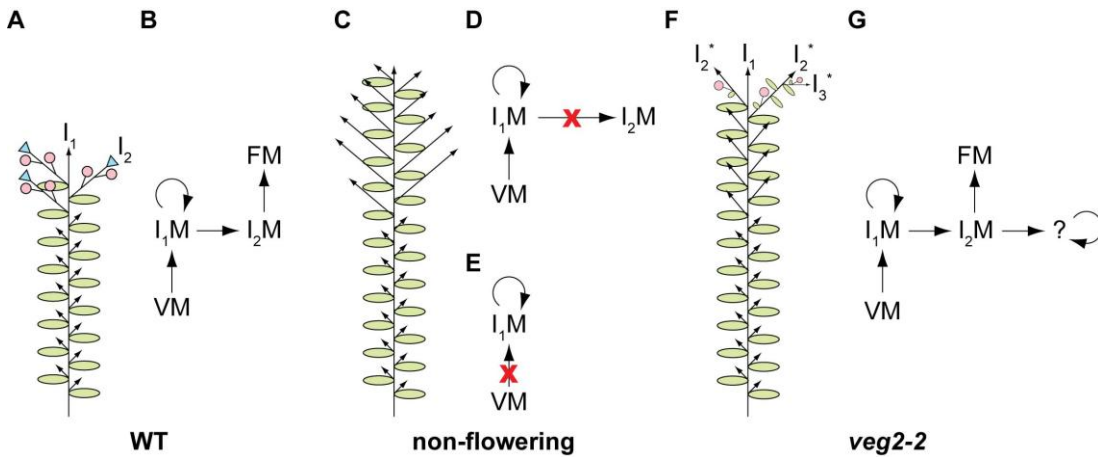


## **CHAPTER 3: The roles of the *VEG2* locus during pea inflorescence development**

### **3.1 Introduction**

#### ***3.1.1 Determination of SAM identity in non-flowering mutants***

Characterisation of mutants which exhibit abnormal meristem identity can enable identification of key genes controlling inflorescence development and can allow inference of the roles of these genes in wild-type plants. The pea inflorescence is in the form of a compound raceme, which involves (i) an indeterminate  $I_1$  meristem that produces the tissues of the main shoot, (ii) axillary  $I_2$  meristems which are leafless with a determinate fate, and (iii) floral meristems (Figure 3.1A). Vegetative and  $I_1$  meristems have distinct identities, and determination for inflorescence development is separable from determination for floral development in pea (Ferguson et al., 1991). Accordingly, a non-flowering pea mutant could potentially undergo the  $V/I_1$  transition from vegetative to inflorescence development but fail to produce recognisable inflorescence or floral structures. Following this reasoning, pea mutants that have a non-flowering phenotype could, in theory, represent two distinct classes, (i)  $I_1$  specification mutants, which remain purely vegetative because of impaired specification of  $I_1$  meristem identity and (ii)  $I_2$  specification mutants, which undergo the transition to inflorescence development but are unable to specify  $I_2$  meristems (Figure 3.1C-E). Identifying which stage of inflorescence development is blocked is an important aspect of mutant phenotype and is thus necessary for characterising the role of the affected locus.



**Figure 3.1.** Inflorescence development in wild-type pea and late- and non-flowering pea mutants.

(A, C, F) Diagrams of plant architecture and (B, D, E, G) schematics of meristem identity transitions are shown for (A-B) wild-type, (C-E) non-flowering mutants (e.g. *veg1*, *veg2-1*, *gigas* under LD conditions), and (F-G) the late-flowering *veg2-2* mutant.

In (A, C, F) locations are indicated for the primary inflorescence ( $I_1$ ), each secondary inflorescence ( $I_2$ ) and a tertiary inflorescence ( $I_3$ , seen in *veg2-2* only). Arrows indicate potential for indeterminate growth, circles are flowers, triangles are terminal  $I_2$  stubs and ovals are compound leaves or bracts (seen in *veg2-2* only).

Meristems in (B, D, E, G) are as follows: vegetative meristem (VM), primary inflorescence meristem ( $I_1M$ ), secondary inflorescence meristem ( $I_2M$ ), and floral meristem (FM). Straight arrows indicate meristem transitions and products. Red crosses indicate that a meristem transition is blocked. Circular arrows indicate meristem indeterminacy.

In wild-type plants, the production of axillary  $I_2$  structures is the clearest sign that the SAM has undergone the V/ $I_1$  transition and adopted final  $I_1$  identity. However, this feature may not be a reliable indicator in inflorescence mutants, especially in non-flowering mutants which fail to produce  $I_2$  structures. It is difficult to distinguish a vegetative SAM from a SAM with  $I_1$  identity based on physical meristem appearance alone (Makasheva, 1983), but two features of  $I_1$  meristems may be useful to allow inference of meristem identity.

Firstly, some genes show expression in only one meristem type in wild-type plants, thus expression of these meristem marker genes can be useful for investigating meristem identity in mutant plants. In pea, *DET* is important for maintaining  $I_1$  meristem identity, and *DET* expression is limited to a narrow range within  $I_1$  meristems (Foucher et al., 2003; Berbel et al., 2012). The occurrence of the V/ $I_1$  transition in the *det* mutant indicates that  $I_1$  identity can be acquired in the absence of *DET* (Singer et al., 1990), thus the absence of *DET* expression does not discount the possibility of  $I_1$  identity. However, the narrow range of *DET* expression means that any meristems that express *DET* can be confidently identified as  $I_1$

meristems, which makes *DET* a useful marker for  $I_1$  identity (Berbel et al., 2012). The idea of using the expression of meristem marker genes to infer meristem identity is not limited to the current aim of determining SAM identity. Expression of marker genes can also be used for axillary meristems, as an early indicator of meristem identity before the distinctive structures produced by  $I_2$  or floral meristems are visible. *VEG1* is a gene critical for specification of  $I_2$  identity that is expressed only in the  $I_2$  meristem, while *PIM* is a gene important for specifying floral meristem identity that is expressed only in floral meristems. Both *VEG1* and *PIM* have previously been used as indicators of  $I_2$  and floral meristem identity, respectively (Taylor et al., 2002; Berbel et al., 2012).

Secondly, the onset of flowering in wild-type pea has previously been linked to an abrupt decrease in the lengths of subsequent internodes (Barber, 1959; Ross and Reid, 1992; Ross et al., 1992). Thus it is possible that this decrease in internode length could be a morphological indicator that the SAM has undergone the V/ $I_1$  transition. Indeed, a previous study has successfully used ontogenetic variation in internode length to detect the influence of early flowering alleles in a non-flowering background (Reid and Murfet, 1984).

In non-flowering mutants, any inference of  $I_1$  identity is reliant on indicators such as *DET* expression and internode length. In late-flowering mutants,  $I_2$  structures are eventually produced, which can be interpreted in one of two ways. The simplest interpretation is that the late-flowering phenotype represents a delay in the V/ $I_1$  transition, and  $I_2$  meristems are specified when the SAM eventually acquires  $I_1$  identity. Using this interpretation, production of  $I_2$  structures could be used as an indicator of the relative timing of the V/ $I_1$  transition. However, if the specification of  $I_2$  structures can be decoupled from the V/ $I_1$  transition (Ferguson et al., 1991), an alternative theory is that there may be an isolated delay in the specification of  $I_2$  structures, after the V/ $I_1$  transition has occurred normally. This second theory relies on the supposition that  $I_2$  specification can be temporarily blocked, which has not yet been shown to be possible in a pea mutant. In this study, multiple indicators will be used for inference of meristem identity.

### 3.1.2 Three pea loci with critical roles in flowering – *GIGAS*, *VEG1* and *VEG2*

Three non-flowering mutants have been described in pea: *gigas*, *veg1* and *veg2-1*. A non-flowering phenotype indicates that the affected loci have critical roles in pea inflorescence development. *gigas* is non-flowering under LD conditions only, but both *veg1* and *veg2-1* are non-flowering under all conditions (Reid and Murfet, 1984; Murfet and Reid, 1993; Beveridge and Murfet, 1996).

Based on expression of the  $I_1$  marker gene *DET*, the non-flowering *veg1* mutant has been found to undergo the  $V/I_1$  transition and acquire  $I_1$  identity at the same time as wild-type under LD conditions (Berbel et al., 2012). Molecular means have not been used to infer meristem identity in the *veg1* mutant under SD conditions. However, ontogenetic variation in internode length in the *veg1* mutant follows the same pattern as wild-type plants, which suggests that the  $V/I_1$  transition also occurs normally in this mutant under SD conditions (Reid and Murfet, 1984). These observations indicate that *veg1* is an  $I_2$  specification mutant (Figure 3.1D).

Although *DET* expression has not previously been used to infer SAM identity in *gigas*, published results show normal expression of *DET* in *gigas-2* under LD conditions (Hecht et al., 2011). Similar to findings for the *veg1* mutant, these results indicate that the non-flowering *gigas* mutant undergoes the  $V/I_1$  transition correctly, but fails to specify  $I_2$  meristems under LD conditions (Figure 3.1D). Under SD photoperiods, normal  $I_2$  structures are eventually produced in the *gigas* mutant (Beveridge and Murfet, 1996), giving clear evidence that the  $V/I_1$  transition also occurs in this mutant under these conditions. The timing of this transition has not previously been investigated in detail, but it has been noted that the weaker *gigas-1* mutant does not show the decrease in internode length associated with flowering time at the same time as wild-type plants under an 8-hour SD photoperiod (Murfet, 1989b). This suggests that the delay in flowering in *gigas* in SD photoperiods may correspond to a delay in the  $V/I_1$  transition, which warrants further investigation and confirmation.

Of the three non-flowering mutants, *veg2-1* has received the least attention in the literature, and has not previously been described in a primary research paper. Neither *DET* expression nor ontogenetic variation in internode length has previously been investigated in the *veg2-1* mutant, so the timing, or even occurrence, of the  $V/I_1$  transition in this mutant cannot be inferred. Preliminary expression results in the late-

flowering *veg2-2* mutant under LD conditions showed a delay in *DET* expression corresponding to the delay in flowering time, suggesting that a delay in the V/I<sub>1</sub> transition may underlie the late-flowering phenotype in this mutant (Sussmilch, 2008). As *veg2-1* is a more severe allele than *veg2-2* (Murfet, 1992; see also Chapter 4), it could be expected that any delay in the V/I<sub>1</sub> transition in *veg2-2* would be reflected with a similar or larger delay in the *veg2-1* mutant, if this transition is not completely blocked.

### 3.1.3 Branching

In pea, axillary meristems form in leaf axils, develop into axillary buds that each comprise several undeveloped phytomers, then become dormant (e.g. Sorokin and Thimann, 1964; Stafstrom and Sussex, 1988; Shimizu and Mori, 1998). In wild-type plants, these axillary buds usually remain dormant for the life of the plant but can be released from dormancy by triggers including non-inductive photoperiods or decapitation, whereby they can develop into branches that reiterate the fate of the main stem (see Beveridge et al., 2003; Dun et al., 2006). In addition, removal of flowers and pods has been linked to increased branching in wild-type pea (Lockhart and Gottschall, 1961; Malik and Berrie, 1975; Murfet, 1985).

A number of different groups of mutants exhibit interesting shoot branching phenotypes in pea. Perhaps the most obvious group of these mutants is those in which the synthesis or reception of hormone signals that regulate bud outgrowth is altered. The non-allelic *ramosus* mutants (*rms*), which are involved in strigolactone signaling, fall into this group (e.g. Arumingtyas et al., 1992; Johnson et al., 2006; Gomez-Roldan et al., 2008). However, mutation to genes involved in the flowering pathway can also result in interesting branching phenotypes.

Mutation to genes within the photoperiod response/circadian clock network, can result in photoperiod-independent branching phenotypes that are characteristic of wild-type plants grown under a specific photoperiod. In wild-type, axillary buds usually remain dormant under LD conditions, but under SD photoperiods, axillary buds at basal nodes develop into large branches (e.g. Beveridge et al., 2003). *sterile nodes* (*sn*), *die neutralis* (*dne*) and *photoperiod* (*ppd*) have a LD branching phenotype, where all buds remain dormant, even when grown under SD conditions (Murfet and Reid, 1985, 1993). In contrast, *late bloomer1* (*late1*) has a SD branching

phenotype, where large branches develop at basal nodes, even in LD photoperiods (Hecht et al., 2007).

The non-flowering *veg1* and *gigas* mutants both develop a distinctive aerial branching phenotype, whereby axillary buds are released from dormancy in upper nodes to develop into lateral branches that in turn bear secondary and tertiary branches in a reiterative pattern (Gottschalk, 1979; Reid and Murfet, 1984; Beveridge and Murfet, 1996). A similar phenotype has been noted for the *veg2-1* mutant, and it has also been observed that late-flowering *veg2-2* mutant plants exhibit branching in the nodes between the wild-type node of floral initiation (NFI) and the *veg2-2* NFI (Murfet and Reid, 1993; Beveridge et al., 2003; Weller, 2007). Increased branching at aerial nodes has been noted in other late-flowering pea lines including the *late5* mutant (see Chapter 6; Sussmilch, 2008) and the extreme late *LF-d* allele (Murfet, 1985; Murfet and Reid, 1985; Murfet, 1992).

The timing of onset of the aerial branching phenotype in *gigas* and *veg1* is described as similar to the timing of flowering in wild-type plants (Reid and Murfet, 1984; Beveridge and Murfet, 1996). Accordingly, it has previously been suggested that the appearance of this phenotype could indicate that the V/I<sub>1</sub> transition has occurred, with non-flowering mutants exhibiting homeotic substitution of I<sub>2</sub> structures with lateral branches (Coen, 1991; Murfet and Reid, 1993; Wiltshire et al., 1994; Reid et al., 1996; Berbel et al., 2012). This is an interesting theory, but it is based only on observations of *veg1* and *gigas* mutants, and has not been tested in the *veg2-1* mutant, or in any late-flowering mutants. Other than basic observation, branching has not previously been characterised in the *veg2-1* or *veg2-2* mutants, but characterisation of this trait could indicate if lateral outgrowth is associated with the V/I<sub>1</sub> transition in these plants, in addition to documenting a new aspect of mutant phenotype for these alleles.

### **3.1.4 The role of *VEG2* in maintaining *I*<sub>2</sub> identity**

The *veg2-2* mutant phenotype allows an insight into the role of the *VEG2* locus beyond the initial role that is blocked in the *veg2-1* mutant. In the late-flowering *veg2-2* mutant, *I*<sub>2</sub> structures bear axillary flowers, but instead of terminating in a wild-type stub, these structures then revert to something resembling the wild-type *I*<sub>1</sub>, bearing compound leaves with dormant axillary buds and retaining an indeterminate apex (Figure 3.1F-G; Sussmilch, 2008). These observations suggest that at least partial specification of *I*<sub>2</sub> meristem identity occurs in the *veg2-2* mutant, but this identity is not retained (Figure 3.1G; Sussmilch, 2008). The final identity of ‘*I*<sub>2</sub>’ meristems in the *veg2-2* mutant requires confirmation.

### **3.1.5 The role of *VEG2* in flower development**

Floral morphology defects have been noted to occur in the *veg2-2* mutant (Murfet, 1992; Murfet and Reid, 1993; Sussmilch, 2008), suggesting that *VEG2* has a role in floral development, but further investigation of this role requires more detailed characterisation of *veg2-2* floral morphology. Based on the ABCE model of floral development in *Arabidopsis*, it is likely that a role in floral development would involve regulation of the MADS-box genes that control floral patterning (e.g. Coen and Meyerowitz, 1991; Theissen and Saedler, 2001). *PIM* has a critical role in correct specification of floral meristems in pea (Berbel et al., 2001; Taylor et al., 2002), and preliminary investigation suggested that the timing and possibly relative level of *PIM* expression could be delayed in the *veg2-2* mutant (Sussmilch, 2008; see also Chapter 5). Whether the observed *veg2-2* floral morphology defects are merely the result of misregulation of *PIM*, or whether these indicate a further role for *VEG2* in floral development beyond upregulation of *PIM*, remained to be established.

### 3.1.6 Chapter aim(s)

The main aim for this chapter was to characterise in detail the roles of the *VEG2* locus during pea inflorescence development. This aim was divided into a series of experimental aims to investigate specific roles of *VEG2* during each stage of inflorescence development. Firstly, in order to examine the potential role of *VEG2* in specification of the  $I_1$  meristem, the occurrence and relative timing of the  $V/I_1$  transition was investigated in *veg2-1* and *veg2-2* using *DET* and ontogenetic variation in internode length as indicators of SAM identity. The timing of this transition was also investigated in the *gigas-2* mutant for the first time under SD conditions, and the *veg1* mutant was included for comparison. Secondly, branching was characterised as a feature of mutant phenotype and as a potential indicator of the  $V/I_1$  transition in the *veg2* mutants for the first time, and also in deflowered wild-type, *veg1*, and *gigas* plants for comparison. Thirdly, expression of the molecular markers *VEG1* and *PIM* were investigated to confirm roles for *VEG2* in specification of  $I_2$  and floral meristem identity. Next, to investigate the role of *VEG2* in maintaining  $I_2$  identity, the nature of the indeterminate *veg2-2* ' $I_2$ ' meristem was confirmed by using *DET* expression as an indicator for  $I_1$  identity. Lastly, to examine the role of *VEG2* during floral development, floral morphology defects in the *veg2-2* mutant were investigated in detail, and the *pim veg2-2* double mutant phenotype was characterised to determine if *VEG2* has a role in floral development beyond upregulation of *PIM*.



## 3.2 Materials and methods

This section contains specific details of materials and methods for studies included in this chapter. General materials and methods also relevant to this chapter are described in Chapter 2.

### 3.2.1 Plant material and growth conditions

Details of plants and plant growth conditions used for the experiments presented in this chapter are outlined in Table 3.1. Plants were either grown in the UTAS phytotron with total photoperiod comprising a base photoperiod of 8 hours of natural daylight extended with fluorescent light for longer photoperiods, or in controlled environment growth cabinets at 20°C under fluorescent light for the full photoperiod. Plants for characterisation of ontogenetic variation in internode length and branching in LD and SD for Sections 3.3.1.2-3.3.2 were grown at the same time, to enable comparison between photoperiods. Plants for the SD expression series in *gigas* and wild-type for qRT-PCR analysis presented in Section 3.3.1.3 were grown and harvested by V. Hecht (Hecht et al., 2011).

Due to sterility of *veg1* and *veg2-1* mutants under all known conditions (Murfet and Reid, 1993), samples from these mutants were obtained by growing segregating populations. As *veg1* and *veg2-1* are gene deletion mutants (Berbel et al., 2012; see Chapter 4), there was no practical means of identifying plants that were heterozygous at these loci, from those that were homozygous, prior to growing their seed. Thus to obtain mutants, seed was grown from wild-type siblings from previous generations, which yielded wild-type families and segregating families. Glasshouse space restrictions limited the number of plants that could be grown at any one time, and the proportion of mutant segregants was sometimes less than expected, so mutant numbers were often limiting for experiments. Seed for *gigas* mutants was easily obtained from mutants previously grown under SD conditions, wherein these mutants flower and set seed (Murfet, 1992). Due to incomplete introgression of *veg1* and *veg2-1* on to a wild-type line NGB5839 background, wild-type siblings from several different wild-type families were used as the wild-type line for these mutants.

**Table 3.1.** Details of plant material and growth conditions for experiments presented in Chapter 3.

For expression studies, tissue type and time-points of tissue harvest (days after sowing) are indicated. Tissues harvested include: main shoot apex (A), apex of the longest branch (B), or secondary inflorescence stub (I<sub>2</sub>S) or apex (I<sub>2</sub>A). For developmental series the range of time-points is shown with the number of time-points indicated in parentheses. Number of plants (*n*) is shown. For expression studies, *n* represents each time-point.

Purpose	Growth conditions	Chapter section(s)	Tissue	Genotypes	Time-point (days)	<i>n</i>
Expression of molecular markers for meristem identity	8h SD (Phytotron)	3.3.1.3 3.3.3	A + B combined	WT (NGB5839)	81	2
				<i>gigas-2</i>	81 112	2 3
				<i>veg2-2</i>	81 112	3 3
				WT ( <i>veg2-1</i> )	81	2
				<i>veg2-1</i>	81	1
				WT ( <i>veg1</i> )	81	2
				<i>veg1</i>	81	2
	8h SD (Cabinet)	3.3.1.3	A	WT (NGB5839)	7-56 (8)	4
				<i>gigas-2</i>	7-105 (10)	4
	18h LD (Phytotron)	3.3.1.1 3.3.3	A (+B where A limiting 74d)	WT (NGB5839)	45	3
				<i>gigas-2</i>	45	3
				<i>veg2-2</i>	45 74	3 3
				WT ( <i>veg1</i> )	45	3
				<i>veg1</i>	45 74	2 2
				WT ( <i>veg2-1</i> )	45	3
				<i>veg2-1</i>	45 74	3 3
		3.3.4	I <sub>2</sub> S	WT (NGB5839)	59	2
			I <sub>2</sub> A	<i>veg2-2</i>	74	3
	24h LD (Cabinet)	3.3.1.1	A	WT (NGB5839)	14 35	4 4
Characterisation of ontogenetic variation in internode length and branching	8h SD (Phytotron)	3.3.1.3 3.3.2		WT (NGB5839)		6
				<i>gigas-2</i>		6
				<i>veg2-2</i>		6
				WT ( <i>veg2-1</i> )		6
				<i>veg2-1</i>		3
				WT ( <i>veg1</i> )		6
				<i>veg1</i>		6
	24h LD (Phytotron)	3.3.1.2 3.3.2		WT (NGB5839) intact		6
				WT (NGB5839) deflowered		6
				<i>gigas-2</i>		6
				<i>veg2-2</i>		6
				WT ( <i>veg2-1</i> )		6
				<i>veg2-1</i>		5
				WT ( <i>veg1</i> )		6
				<i>veg1</i>		6
Characterisation of <i>veg2-2</i> floral morphology	24h LD (Phytotron)	3.3.5.1		<i>veg2-2</i>		7
	8h SD (Phytotron)					8
Characterisation of <i>pim veg2-2</i> phenotype	18h LD (Phytotron)	3.3.5.2		WT (NGB5839)		20
				<i>veg2-2</i>		17
				<i>pim-2</i>		14
				<i>pim-2 veg2-2</i>		23

### 3.2.2 Genotyping

*veg1* and *veg2-1* mutants were identified by genotyping plants in segregating populations with PCR-based markers. Putative *veg1* mutants were identified by absence of PCR product from primer pair PsFULc-2F and PsFULc-2R (see Appendix 1), which amplify a 950-bp fragment from the *VEG1* gene in homozygous wild-type and heterozygous plants (Berbel et al., 2012). Putative *veg2-1* mutants were identified by absence of PCR product from primer pair PsFD-7F and PsFD-6R, which amplify a 1.2kb fragment from the *FDA* gene in homozygous wild-type and heterozygous plants. Identity of putative mutants was confirmed by growing plants until appearance of distinctive aerial branching phenotype. Putative mutants used for harvest of material at early time-points for expression analysis, were kept until confirmation of identity by plant phenotype before apical samples were included in expression experiments.

Prior to characterisation of *pim-2 veg2-2* double mutant phenotype, double mutant plants and single mutants heterozygous for the second mutant allele were identified using CAPS markers designed for both *pim-2* and *veg2-2* mutant alleles. For the *PIM* locus, the 185bp PCR product from primer pair PsPIM-F1 and PsPIM-R1 was digested with XmnI (New England Biolabs, Inc., Ipswich, MA), according to the manufacturer's instructions. This enzyme digests PCR product containing the *pim-2* mutation, yielding 19bp and 166bp digestion products, but does not cut wild-type PCR products. For the *VEG2* locus, the 732bp PCR product from primer pair PsFD-4F and PsFD-5R was digested with BspHI (New England Biolabs, Inc., Ipswich, MA), according to the manufacturer's instructions. This enzyme digests PCR product containing the *veg2-2* mutation, yielding 246bp and 486bp digestion products, but does not cut wild-type PCR products. For this study, plants were grown only from single mutants heterozygous for the second mutant allele, and double mutants were identified by distinctive phenotype.

### 3.2.3 *qRT-PCR analysis*

For expression experiments included in Sections 3.3.1, 3.3.3 and 3.3.4, expression levels of *DET*, *VEG1* and *PIM* relative to *ACTIN* were measured by qRT-PCR (see Appendix 1 for primer details). Samples comprised dissected apical buds (2mm<sup>2</sup>; main shoot apex, branch apex, or indeterminate apex of the *veg2-2* I<sub>2</sub>,) or I<sub>2</sub> stubs (approximately 1cm of tissue from the last I<sub>2</sub> node to stub tip) as indicated in figure legends. For growth conditions and time-points, see Table 3.1. Time-points for *DET* expression experiments included in Sections 3.3.1.1 and 3.3.1.3 were chosen to coincide with the expected peaks in *DET* expression for wild-type and *veg2-2* plants, which corresponded to approximately one week after the first macroscopic appearance of developing flower buds in the apex of each genotype, as determined from preliminary expression studies (Sussmilch, 2008; see also Chapter 5). The SD *gigas* expression series shown in Section 3.3.1.3 was harvested and processed with expression of *ACT* in RT- and RT+ and expression of *DET* on a single replicate determined by V. Hecht, prior to this study. During the time-frame of this study, *DET* expression on a second replicate of these existing samples was determined, and all other experiments were conducted without technical assistance.

### 3.2.4 *Plant measurements*

For characterisation of ontogenetic variation in internode length for Sections 3.3.1.2 and 3.3.1.3, internode length was measured for all nodes on the stem with expanded leaves present at time of harvest of dried wild-type plants (LD: 97 days after sowing; SD: 152 days after sowing). To determine days to first open flower (DTF), plants were checked for flowers every 1-3 days, and stage of flower development was used to estimate date of flower opening.

To characterise branching during development, the total length and number of nodes were measured for the main lateral present at each stem node, in LD and SD conditions. Six weekly time-points covered early branch outgrowth and establishment of the aerial branching phenotype in the mutants (36-71 days after sowing in LD; 64-99 days after sowing in SD). A final seventh time-point was taken at plant harvest after seed maturation and drying in the wild-type plants (97 days after sowing in LD; 152 days after sowing in SD). The increase in length and number of nodes was calculated by subtracting the measurement of the previous time-point

from each new measurement, separately for each lateral. Decrease in lateral length due to drying was considered to represent a 0mm increase in lateral length. Mean values for each node were calculated for each genotype.  $I_2$  structures were not included in lateral measurements. Data for the first time-point for branching measurements under LD conditions (36 days after sowing) is not included for *veg2-2*.

In order to characterise timing of lateral outgrowth, two arbitrarily defined stages of this process were investigated in the same experiment described above. The length of each lateral on the main stem was measured each week from the first time-point (LD: 36 days after sowing; SD: 64 days after sowing) until the first ‘enlarged aerial bud’ (axillary bud  $\geq 5$ mm at node 8 or above) and the first ‘aerial branch’ (lateral with one or more expanded leaves at node 8 or above) had developed. Some plants had not shown aerial lateral outgrowth by these measures before the time of plant harvest (LD: 97 days after sowing; SD: 152 days after sowing). If multiple nodes had developed enlarged aerial buds or aerial branches between time-points, the node with the longest lateral was counted as the node of first enlarged aerial bud or aerial branch, respectively. Mean plant age at time of aerial bud outgrowth or branch development was calculated using the midpoint of measurement time-point intervals. The first interval was defined as ranging from 21 days after sowing until the first branching measurement time-point (LD: 36 days after sowing; SD: 64 days after sowing) to account for the time required for plants to develop 8 expanded nodes.

Measurements for comparison of total branching included all vegetative laterals that were 5mm or longer in length. The portion of each indeterminate  $I_2$  structure in *veg2-2* after the  $I_2$  structure had reverted to  $I_1$  appearance was also included in measurements of total branching. For this trait, laterals and the main stem were measured at time of plant harvest (LD: 97 days after sowing; SD: 152 days after sowing).

### 3.3 Results

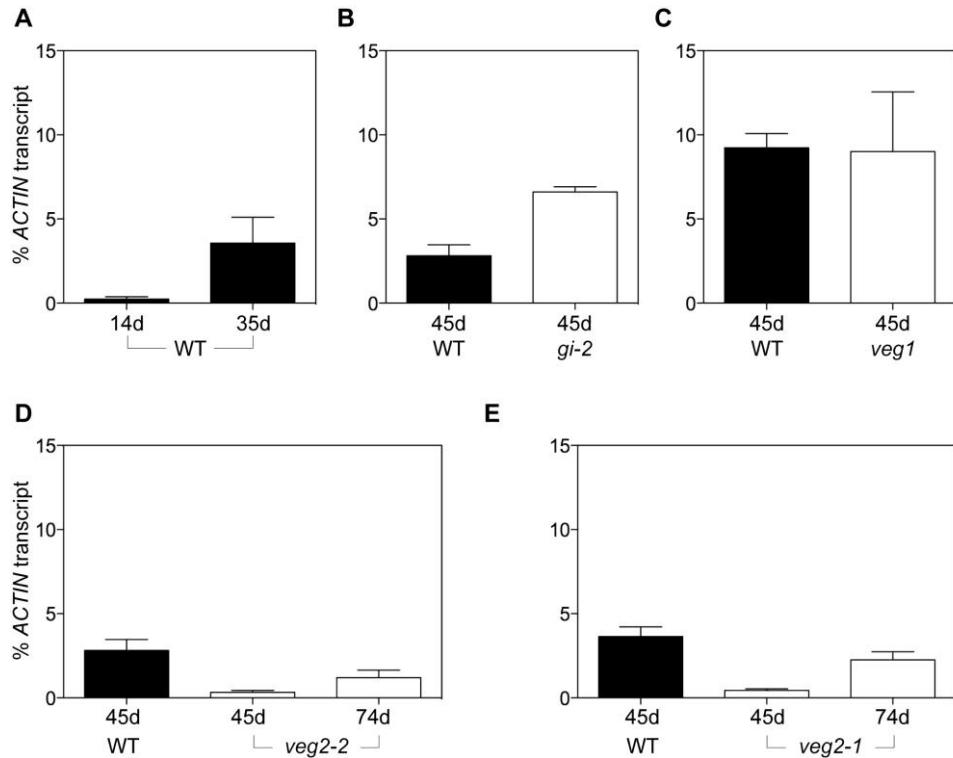
#### 3.3.1 Examining a possible role for *VEG2* in *I*<sub>1</sub> meristem specification

##### 3.3.1.1 Investigating SAM identity under LD conditions

In a preliminary experiment, induction of *DET* was measured by qRT-PCR in wild-type apices before and after the V/*I*<sub>1</sub> transition. Figure 3.2A shows that in wild-type plants, *DET* expression in the shoot apex was negligible before completion of the V/*I*<sub>1</sub> transition, but *DET* was upregulated in *I*<sub>1</sub> meristems, as previously described (Berbel et al., 2012). Next, the identity of the *veg2-1* SAM was examined for the first time under LD conditions by using expression of *DET* as a marker for *I*<sub>1</sub> meristem identity, and SAM identity in *veg1*, *gigas-2* and *veg2-2* mutants and corresponding wild-type plants was also investigated for comparison. *veg1* and *gigas* mutants were expected to show expression of *DET* at the same time as wild-type and *veg2-2* was expected to show delayed *DET* upregulation (Sussmilch, 2008; Hecht et al., 2011; Berbel et al., 2012). Time-points of 45 and 74 days after sowing were chosen to coincide with the expected peaks in *DET* expression for wild-type and *veg2-2* plants (Sussmilch, 2008; see also Chapter 5).

After the time of the wild-type V/*I*<sub>1</sub> transition, when *DET* expression had been upregulated in wild-type apices, a high level of *DET* expression was seen in apices of *veg1* and *gigas* mutants (Figure 3.2B-C). This finding is in agreement with the results of previous studies, and is consistent with the V/*I*<sub>1</sub> transition occurring in *veg1* and *gigas* at a similar time to wild-type plants (Hecht et al., 2011; Berbel et al., 2012). The *veg2-2* mutant showed a very low level of *DET* expression at the first time-point (Figure 3.2D), that was similar to levels seen in wild-type apices prior to completion of the V/*I*<sub>1</sub> transition (Figure 3.2A). *DET* was upregulated in *veg2-2* by the time of macroscopic appearance of floral buds in the *veg2-2* apex, 74 days after sowing (Figure 3.2D). This result is in agreement with preliminary findings (Sussmilch, 2008; see also Chapter 5), and suggests that completion of the V/*I*<sub>1</sub> transition may be delayed in the *veg2-2* mutant. The *veg2-1* mutant showed a similar pattern of *DET* expression to that seen in the *veg2-2* mutant (Figure 3.2D-E). In *veg2-1*, *DET* was expressed at a low level 45 days after sowing, but was upregulated by the time the *veg2-2* V/*I*<sub>1</sub> transition had occurred, 74 days after sowing (Figure 3.2E). The finding that *DET* is eventually expressed in the *veg2-1* mutant reveals that the V/*I*<sub>1</sub> transition

does occur in this mutant under LD conditions. The delay in the timing of *DET* upregulation, relative to wild-type, *gigas* and *veg1*, suggests that completion of this transition may be delayed in *veg2-1*, similar to findings for *veg2-2*. Overall, these results suggest that *VEG2* may play an important role in the V/I<sub>1</sub> transition under LD conditions, and that *FTa1/GIGAS* and *VEG1* either do not act during this transition or share functional redundancy with other genes.



**Figure 3.2.** Expression of *DET* as an indicator of I<sub>1</sub> meristem identity, under LD conditions.

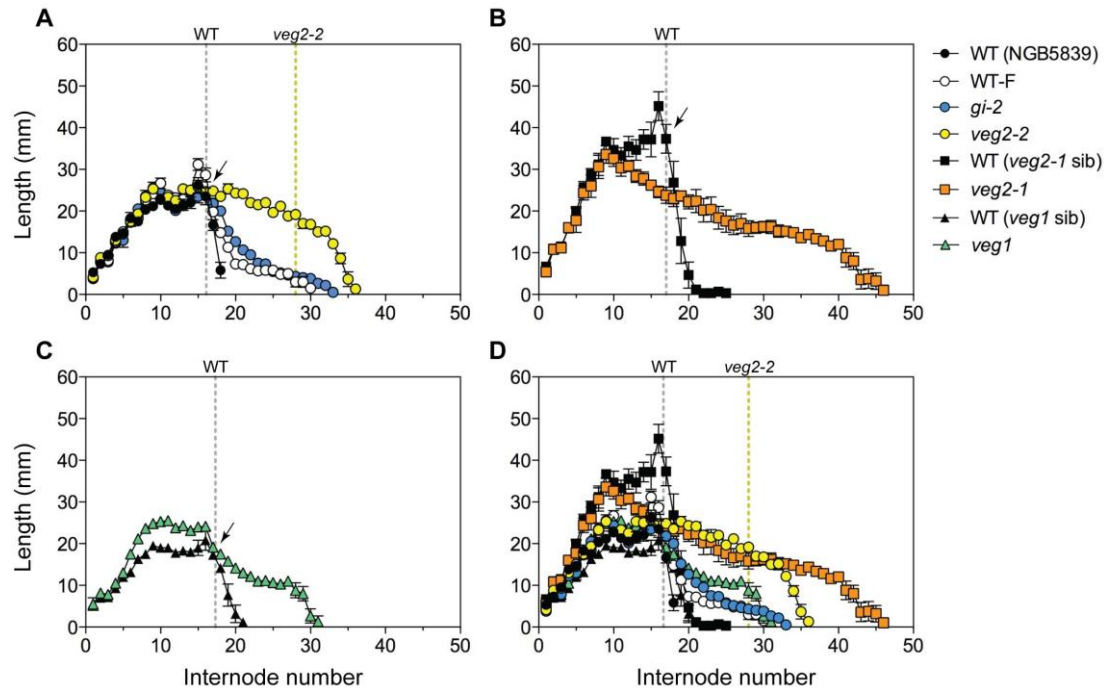
*DET* expression in the shoot apex of wild-type plants, the non-flowering mutants *veg1*, *gigas* (*gi-2*) and *veg2-1*, and the late-flowering mutant *veg2-2*. (A) Wild-type *DET* expression prior to completion of the V/I<sub>1</sub> transition (14 days after sowing), and after the V/I<sub>1</sub> transition (35 days after sowing). (B-E) *DET* expression in the mutants after the occurrence of the V/I<sub>1</sub> transition in wild-type (45 days after sowing), and in *veg2-2* (74 days after sowing), in a separate experiment to that shown in (A). Wild-type lines are as follows: (A, B, D) NGB5839, (C) wild-type sibling of *veg1*, (E) wild-type sibling of *veg2-1*. For relative transcript levels, values have been normalised to the transcript level of *ACTIN* and are shown as mean percentage *ACTIN*  $\pm$  standard error for 2-3 biological replicates.

### 3.3.1.2 Characterisation of ontogenetic variation in internode length under LD conditions

The decrease in internode length that occurs in wild-type pea at the onset of flowering could be a morphological indicator that the SAM has undergone the V/I<sub>1</sub> transition. In support of this, non-flowering *gigas* and *veg1* mutants, which appear to undergo the V/I<sub>1</sub> transition at a similar time to wild-type (Figure 3.2B-C; Hecht et al., 2011; Berbel et al., 2012), are described as entering a stable compact aerial phase, characterised by a dramatic decrease in internode length, which begins at a comparable node to flowering in corresponding wild-type plants (Reid and Murfet, 1984; Beveridge and Murfet, 1996; Beveridge et al., 2001). In this study, ontogenetic variation in internode length was investigated as a morphological indicator of the V/I<sub>1</sub> transition in *veg2-1* and *veg2-2* in comparison with *gigas* and *veg1* mutants, deflowered wild-type plants in which flowers were removed after anthesis, and corresponding intact wild-type plants under LD conditions.

All wild-type lines showed a similar pattern of ontogenetic variation in internode length. Internode length increased between successive nodes during vegetative development, generating a double peak (Figure 3.3). The first peak occurred at approximately internode 10 and the second peak occurred immediately prior to flowering at approximately internode 15 (Figure 3.3). Internode length then decreased rapidly between successive reproductive nodes until the complete cessation of stem growth associated with apical senescence (Figure 3.3). This pattern matched previous descriptions of wild-type ontogenetic variation in internode length (e.g. Reid and Murfet, 1984; Ross and Reid, 1992; Ross et al., 1992). In deflowered wild-type plants, more reproductive nodes were produced relative to intact wild-type plants and the decrease in internode length during reproductive development occurred more gradually (Figure 3.3A). This may be explained by delayed apical senescence in the absence of developing fruit (see also Section 3.3.2.5). Regardless of rate, internode length decreased at a similar time between intact and deflowered wild-type plants, at approximately internode 16 (Figure 3.3A), which corresponded to the start of reproductive development (mean NFI  $\pm$  SE: intact wild-type =  $16.0 \pm 0.0$ , deflowered wild-type =  $16.2 \pm 0.2$ ).





**Figure 3.3.** Ontogenetic variation in internode length under LD conditions.

(A) Plants with NGB5839 background: intact wild-type (NGB5839), deflowered wild-type (WT-F; NGB5839) with all flowers removed after anthesis, late-flowering *veg2-2* and non-flowering *gigas* (*gi-2*) plants.

(B) *veg2-1* segregants: wild-type siblings and non-flowering *veg2-1* mutant plants.

(C) *veg1* segregants: wild-type siblings and non-flowering *veg1* mutant plants.

(D) All genotypes from (A) to (C) on the same plot.

Internodes are numbered with internode 1 between the first and second scale leaf. Data points represent mean  $\pm$  standard error for  $n = 5-6$  plants per genotype. Broken vertical lines indicate the mean internode between the first and second reproductive nodes for the genotypes which flowered during this experiment: wild-type (grey; combined mean shown where multiple wild-types are present in one graph) and *veg2-2* (yellow). Arrows point to the first node of decreased internode length in intact wild-type plants.

Similar to deflowered wild-type plants, *gigas-2* and *veg1* mutants also exhibited a gradual decrease in internode length that began at a similar time to corresponding wild-type plants (approximately internode 16-17; Figure 3.3A and C). Curiously, maximum internode length was higher in *veg1* than for wild-type siblings (Figure 3.3C). These findings match the results for *veg1* and *gigas-1* from previous studies (Reid and Murfet, 1984; Beveridge and Murfet, 1996).

Ontogenetic variation in internode length in the *veg2* mutants followed a different pattern to that seen in wild-type, *gigas* and *veg1* (Figure 3.3). In *veg2-2*, there was a gradual decline in internode length started at approximately internode 22, which preceded the first reproductive node (Figure 3.3A). There was a significant

delay in flowering time in the *veg2-2* mutant relative to wild-type (mean NFI  $\pm$  SE: wild-type =  $16.0 \pm 0.0$ , *veg2-2* =  $28.0 \pm 0.4$ ;  $p < 0.001$ ). After flowering, a rapid decline started at approximately internode 33 in *veg2-2*, which was possibly associated with monocarpic senescence (Figure 3.3A). Both phases of decreasing internode length in *veg2-2* were delayed relative to the decrease seen in wild-type which occurred at approximately internode 16 (Figure 3.3A). However, unlike wild-type, *gigas* and *veg1*, there was no rapid decrease in internode length associated with the expected timing of the V/I<sub>1</sub> transition, immediately prior to flowering (Figure 3.3). In the *veg2-1* mutant, there was a gradual decline in internode length after the first peak at approximately node 10, with a plateau or a second peak seen in some plants between internodes 30 and 40, and a more rapid decline thereafter (Figure 3.3B). Without a rapid decline in internode length at the expected time of the V/I<sub>1</sub> transition in the *veg2-2* mutant, this trait could not be used to infer the exact timing of the transition. However, the patterns seen in *veg2-1* and *veg2-2* were clearly distinct from those seen in wild-type (Figure 3.3).

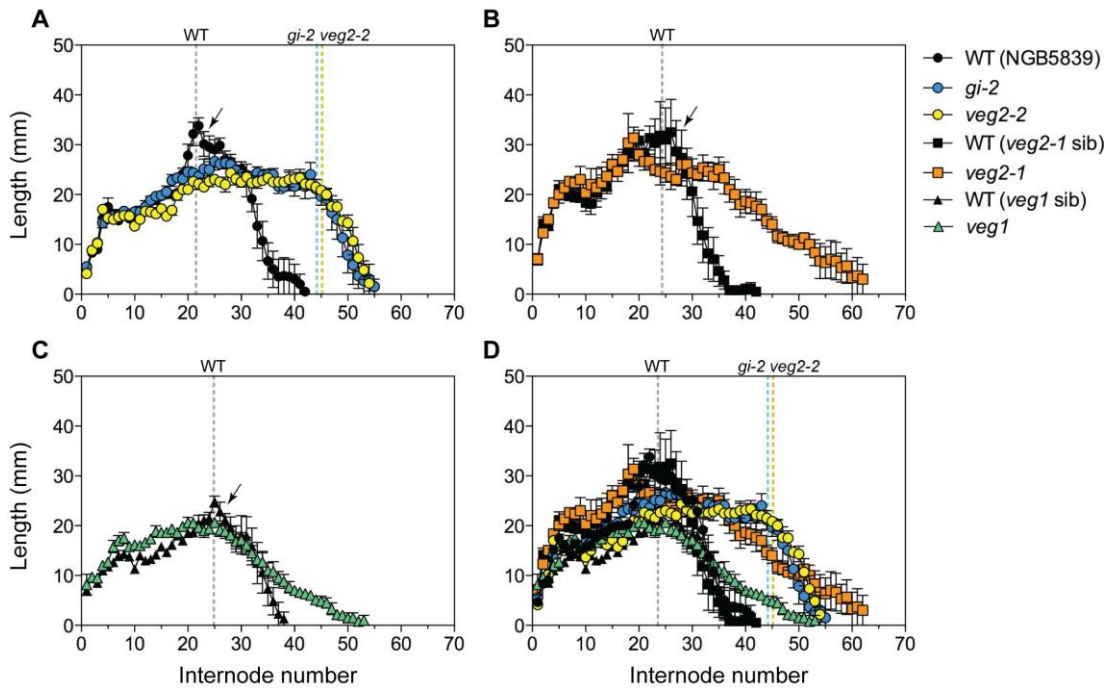
In summary, both *gigas* and *veg1* mutants showed similar timing of *DET* upregulation and similar pattern of ontogenetic variation in internode length, relative to wild-type (Figures 3.2 and 3.3). In contrast, both *veg2* mutants showed delayed *DET* upregulation and a different pattern of ontogenetic variation in internode length to wild-type (Figures 3.2 and 3.3). As both of these traits are normally associated with the V/I<sub>1</sub> transition, it seems likely that this transition is altered in the *veg2* mutants under LD conditions. This data suggests that *VEG2* is important for the correct timing of the V/I<sub>1</sub> transition under LD conditions.

### 3.3.1.3 *Investigating the relative timing of the V/I<sub>1</sub> transition under SD conditions*

When wild-type pea plants are grown in non-inductive SD conditions, flowering occurs later than under LD photoperiods, floral initials are often aborted at the first few reproductive nodes, and pod development and monopodial senescence is delayed (Haupt, 1969; Gianfagna and Davies, 1981; Murfet, 1985). Changing photoperiod from LD to non-inductive SD conditions does not alter the basic phenotypes of the non-flowering *veg2-1* mutant, late-flowering *veg2-2* mutant, or non-flowering *veg1* mutant (Reid and Murfet, 1984; Murfet and Reid, 1993). However, photoperiod has a large effect on the *gigas* mutant phenotype, which is non-flowering under LD conditions but does flower, albeit considerably later than wild-type under SD conditions (e.g. Murfet, 1992). In this study, ontogenetic variation in internode length was investigated in conjunction with expression of the I<sub>1</sub> meristem marker *DET* to investigate the relative timing of the V/I<sub>1</sub> transition in *veg2-1*, *veg2-2* and *gigas-2*, in comparison with wild-type and *veg1* under a SD (8h) photoperiod.

Similar to observations for LD conditions (Figure 3.3), internode length in wild-type plants grown under SD conditions exhibited an increase with development (Figure 3.4). In wild-type plants, a small peak was present between internodes 5 and 10, and a second larger peak occurred immediately prior to flowering, between internodes 22 and 27 (Figure 3.4). A decrease in internode length coincided with the onset of reproductive development (Figure 3.4). The *veg1* mutant showed a decrease in internode length at the same time as wild-type (Figure 3.4C), as occurred under LD conditions (Figure 3.3C). The *veg2-2* mutant showed a delay in the timing of internode length decrease, relative to wild-type under SD conditions, with internode length decreasing in *veg2-2* mutants at approximately internode 42 (Figure 3.4A). This decrease began prior to the first flowering node in *veg2-2* (mean NFI  $\pm$  SE =  $45.2 \pm 0.5$ ), similar to findings from LD conditions (Figures 3.3A and 3.4A). Interestingly, in contrast to findings under LD conditions (Figure 3.3A), the *gigas* mutant showed a delay in the timing of internode length decrease under SD conditions, sharing the pattern shown by the *veg2-2* mutant (Figure 3.4A). This reflected the similarity in flowering time between *gigas* and *veg2-2* under SD conditions (mean NFI  $\pm$  SE: *gi-2* =  $44.2 \pm 2.0$ ;  $p = 0.830$ ), which both flowered significantly later than wild-type (wild-type NFI =  $21.5 \pm 0.4$ ;  $p < 0.001$ ). The *veg2-1*

mutant showed a different pattern under SD than seen in LD (Figure 3.3B), with three peaks, the first similar to the small wild-type peak at approximately internode 9, the second, and largest, prior to that in wild-type at approximately internode 19, and the third small peak at approximately internode 29 (Figure 3.4B). These results suggest that the  $V/I_1$  transition in *gigas* and *veg2* mutants may be altered relative to wild-type and the *veg1* mutant under SD conditions.



**Figure 3.4.** Ontogenetic variation in internode length under SD conditions.

(A) Plants with NGB5839 background: wild-type (NGB5839), late-flowering *veg2-2* and late-flowering *gigas* (*gi-2*) plants.

(B) *veg2-1* segregants: wild-type siblings and non-flowering *veg2-1* mutant plants.

(C) *veg1* segregants: wild-type siblings and non-flowering *veg1* mutant plants.

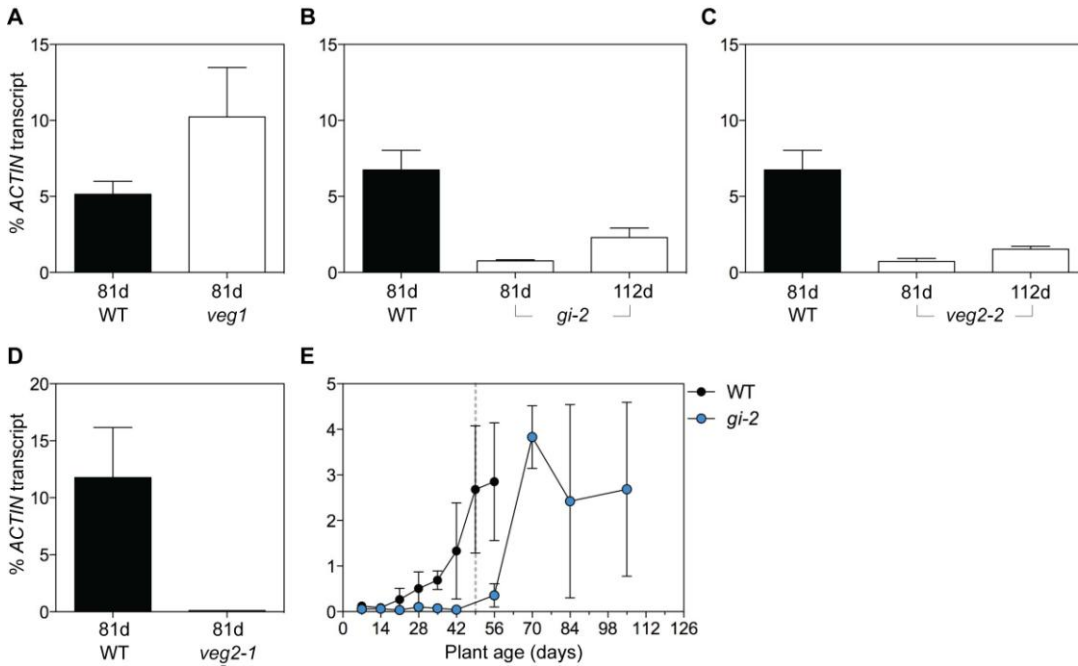
(D) All genotypes from (A) to (C) on the same plot.

Internodes are numbered with internode 1 between the first and second scale leaf. Data points represent mean  $\pm$  standard error for  $n = 3-6$  plants per genotype. Broken vertical lines indicate the mean internode between the first and second reproductive nodes for the genotypes which flowered during this experiment: wild-type (grey; combined mean shown where multiple wild-types are present in one graph), *gi-2* (blue) and *veg2-2* (yellow). Arrows point to the first node of decreased internode length in wild-type plants.

As an additional means of investigating timing of the V/I<sub>1</sub> transition, expression of the I<sub>1</sub> marker gene *DET* was examined in apices of *veg1*, *gigas-2*, *veg2-1* and *veg2-2* mutants. In the first experiment, two time-points were chosen to coincide with the expected peaks in *DET* expression for wild-type and *gigas/veg2-2* plants. Similar to wild-type plants, *veg1* exhibited a high level of *DET* expression after the wild-type V/I<sub>1</sub> transition had occurred, 81 days after sowing (Figure 3.5A). Both *gigas* and *veg2-2* mutants showed a low level of *DET* expression at this first time-point, but a higher level of *DET* expression after the V/I<sub>1</sub> transition had occurred (Figure 3.5B-C). *DET* had not been upregulated in the *veg2-1* mutant after the time of the wild-type V/I<sub>1</sub> transition (Figure 3.5D). Insufficient numbers of *veg2-1* mutants among segregants prevented testing of *DET* expression at a second, later time-point to show if *DET* is upregulated in the *veg2-1* mutant by the time of the *veg2-2* V/I<sub>1</sub> transition.

*DET* expression was further analysed in wild-type and *gigas* shoot apices over a developmental series in a second experiment covering time-points from 1-8 weeks after sowing in wild-type, and from 1-15 weeks after sowing in *gigas* (Figure 3.5E). In the developmental series, there was a clear delay (of approximately 5 weeks) in the expression of *DET* in *gigas* compared to wild-type plants (Figure 3.5E).

Using *DET* as a marker of I<sub>1</sub> meristem identity, these results provide further evidence suggesting that the V/I<sub>1</sub> transition occurs at a similar time to wild-type in the *veg1* mutant, but is delayed in the *veg2-2* and *gigas* mutants under SD conditions. This suggests that *VEG2* and *GIGAS* are both important for correct timing of the V/I<sub>1</sub> transition under SD conditions.



**Figure 3.5.** Expression of *DET* as an indicator of  $I_1$  meristem identity, under SD conditions.

*DET* expression in shoot apices (main stem or longest branch) in (A) *veg1*, (B) *gigas* (*gi-2*), (C) *veg2-2*, and (D) *veg2-1* after the time of the V/ $I_1$  transition in wild-type (81 days after sowing), and in late-flowering *gigas* and *veg2-2* mutants (112 days after sowing). (E) *DET* expression in wild-type and *gigas* across a developmental series covering time-points from 1 to 15 weeks after sowing, in a separate experiment to that shown in (A–D). Dashed line in (E) indicates the timing of first macroscopic appearance of floral buds in the wild-type apex. Wild-type lines are as follows: (A) wild-type siblings of *veg1* and (B, C, E) NGB5839, (D) wild-type siblings of *veg2-1*. For relative transcript levels, values have been normalised to the transcript level of *ACTIN* and are shown as mean percentage *ACTIN*  $\pm$  standard error for 1–3 biological replicates.

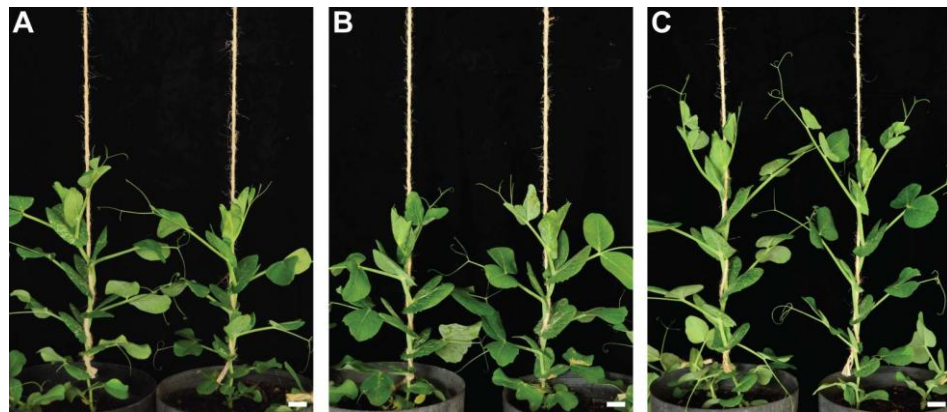
### 3.3.2 Characterisation of aerial branching

It has previously been proposed that the onset of the aerial branching phenotype in non-flowering pea mutants could be an indicator of the V/ $I_1$  transition, with these lateral branches interpreted as homeotic substitutions for  $I_2$  structures (Coen, 1991; Murfet and Reid, 1993; Wiltshire et al., 1994; Reid et al., 1996; Berbel et al., 2012). Aerial branching has not previously been characterised in any detail in the *veg2* mutants. Based on results from earlier experiments investigating relative timing of the V/ $I_1$  transition (Figures 3.2–3.5), it could be expected that timing of branch outgrowth may be delayed in *veg2-1* and *veg2-2* in LD and SD, and in *gigas* in SD, relative to wild-type and *veg1*, if this trait reflects timing of the V/ $I_1$  transition. As increased branching can result from removing flowers from wild-type plants (e.g. Lockhart and Gottschall, 1961), an alternative possibility is that branch outgrowth may be associated with the delayed timing or absence of flowering and pod

development in late- and non-flowering mutants, respectively. In this study, branching was characterised in *veg2-1*, *veg2-2*, *gigas-2* and *veg1* mutants, deflowered wild-type plants (all flowers removed after anthesis; LD only) and corresponding intact wild-type plants, with reference to flowering time, under both LD and SD conditions.

### 3.3.2.1 *Flowering time*

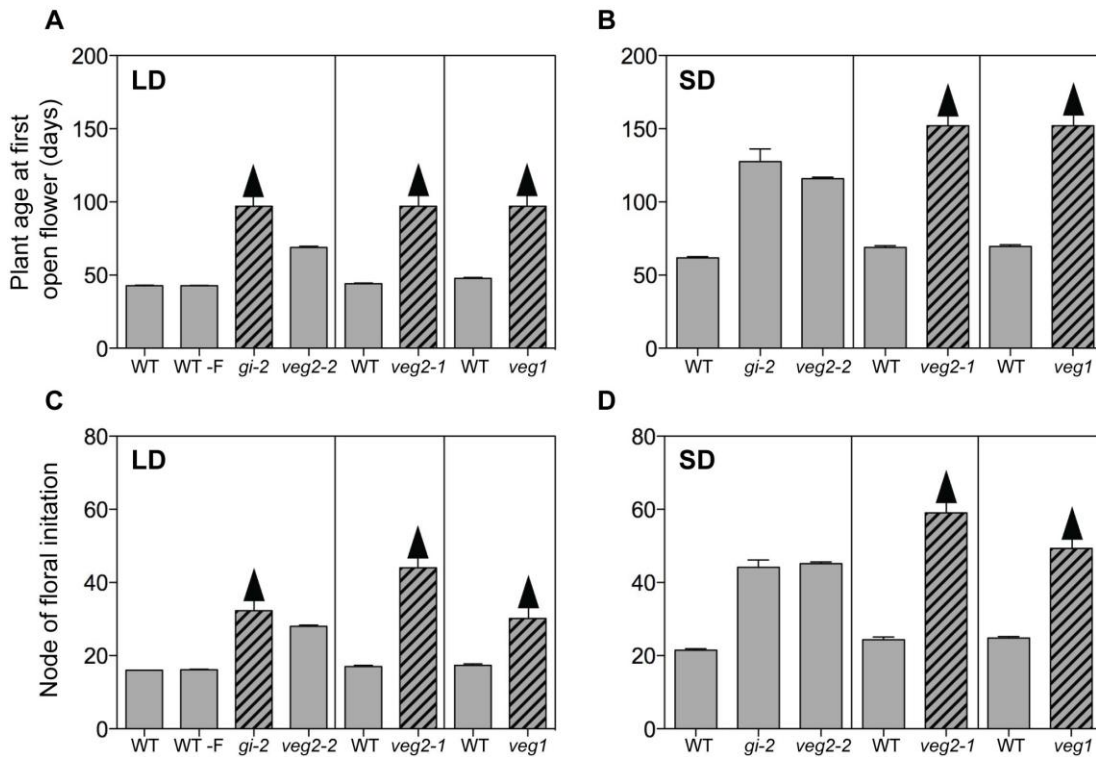
During vegetative development, before the appearance of flowers in wild-type plants, all mutants shared a wild-type appearance (Figure 3.6), under both photoperiods, as previously described (Murfet, 1985; Beveridge et al., 2003; Berbel et al., 2012). Flowering time was measured in wild-type and late-flowering mutants, using two measures commonly employed in pea: plant age at first open flower (DTF) and NFI. Flowering was significantly delayed in *veg2-2* relative to wild-type under both LD and SD conditions, both in terms of DTF and NFI (Figure 3.7;  $p < 0.001$ ), in accordance with previous observation (Murfet, 1992). *gigas* was non-flowering under LD conditions and flowered significantly later than wild-type under SD conditions both in terms of DTF and NFI (Figure 3.7;  $p < 0.001$ ). *veg2-1* and *veg1* plants were non-flowering under both photoperiods (Figure 3.7).



**Figure 3.6.** Similar appearance of non-flowering mutants and wild-type pea plants during vegetative growth.

(A) Wild-type (NGB5839, left) and *gigas-2* mutant (right). (B) Wild-type (sibling, left) and *veg1* mutant (right). (C) Wild-type (sibling, left) and *veg2-1* mutant (right). Plants depicted were grown under LD conditions and are shown 33 days after sowing. Scale bars represent 1 cm.





**Figure 3.7.** Flowering time in plants used for characterisation of lateral outgrowth.

(A-B) Plant age at first open flower (DTF; days after sowing) under (A) LD and (B) SD photoperiods.

(C-D) Node of floral initiation (NFI) under (C) LD and (D) SD photoperiods.

For non-flowering *gigas* (*gi-2*; non-flowering LD only), *veg2-1* and *veg1* mutants, bars with diagonal hatching and arrow show (A-B) harvest date or (C-D) total nodes with expanded leaves at harvest date, to indicate that these plants did not flower during this experiment. Wild-type lines are as follows, left to right for each photoperiod: intact NGB5839, deflowered NGB5839 (WT-F: LD only), wild-type sibling of *veg2-1*, wild-type sibling of *veg1*. Values represent mean  $\pm$  standard error for  $n = 3-6$  plants per genotype.

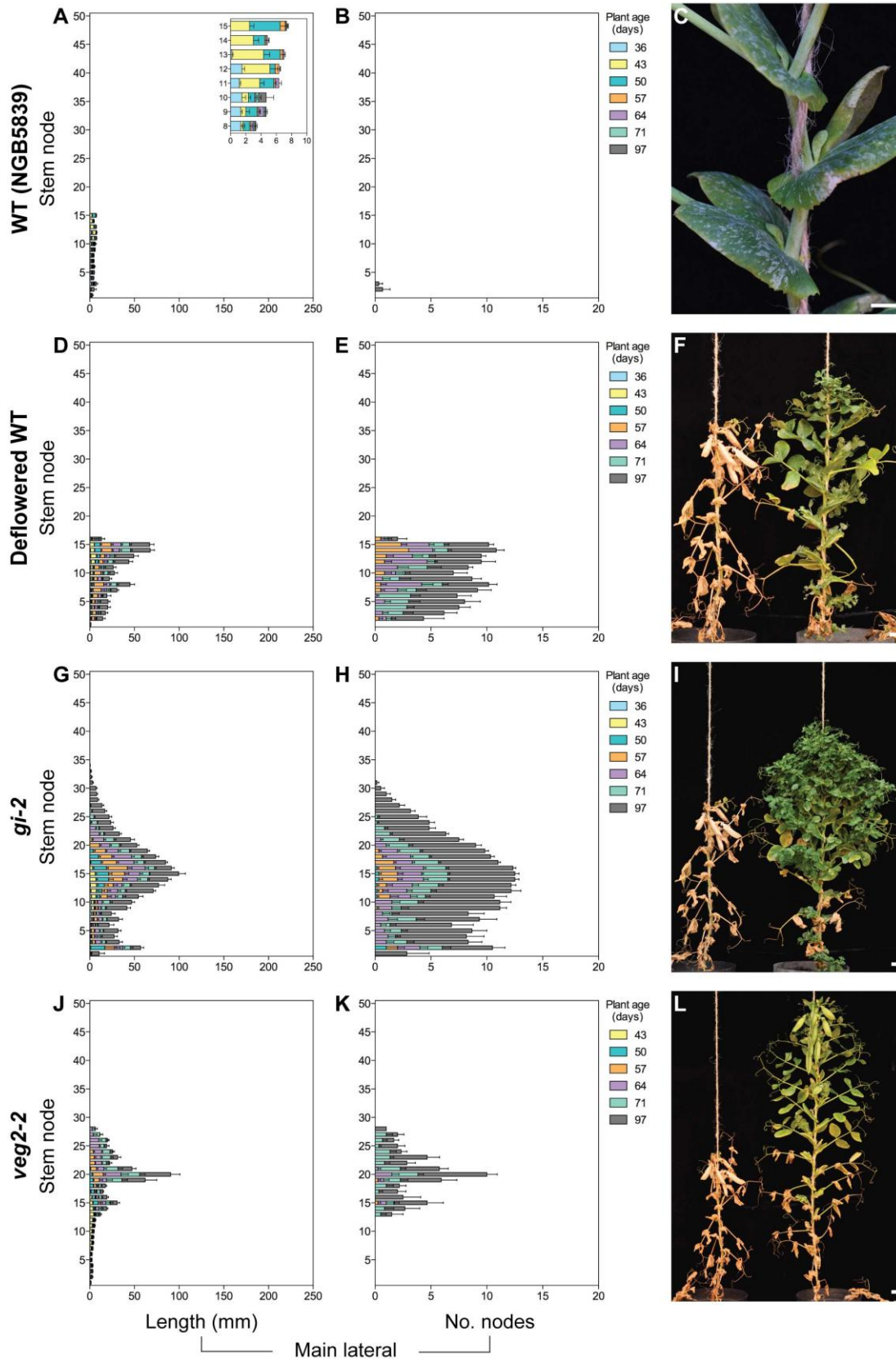
### 3.3.2.2 Characterisation of lateral outgrowth

To characterise branching, the length and number of nodes with expanded leaves was measured for the main lateral at each node on the plant stem during plant development under LD and SD conditions. Six weekly time-points covered early lateral outgrowth and establishment of the aerial branching phenotype in the mutants, and a final seventh time-point was taken at plant harvest after seed maturation and drying had occurred in wild-type plants. In SD conditions, laterals with expanded leaf nodes were already well established at basal nodes prior to the first time-point.

As the plants developed, all genotypes (wild-type and mutant) showed some lateral outgrowth under both LD and SD photoperiods (Figures 3.8 and 3.9). Across genotypes there was a general trend for initial outgrowth to occur within a variable zone of aerial nodes, sometimes over several nodes simultaneously, with outgrowth radiating to higher nodes and lower nodes with time (Figures 3.8 and 3.9). This trend

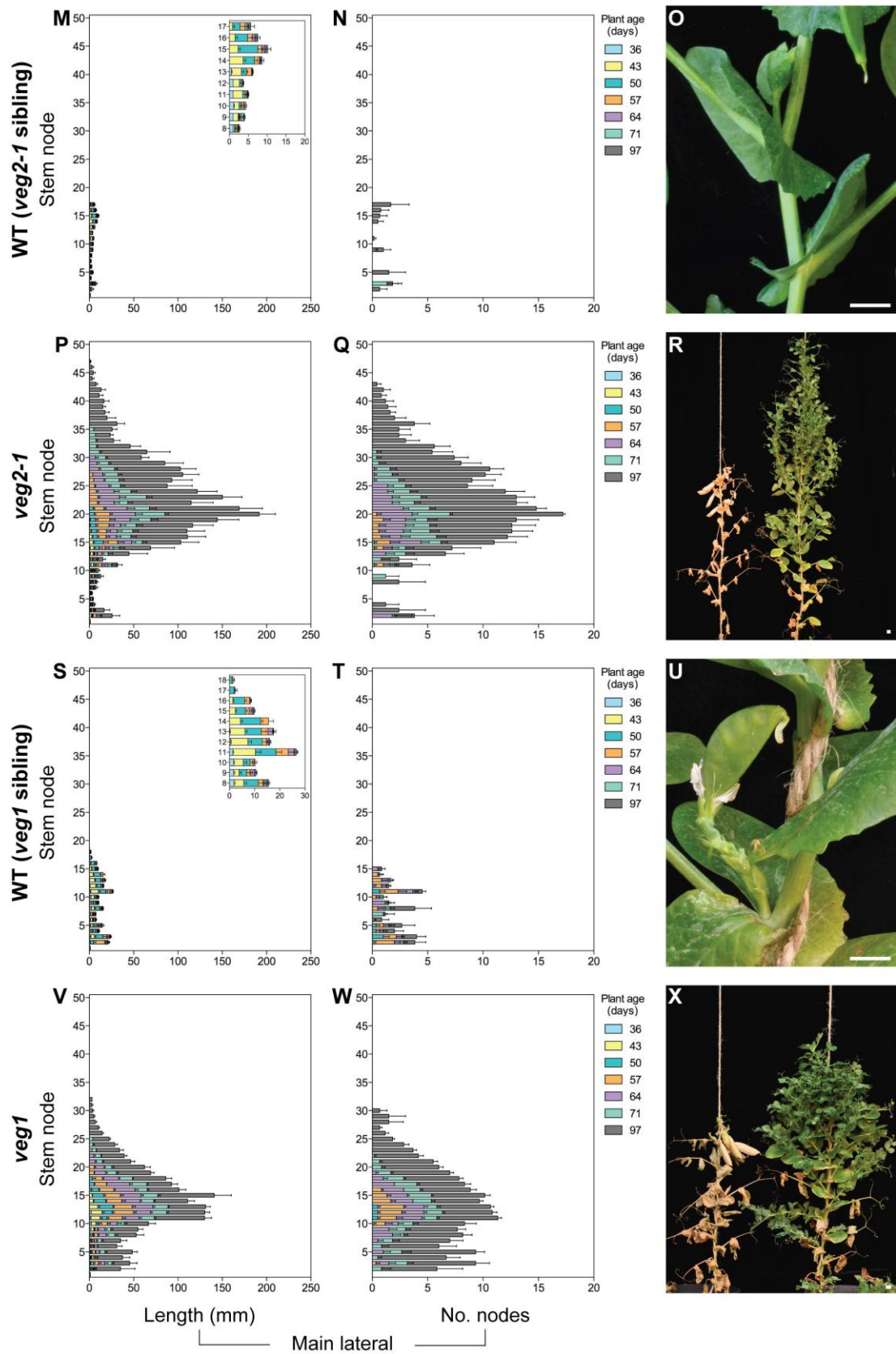


was clearest in the non-flowering mutants. Once an axillary bud exhibited outgrowth (evident as an increase in length), one of two fates was adopted. Fate 1: development arrested at the new, larger size until the bud yellowed and dried as the plant senesced (e.g. Figure 3.8C). Fate 2: development continued and a branch with expanded leaves was formed (e.g. Figure 3.9L). The second fate was usually adopted in deflowered wild-type plants and all mutants under LD conditions, and in all plants under SD conditions (Figures 3.8 and 3.9). Branches shared the fate of the main stem, flowering and undergoing senescence several weeks after the main stem in wild-type plants and the late-flowering mutants (*veg2-2*, *gigas* in SD only), and exhibiting indefinite vegetative growth until plant harvest in the non-flowering mutants (Figures 3.8 and 3.9). In Figures 3.8 and 3.9, branches appear at higher nodes in the mutants relative to wild-type plants (both intact and deflowered), and in the non-flowering mutants relative to the late-flowering mutants. This can be explained by the presence of an  $I_2$  as the main lateral at each reproductive node in the plants that flowered, and  $I_2$  structures were not measured for this portion of the study.



**Figure 3.8.** Characterisation of lateral outgrowth under LD conditions.

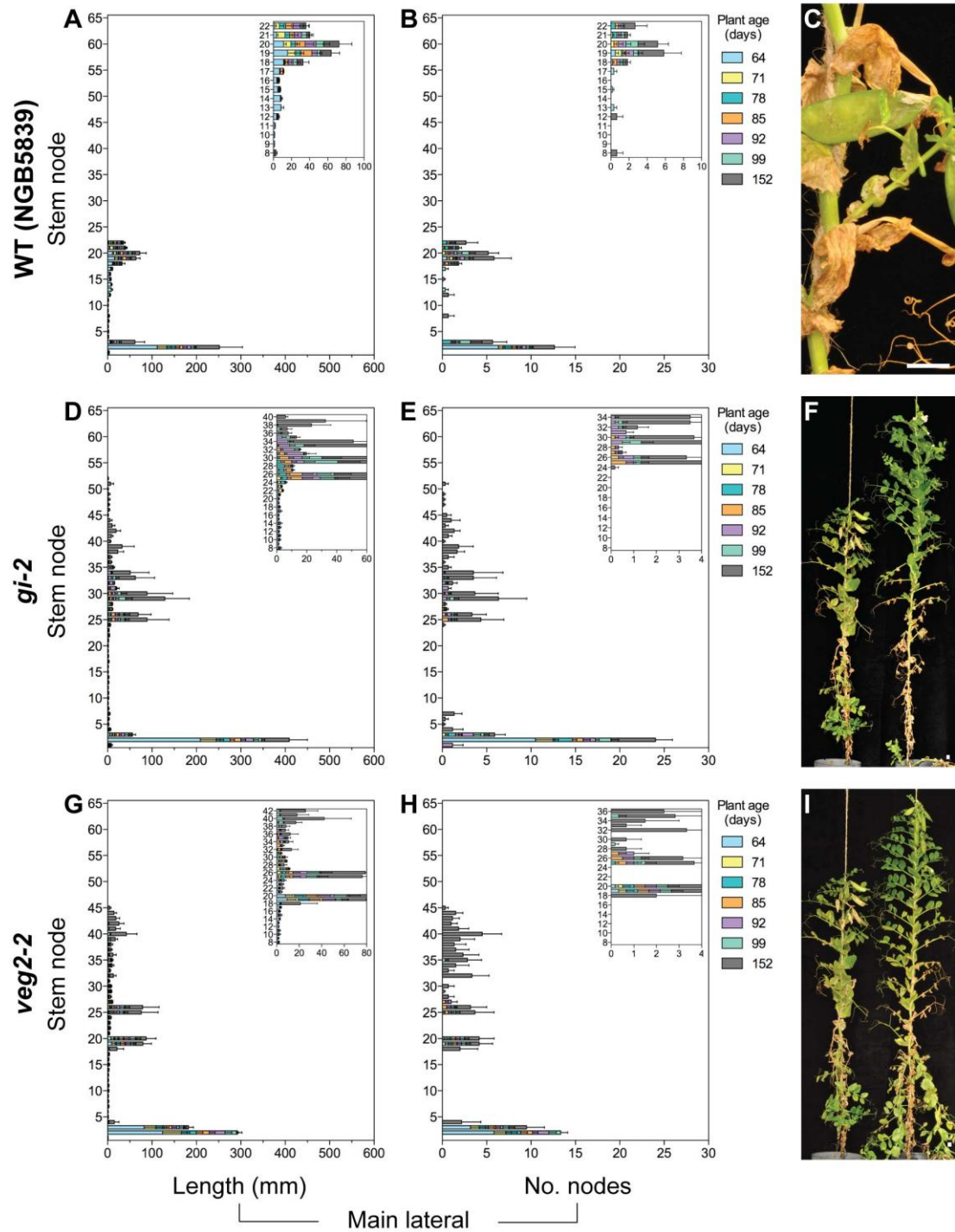
(A-C) Intact wild-type (NGB5839), (D-F) deflowered wild-type (NGB5839), (G-I) non-flowering *gigas* (*gi-2*) mutant, (J-L) late-flowering *veg2-2* mutant. (continued on next page)



**Figure 3.8. (continued) (M-O)** Wild-type for *veg2-1*, **(P-R)** non-flowering *veg2-1* mutant, **(S-U)** wild-type for *veg1*, **(V-X)** non-flowering *veg1* mutant. (continued on next page)

**Figure 3.8. (continued)** (A, B, D, E, G, H, J, K, M, N, P, Q, S, T, V, W) Graphical representations of lateral development based on measurements taken at six weekly time-points (36-71 days after sowing) and at plant harvest (97 days after sowing). (A, D, G, J, M, P, S, V) Increase in lateral length. (B, E, H, K, N, Q, T, W) Increase in number of nodes on the main lateral at each node.  $I_2$  structures are not included in lateral measurements. Stem node (main stem only) is shown on the y-axis and lateral length on the x-axis to represent an upright plant. Mean values  $\pm$  standard error for  $n = 5-6$  plants are shown. For A, M and S aerial nodes from node 8 upwards are magnified inset for clarity.

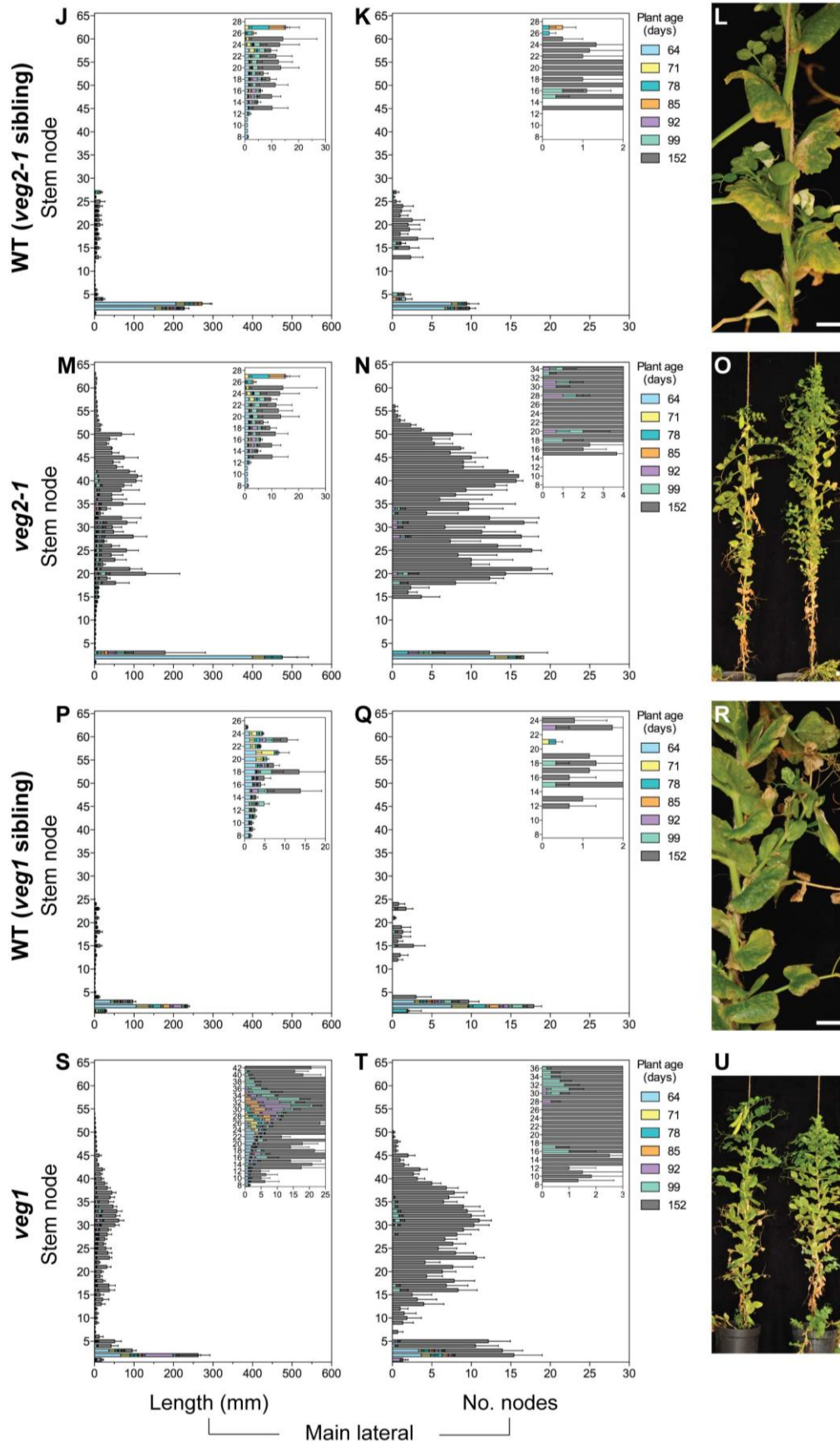
Plant photos are as follows: (C) wild-type (NGB5839) axillary buds that showed initial outgrowth ( $>5\text{mm}$ ) before development arrested shown 97 days after sowing, (F) intact (left) and deflowered (right) wild-type (NGB5839) plants shown 93 days after sowing, (I) wild-type (NGB5839; left) and *gi-2* mutant (right) shown 93 days after sowing, (L) wild-type (NGB5839; left) and *veg2-2* mutant (right) shown 93 days after sowing, (O) developing branch in wild-type sibling of *veg2-1* shown 53 days after sowing, (R) wild-type (sibling; left) and *veg2-1* mutant (right) shown 93 days after sowing, (U) flowering branch in wild-type sibling of *veg1* shown 73 days after sowing, (X) wild-type (sibling; left) and *veg1* mutant (right) shown 93 days after sowing. Scale bars represent 1cm.



**Figure 3.9.** Characterisation of lateral outgrowth under SD conditions.

(A-C) Wild-type (NGB5839), (D-F) late-flowering *gigas* (*gi-2*) mutant, (G-I) late-flowering *veg2-2* mutant. (continued on next page)





**Figure 3.9. (continued)** (J-L) Wild-type for *veg2-1*, (M-O) non-flowering *veg2-1* mutant, (P-R) wild-type for *veg1*, (S-U) non-flowering *veg1* mutant. (continued on next page)

**Figure 3.9. (continued)** (A, B, D, E, G, H, J, K, M, N, P, Q, S, T) Graphical representations of lateral development based on measurements taken at six weekly time-points (64-99 days after sowing) and at plant harvest (152 days after sowing). (A, D, G, J, M, P, S) Increase in lateral length. (B, E, H, K, N, Q, T) Increase in number of nodes on the main lateral at each node.  $I_2$  structures are not included in lateral measurements. Stem node (main stem only) is shown on the y-axis and lateral length on the x-axis to represent an upright plant. Mean values  $\pm$  standard error for  $n = 3-6$  plants are shown. Aerial nodes from node 8 upwards are magnified inset for clarity.

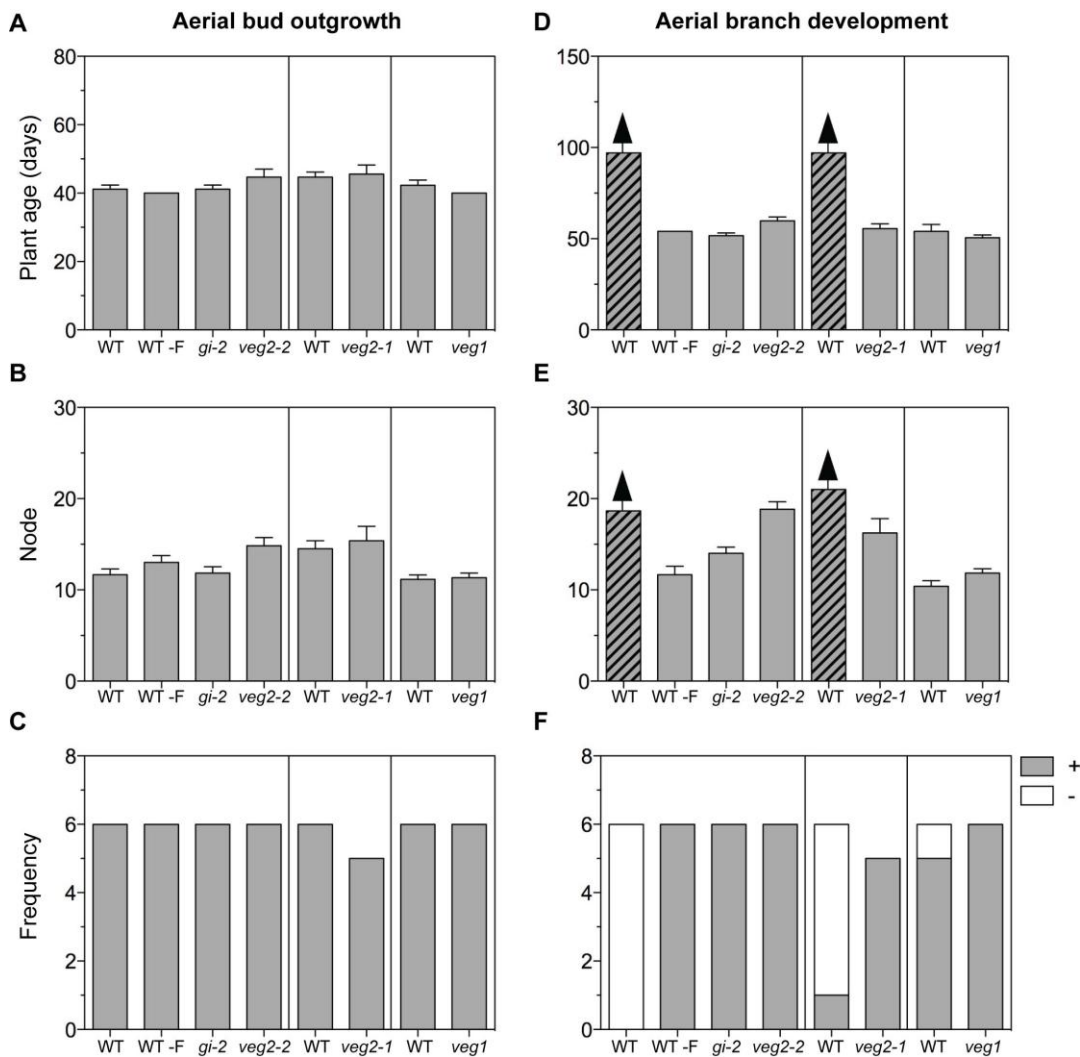
Plant photos are as follows: (C) flowering branch in wild-type (NGB5839) shown 135 days after sowing, (F) wild-type (NGB5839; left) and *gi-2* mutant (right) shown 134 days after sowing, (I) wild-type (NGB5839; left) and *veg2-2* mutant (right) shown 134 days after sowing, (L) flowering branches in wild-type sibling of *veg2-1* shown 134 days after sowing, (O) wild-type (sibling; left) and *veg2-1* mutant (right) shown 135 days after sowing, (R) flowering branch in wild-type sibling of *veg1* shown 134 days after sowing, (U) wild-type (sibling; left) and *veg1* mutant (right) shown 134 days after sowing. Scale bars represent 1cm.

### 3.3.2.3 *Timing of lateral outgrowth*

In the same experiment, the timing of lateral outgrowth was investigated in more detail. Two stages of lateral development were arbitrarily defined. The term ‘enlarged aerial bud’ was used to describe laterals which developed to a length of 5mm or longer, at node 8 or above on the main stem. The term ‘aerial branch’ was used to describe laterals that had developed one or more nodes with fully expanded leaves, at node 8 or above on the main stem. The timing of each of these two stages was investigated in terms of both plant age at first occurrence and node of first occurrence.

Under LD conditions, one or more enlarged aerial buds developed in all plants, allowing comparison of lateral outgrowth between all genotypes (Figure 3.10A-C). Enlarged aerial buds first developed between 37 and 57 days after sowing, between nodes 9 and 20 on the main stem (Figure 3.10A-C). In terms of plant age, this timing of outgrowth overlapped with the wild-type timing of floral development, but not with that of the late-flowering *veg2-2* mutant (Figure 3.7A). There was no significant difference between the genotypes in terms of mean plant age at first enlarged aerial bud development (Figure 3.10A;  $p = 0.058$ ). In *veg2-2*, enlarged aerial buds first developed at a significantly later node than in intact wild-type (Figure 3.10B;  $p = 0.036$ ). However, there was no significant difference between *veg2-2* and deflowered wild-type plants (Figure 3.10B;  $p = 0.345$ ), which shared the same flowering time as intact wild-type plants (Figure 3.7A-B). There was also no significant difference between *veg2-1* and wild-type in terms of node of first enlarged aerial bud (Figure 3.10B;  $p = 0.614$ ). In summary, there were no consistent

differences in timing of first enlarged aerial bud development between wild-type and both *veg2* mutants (Figure 3.10).



**Figure 3.10.** Timing of lateral outgrowth under LD conditions.

(A-C) Development of an axillary bud  $\geq 5$ mm, at node 8 or higher on the plant stem ('enlarged aerial bud'). (A-B) Timing of first development in terms of (A) plant age (days after sowing) at first occurrence and (B) node of first occurrence. (C) The frequency of plants with presence (+; grey) or absence (-; white) of any enlarged aerial buds on the main stem at time of plant harvest (97 days after sowing).

(D-F) Development of an 'aerial branch' with an expanded leaf, at node 8 or higher on the plant stem. (D-E) Timing of first development, in terms of (D) plant age (days after sowing) and (E) node of first appearance. (F) Frequency of plants with presence (+; grey) or absence (-; white) of any aerial branches on the main stem at time of plant harvest. Genotypes for which aerial branches did not develop in a majority of plants within the time-frame of this experiment are represented by bars with diagonal hatching showing (D) plant age at harvest, or (E) mean total nodes with expanded leaves at harvest.

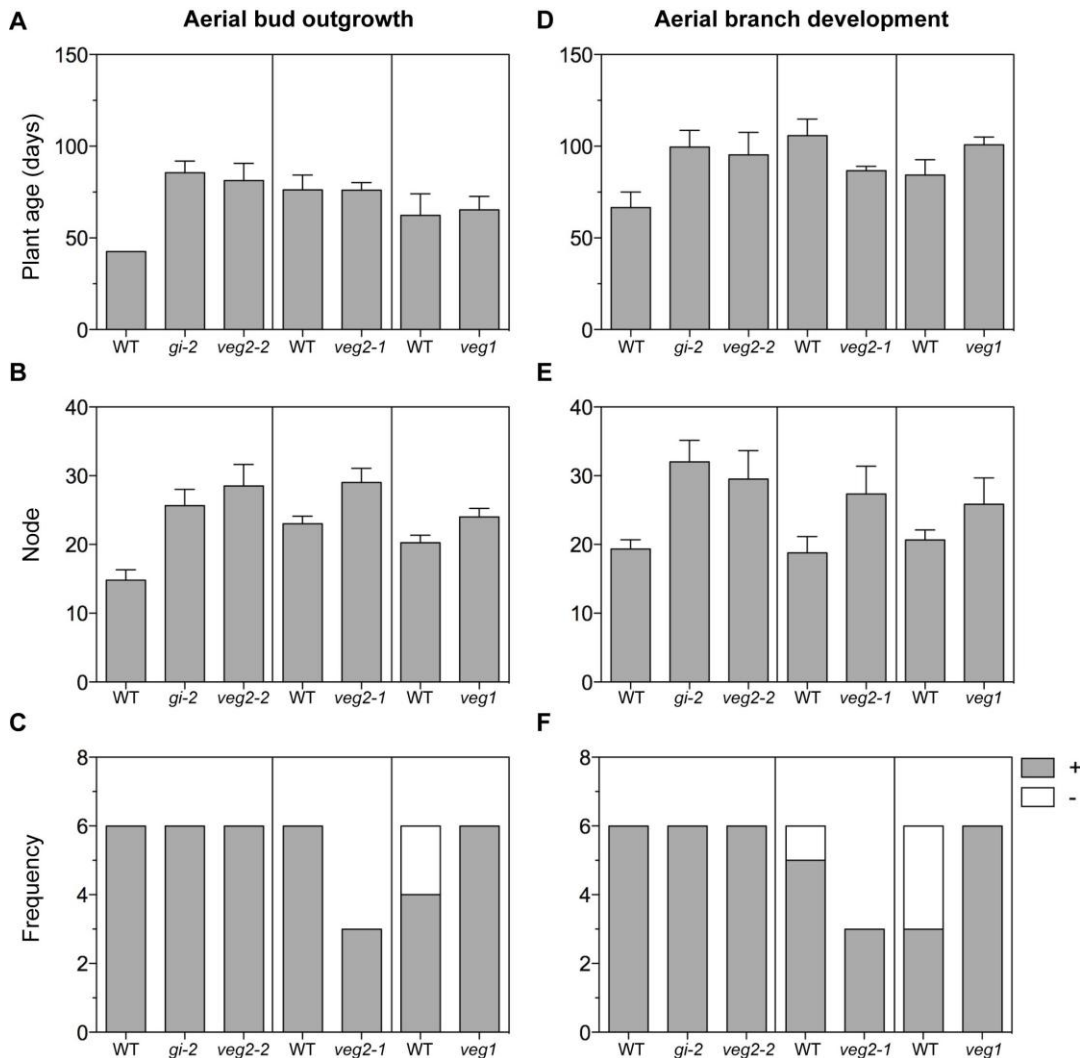
Wild-type lines are as follows, from left to right: intact wild-type line NGB5839, deflowered wild-type (WT-F; NGB5839), wild-type siblings of *veg2-1*, wild-type siblings of *veg1*.  $n = 5-6$  plants per genotype. For (A, B, D, E) values represent mean  $\pm$  standard error.



To focus on laterals that developed further, the timing of first aerial branch development was also investigated under LD conditions (Figure 3.10D-F). Aerial branches developed in a majority of deflowered wild-type, *gigas*, *veg2-2*, *veg2-1*, wild-type for *veg1*, and *veg1* plants, allowing comparison of timing of aerial branch development between these genotypes (Figure 3.10D-F). However the majority of plants for wild-type line NGB5839 and wild-type *veg2-1* siblings did not develop any aerial branches prior to the time of plant harvest (Figure 3.10F), so these genotypes were excluded from further comparisons. There was no significant difference in plant age at time of first aerial branch development between any of the genotypes with aerial branches (Figure 3.10D;  $p = 0.053$ ). Node of first branch development was significantly later in *veg2-1* compared to deflowered wild-type ( $p = 0.016$ ) and *veg1* ( $p = 0.022$ ), and in *veg2-2* compared to deflowered wild-type ( $p < 0.001$ ), *gigas* ( $p = 0.006$ ) and *veg1* ( $p < 0.001$ ; Figure 3.10E). However, there was no significant difference in node of first branch development between *veg2-1* and *gigas* (Figure 3.10E;  $p = 0.531$ ). Overall, the timing of aerial branch development was not consistently delayed in the *veg2* mutants relative to wild-type, *gigas* and *veg1*. Based on earlier results that suggest a delay in the timing of the V/I<sub>1</sub> transition in *veg2-1* and *veg2-2* relative to wild-type, *gigas* and *veg1* (Figures 3.2 and 3.3), the timing of aerial branch outgrowth did not appear to reflect the timing of the V/I<sub>1</sub> transition under LD conditions.

Next, the timing of lateral outgrowth was investigated under SD conditions. At least one enlarged axillary bud developed in a majority of plants of each genotype under SD conditions (Figure 3.11C). There was no significant difference in plant age at first enlarged axillary bud development between *veg2-1* and wild-type ( $p = 1.000$ ), or between *veg2-2* and wild-type (Figure 3.11A;  $p = 0.070$ ). Node of first enlarged aerial bud was significantly later in *veg2-2* and *gigas* than in wild-type ( $p = 0.05$ ,  $p = 0.041$ , respectively), but there was no significant difference between *veg2-1* and wild-type (Figure 3.11B;  $p = 0.361$ ). Under SD conditions, at least one aerial branch developed in a majority of plants of each genotype (Figure 3.11F), but there was no significant difference between genotypes in plant age at first aerial branch development (Figure 3.11D;  $p = 0.059$ ) or in node of first aerial branch (Figure 3.11E;  $p = 0.065$ ). Overall, the timing of lateral outgrowth by these measures did not

reflect the differences in the timing of the V/I<sub>1</sub> transition between mutants under SD conditions, inferred from earlier results (Figures 3.4 and 3.5).



**Figure 3.11.** Timing of lateral outgrowth under SD conditions.

(A-C) Development of an axillary bud  $\geq 5$ mm, at node 8 or higher on the plant stem ('enlarged aerial bud'). (A-B) Timing of first development in terms of (A) plant age (days after sowing) at first occurrence and (B) node of first occurrence. (C) The frequency of plants with presence (+; grey) or absence (-; white) of any enlarged aerial buds on the main stem at the time of plant harvest (152 days after sowing).

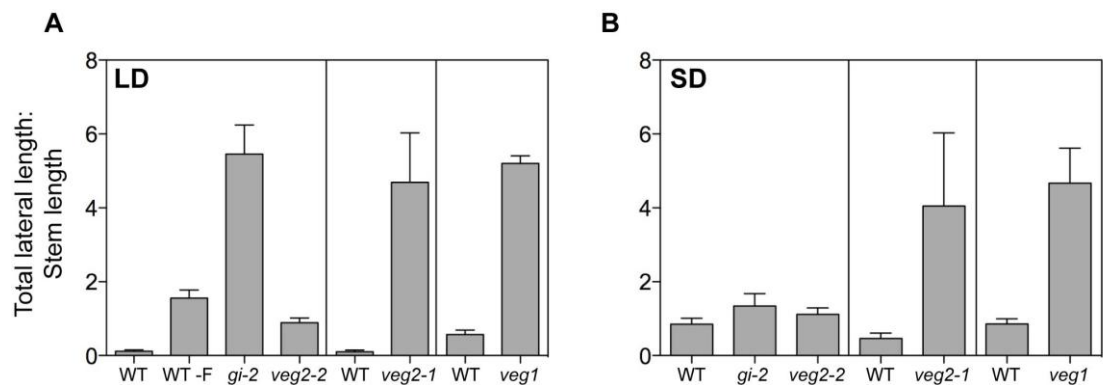
(D-F) Development of an 'aerial branch' with an expanded leaf, at node 8 or higher on the plant stem. (D-E) Timing of first development, in terms of (D) plant age (days after sowing) and (E) node of first appearance. (F) Frequency of plants with presence (+; grey) or absence (-; white) of any aerial branches on the main stem at time of plant harvest.

Wild-type lines are as follows, from left to right: wild-type line NGB5839, wild-type siblings of *veg2-1*, wild-type siblings of *veg1*.  $n = 3-6$  plants per genotype. For (A, B, D, E) values represent mean  $\pm$  standard error.

### 3.3.2.4 *Total branching*

The measurements of lateral outgrowth presented so far in this study have focused on the main lateral bud or branch present at each node. Some plants in this experiment had up to three vegetative laterals arising directly from the main stem at a single node, and exhibited compound branching with up to four orders of branching. In order to characterise the extent of branching in the *veg2* mutants for the first time, total branching in the *veg2* mutants relative to *gigas*, *veg1*, deflowered wild-type and intact wild-type plants, was also compared in this study. To do this, the total length of all laterals was divided by the stem length, giving a ratio that alleviated the effects of differences in internode length between genotypes.

Under LD conditions, total branching was significantly higher ( $p > 0.05$ ) in deflowered wild-type plants, and all late- and non-flowering mutants relative to corresponding intact wild-type plants (Figure 3.12A). Total branching was equally high in all non-flowering mutants (Figure 3.12A;  $p = 0.892$ ). Total branching was also comparable between deflowered wild-type plants and the late-flowering *veg2-2* mutant (Figure 3.12A;  $p = 0.113$ ). Under SD conditions, total branching was comparable between non-flowering *veg2-1* and *veg1* mutants ( $p = 1.000$ ), but not significantly different between the late-flowering mutants (*gigas* and *veg2-2*) and their wild-type line (Figure 3.12B;  $p = 0.371$ ). In summary, total branching was found to be higher in late-flowering mutants than wild-type under LD conditions only, and higher in non-flowering mutants than late-flowering mutants and wild-type under both LD and SD conditions.



**Figure 3.12.** Total branching in wild-type, late- and non-flowering pea mutants.

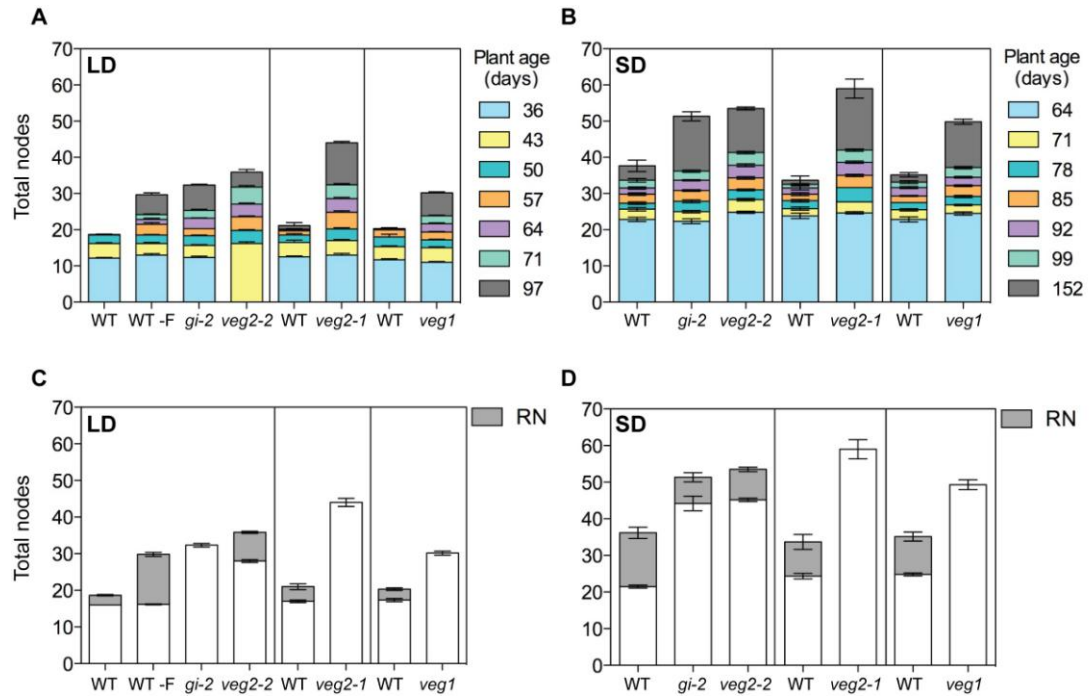
Ratio of total length of all lateral buds or branches  $\geq 5$ mm in length, including additional primary branches and higher order branches, divided by stem length, as measured at time of plant harvest under (A) LD (97 days after sowing) and (B) SD (152 days after sowing) photoperiods.

(continued on next page)

**Figure 3.12. (continued)** Wild-type lines are as follows, from left to right: wild-type line NGB5839, deflowered wild-type (WT-F; NGB5839; LD only), wild-type siblings of *veg2-1*, wild-type siblings of *veg1*. Values represent mean  $\pm$  standard error for  $n = 3-6$  plants per genotype.

### 3.3.2.5 *Apical senescence*

The total number of nodes with expanded leaves was measured at each time-point of the branching experiment, which allows comparison of the relative timing of apical senescence between genotypes. At early time-points, the total number of nodes was comparable between genotypes, indicating that growth rates were similar (Figure 3.13A-B). A significant increase in total nodes was detected in deflowered wild-type and each late- and non-flowering mutant relative to intact wild-type plants ( $p < 0.05$ ), indicating delayed senescence, under both LD and SD photoperiods (Figure 3.13). Under LD conditions, the difference in total nodes relative to intact wild-type plants became significant after wild-type flowering (Figure 3.7A), between 50 and 64 days after sowing (Figure 3.13A). Under SD conditions the difference became significant before the first time-point at 64 days after sowing in *veg2-2* (Figure 3.13B). In the other mutants, the difference also became significant after wild-type flowering (Figure 3.7B), between 78 and 92 days after sowing (Figure 3.13B). Total nodes at plant harvest was higher for wild-type and late-flowering mutants under SD conditions than LD conditions (Figure 3.13). As NFI was not significantly different between intact and deflowered wild-type plants under LD conditions (Figure 3.7C;  $p = 0.868$ ), an increase in total nodes equated to a significantly higher number of reproductive nodes in deflowered wild-type plants relative to intact wild-type plants (Figure 3.13C;  $p < 0.001$ ).



**Figure 3.13.** Total nodes as a measure for relative timing of plant senescence.

(A-B) Total nodes with expanded leaves under (A) LD and (B) SD photoperiods measured at six weekly time-points during flower and branch development (LD: 36-71 days after sowing; SD: 64-99 days after sowing) and a seventh time-point at plant harvest after seed development in intact wild-type plants (LD: 97 days after sowing; SD: 152 days after sowing). Data for the first LD time-point (36d) is not included for *veg2-2*.

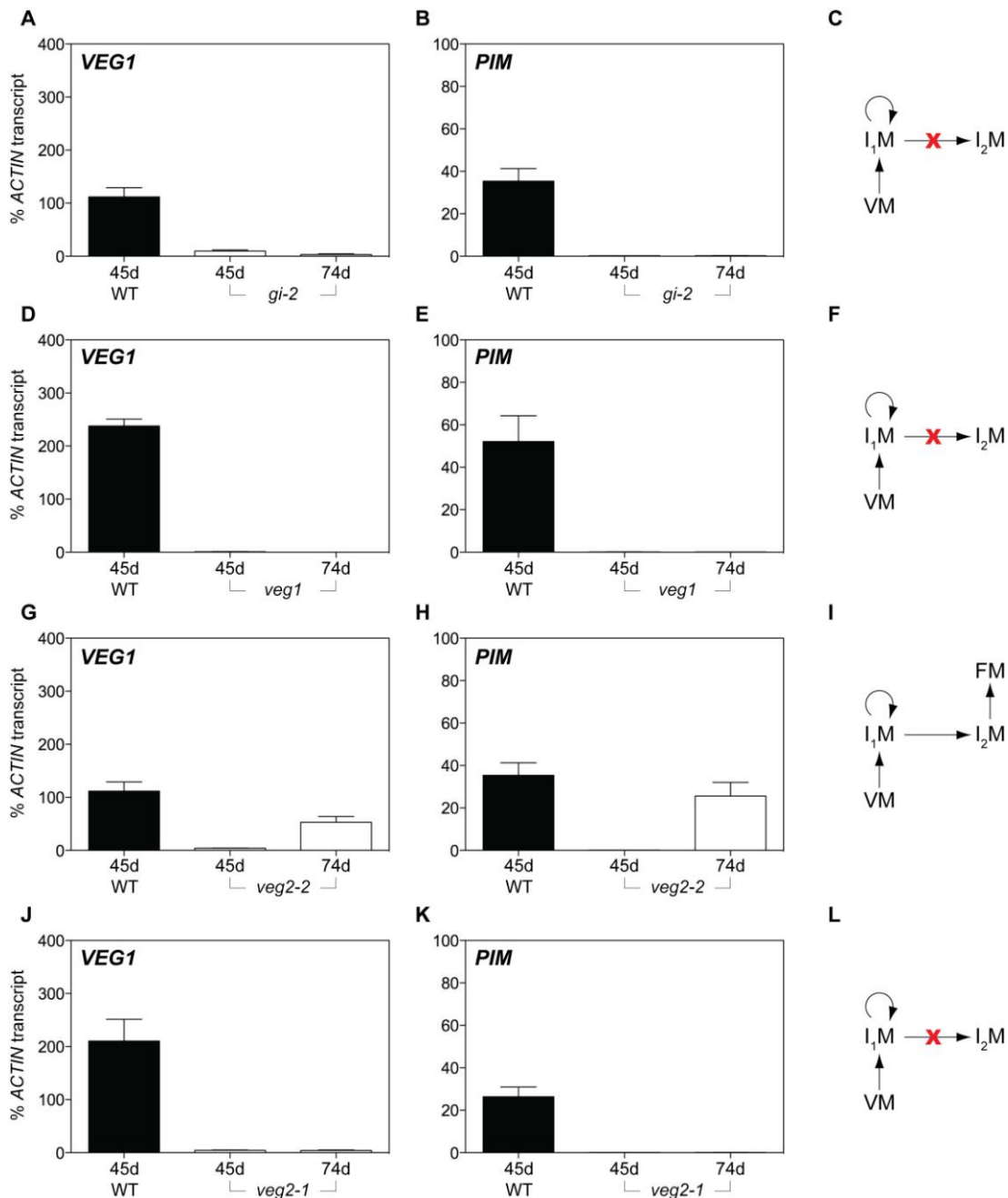
(C-D) Total nodes measured at time of plant harvest in (C) LD and (D) SD photoperiods, with reproductive nodes (RN; bearing an axillary  $I_2$ , starting at the NFI, indicated for genotypes that flowered).

Wild-type lines are as follows, from left to right: wild-type line NGB5839, deflowered wild-type (WT-F; NGB5839; LD only), wild-type siblings of *veg2-1*, wild-type siblings of *veg1*. Values represent mean  $\pm$  standard error for  $n = 3-6$  plants per genotype.

### 3.3.3 Investigating the role of *VEG2* in specification of $I_2$ and floral meristem identity

Expression of *VEG1* and *PIM*, marker genes for  $I_2$  and floral meristem identity, respectively, were investigated in *veg2-1* and *veg2-2* mutants, compared with *gigas-2* and *veg1* mutants, in the same experiment shown earlier in Sections 3.3.1.1 and 3.3.1.3. Neither *VEG1*, nor *PIM* were expressed in the apices of the non-flowering mutants, which is consistent with the lack of visible  $I_2$  or floral structures in these plants (Figure 3.14A-B, D-E, J-K; Figure 3.15D-E, J-K). Upregulation of *VEG1* and *PIM* was delayed in the late-flowering mutants, *veg2-2* and *gigas*, relative to wild-type plants, in accordance with the delayed appearance of  $I_2$  and floral structures seen in these plants (Figure 3.14G-H; Figure 3.15A-B, G-H). The lack of expression of *VEG1* and *PIM* in *veg2-1* confirms that  $I_2$  specification and subsequent

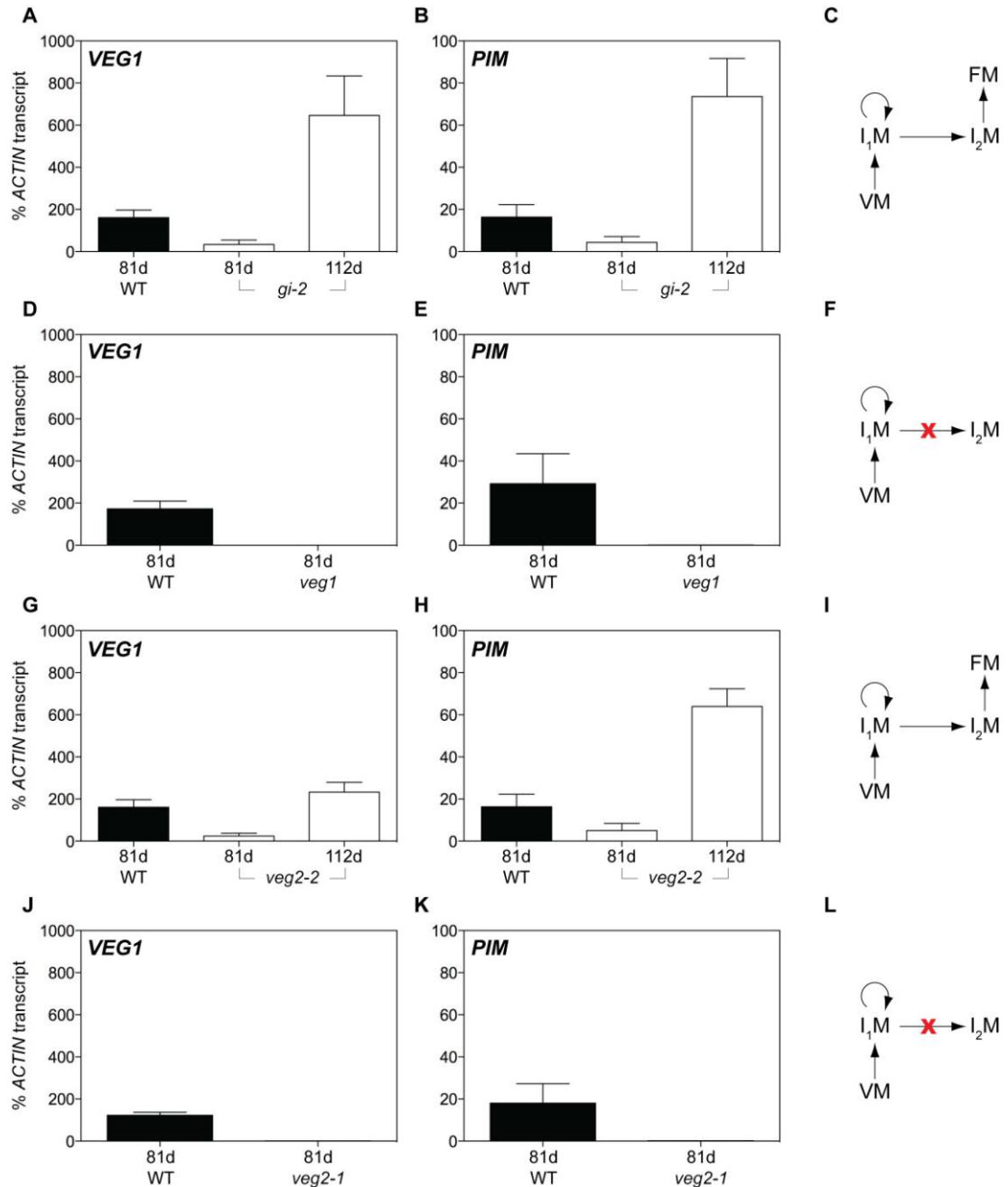
specification of floral meristems is blocked in this mutant. These results support a role for *VEG2* in the specification of  $I_2$  and floral meristems in both LD and SD photoperiods.



**Figure 3.14.** Expression of *VEG1* and *PIM* as indicators for the presence of  $I_2$  and floral meristems, under LD conditions.

(A, B, D, E, G, H, J, K) Expression of *VEG1* and *PIM* in shoot apices of *gigas* (*gi-2*), *veg1*, *veg2-2*, and *veg2-1* mutants and corresponding wild-type plants at time-points 45 and 74 days after sowing. Wild-type lines are as follows: (A, B, G, H) NGB5839, (D, E) wild-type sibling of *veg1*, (J, K) wild-type sibling of *veg2-1*. For relative transcript levels, values have been normalised to the transcript level of *ACTIN* and are shown as mean percentage *ACTIN*  $\pm$  standard error for 2-3 biological replicates. Apices include a mixture of main shoot and branch apices (74d only; expression comparable). (continued next page)

**Figure 3.14. (continued) (C, F, I, L)** Schematics of the meristem transitions inferred from expression results in (C) *gigas*, (F) *veg1*, (I) *veg2-2*, (L) *veg2-1*. The V/I<sub>1</sub> transition from vegetative meristem (VM) to primary inflorescence meristem (I<sub>1</sub>M) and production of axillary floral meristems (FM) from secondary inflorescence meristems (I<sub>2</sub>M; where relevant) are shown. Straight arrows indicate meristem transitions. Circular arrows indicate meristem indeterminacy. Blocked transitions are indicated with a red cross.



**Figure 3.15.** Expression of *VEG1* and *PIM* as indicators for the presence of I<sub>2</sub> and floral meristems, under SD conditions.

(A, B, D, E, G, H, J, K) Expression of *VEG1* and *PIM* in shoot apices of *gigas* (*gi-2*), *veg1*, *veg2-2*, and *veg2-1* mutants and corresponding wild-type plants at time-points 81 and 112 days after sowing. Wild-type lines are as follows: (A, B, G, H) NGB5839, (D, E) wild-type sibling of *veg1*, (J, K) wild-type sibling of *veg2-1*. For relative transcript levels, values have been normalised to the transcript level of *ACTIN* and are shown as mean percentage *ACTIN* ± standard error for 1-5 biological replicates. (continued next page)

**Figure 3.15. (continued)** Apices include a mixture of main shoot and branch apices (expression comparable). (C, F, I, L) Schematics of the meristem transitions inferred from expression results in (C) *gigas*, (F) *veg1*, (I) *veg2-2*, (L) *veg2-1*. The V/I<sub>1</sub> transition from vegetative meristem (VM) to primary inflorescence meristem (I<sub>1</sub>M) and production of axillary floral meristems (FM) from secondary inflorescence meristems (I<sub>2</sub>M; where relevant) are shown. Straight arrows indicate meristem transitions. Circular arrows indicate meristem indeterminacy. Blocked transitions are indicated with a red cross.

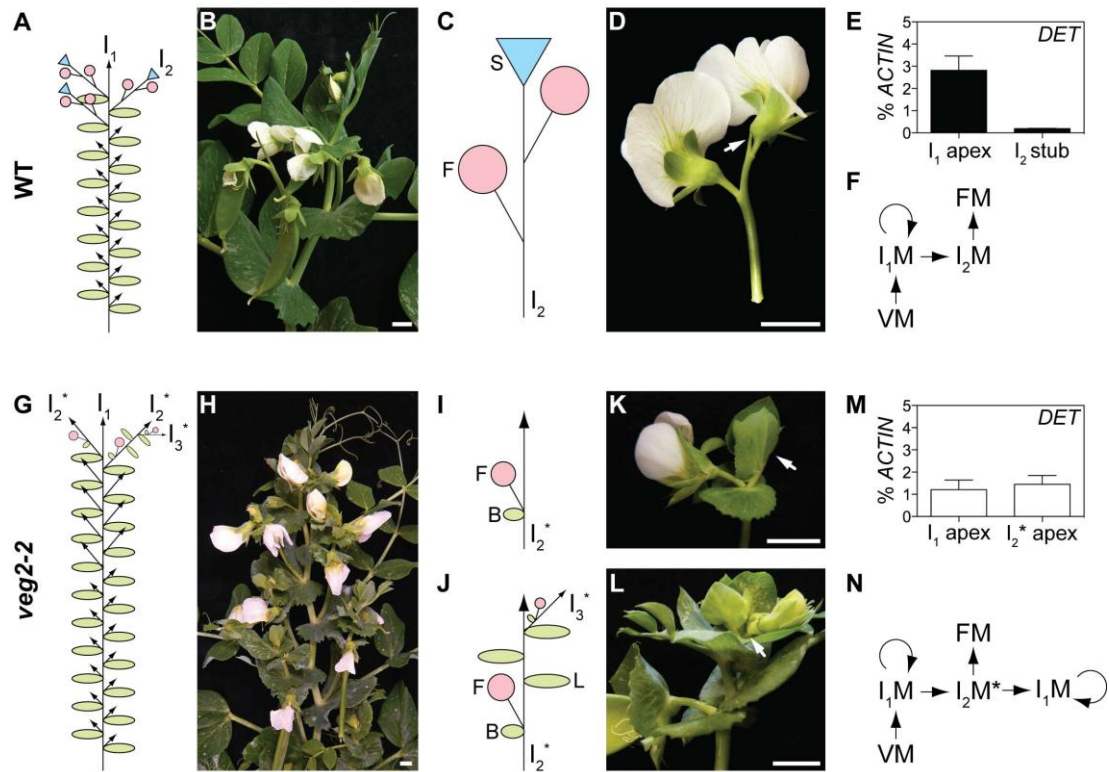
### 3.3.4 Investigating the role of *VEG2* in maintaining I<sub>2</sub> meristem identity

The weaker *veg2-2* allele does not prevent I<sub>2</sub> and subsequent floral meristem specification under LD or SD conditions, thus it can be used to study the roles of *VEG2* beyond I<sub>2</sub> specification, which is blocked in the *veg2-1* mutant (Figures 3.7, 3.14 and 3.15). After the V/I<sub>1</sub> transition in the *veg2-2* mutant, axillary structures develop that bear one or two axillary flowers, thus meeting the key criterion of an I<sub>2</sub>. Wild-type I<sub>2</sub> structures exhibit complete suppression of leaves and each terminates in a hairy stub (Figure 3.16C-D). In contrast, in the *veg2-2* mutant each flower is subtended by a bract comprising stipule-like leaf tissue but lacking the rachis and leaflets of a full pea compound leaf (Figures 3.16I-L and 3.17). This suggests that only partial I<sub>2</sub> identity may be specified in the *veg2-2* mutant. In addition, the *veg2-2* ‘I<sub>2</sub>’ retains an indeterminate apex (Figure 3.16I-L) and reverts to a structure resembling the wild-type I<sub>1</sub> (Figure 3.16A-B), bearing full compound leaves often with dormant axillary buds as would be observed on the main stem (Figure 3.17). Any flower present at a subsequent node on the *veg2-2* ‘I<sub>2</sub>’ after this apparent reversion, is not borne directly in a leaf axil, but instead arises from an indeterminate tertiary inflorescence structure (I<sub>3</sub>) that resembles the ‘I<sub>2</sub>’ (Figure 3.16J and L). The *veg2-2* ‘I<sub>2</sub>’ is distinct from a flowering branch due to the presence of axillary flowers and subtending bracts. This phenotype of indeterminate ‘I<sub>2</sub>’ structures is seen at every reproductive node of *veg2-2* mutants under both LD and SD conditions (Sussmilch, 2008).

These observations indicate that the final identity of the *veg2-2* ‘I<sub>2</sub>’ is that of an I<sub>1</sub> meristem. To test this, expression of the I<sub>1</sub> meristem marker *DET* was investigated in the indeterminate apex of the *veg2-2* ‘I<sub>2</sub>’, after axillary flower production. *DET* was found to be expressed in I<sub>1</sub> meristems and the *veg2-2* ‘I<sub>2</sub>’ apex, but not in wild-type I<sub>2</sub> stubs (Figure 3.16E and M). These results combined with observations of *veg2-2* morphology, indicate that each ‘I<sub>2</sub>’ structure in *veg2-2* fails to maintain I<sub>2</sub> identity after the production of axillary flowers (Figures 3.14 and 3.15), and reverts



to  $I_1$  identity (Figure 3.16N). These findings indicate that *VEG2* has an important role in maintenance of  $I_2$  meristem identity.



**Figure 3.16.** Characterisation of  $I_2$  identity defects in the *veg2-2* mutant.

(A-F) The wild-type pea inflorescence. (A) Diagram of wild-type plant architecture. (B) Photo of reproductive nodes on the wild-type stem. (C) Diagram and (D) photo of the wild-type  $I_2$  which bears axillary flowers (F) and terminates in a stub (S; arrow). (E) Relative expression of *DET* transcript as an indicator of  $I_1$  identity in wild-type (NGB5839) in the shoot apex (dissected to 2mm<sup>2</sup>) after the V/ $I_1$  transition (45 days after sowing) and in the  $I_2$  stub (approximately 1 cm from the last  $I_2$  node to stub tip) during early flower development (59 days after sowing). (F) Schematic of meristem transitions involved in wild-type inflorescence development.

(G-N) The *veg2-2* mutant inflorescence. (G) Diagram of plant architecture in the *veg2-2* mutant. (H) Photo of reproductive nodes on the *veg2-2* stem. (I) Diagram and (K) photo of a typical *veg2-2* mutant ' $I_2$ ', which bears an axillary flower with subtending bract (B) and retains an indeterminate apex (arrow). (J) Diagram and (L) photo of an older ' $I_2$ ', which has remnants of a flower with an abnormal sepal whorl and subtending bract at the first node, three nodes with full compound leaves (L) and then another flower on an axillary tertiary inflorescence ( $I_3$ ; arrow in photo). (M) Relative expression of *DET* transcript as an indicator of  $I_1$  identity in *veg2-2* in the shoot ( $I_1$ ) apex and indeterminate ' $I_2$ ' apex during early flower development (74 days after sowing). (N) Schematic of meristem identity transitions during inflorescence development in the *veg2-2* mutant.

In diagrams, arrows indicate indeterminate growth, circles are flowers, triangles are terminal stubs, and ovals are leaves or bracts. Asterisks indicate abnormal nature of structures. In photos, scale bars indicate 1cm. In graphs, mean percentage *ACTIN*  $\pm$  standard error for 2-3 biological replicates is shown. In schematics, meristems are: vegetative meristem (VM), primary inflorescence meristem ( $I_1M$ ), secondary inflorescence meristem ( $I_2M$ ), floral meristem (FM). Straight arrows indicate meristem transitions and products. Circular arrows indicate meristem indeterminacy.



**Figure 3.17.** Bracts and leaves on the *veg2-2* 'I<sub>2</sub>' structure.

(A) Photo of full pea compound leaf with stipules (S), rachis (R), leaflets (L) and tendrils (T).

(B-D) Photos of a typical 'I<sub>2</sub>' from the *veg2-2* mutant, with an axillary pod with subtending bract at the first node, a full compound leaf at the second node and an indeterminate apex. (B) Whole 'I<sub>2</sub>' structure. (C) Bract with axillary pod (left) and full compound leaf with small axillary tertiary inflorescence (I<sub>3</sub>; right). (D) Bract comprising serrated leaf tissue (resembling the serrated base of wild-type stipules) fused to a point at the tip, with axillary pod removed for clarity.

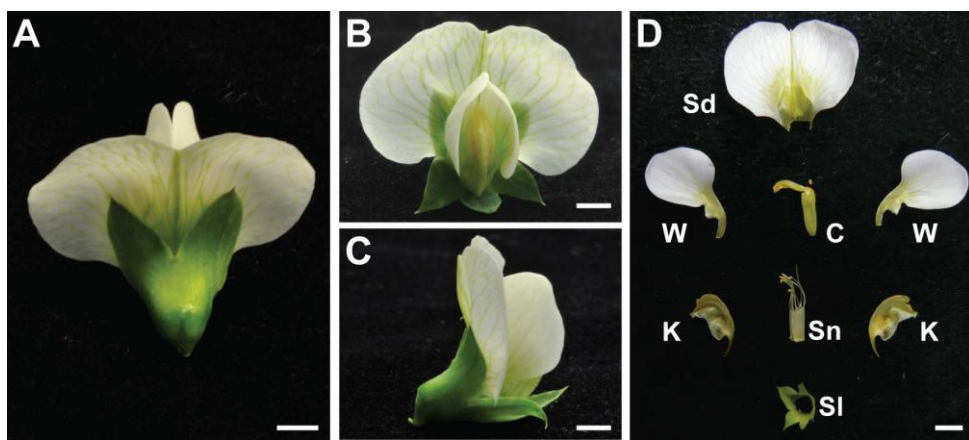
Scale bars indicate 1mm.

### 3.3.5 Examining the role of *VEG2* in floral development

#### 3.3.5.1 *Floral morphology in the veg2-2 single mutant*

Floral morphology defects in the *veg2-2* mutant were characterised in this study in order to examine the role of *VEG2* in floral development. As shown in Figure 3.18, wild-type pea flowers are zygomorphic, comprising five sepals (two adaxial and three abaxial, fused at the base), five petals (one standard, two wings and two fused keel petals), 10 stamens and a single central carpel. Flowers in the *veg2-2* mutant ranged from severely abnormal with all whorls affected, to completely normal flowers (Figure 3.19A). Looking first at floral morphology under LD conditions, defects were most common in outer whorls (sepal and petal; Figure 3.19B). All abnormal *veg2-2* flowers were affected in the sepal whorl as a minimum (Figure 3.19B). Within the sepal whorl, all abnormal flowers had defects affecting the adaxial sepals and 15% of abnormal flowers had additional defects affecting abaxial sepals as well (Figure 3.19C). Defects in the petal whorl, in addition to the sepal whorl, were also present in 77% of abnormal flowers (Figure 3.19B). The majority of flowers that had defects affecting the petal whorl, had defects in the standard petal alone (40%) or in both standard and wing petals (40%; percentage of flowers with petal defects indicated; Figure 3.19C). Defects in stamen and carpel whorls were rare, occurring in only 8% and 4% of abnormal flowers, respectively (Figure 3.19B). The nature of defects varied between flowers and according to organ type (Figure 3.19D). Common defects included fusion to leaf or petal tissue, a reduced number of organs, and organ displacement or malformation (Figure 3.19D).

Fusion to leaf tissue occurred in all four floral whorls, and a reduction in organ number was observed in sepal, petal and stamen whorls (Figure 3.19D). Both the occurrence and severity of floral defects decreased acropetally (Figure 3.19E-G). Abnormal flowers were observed more frequently at lower reproductive nodes on the main stem and all flowers were normal at the highest reproductive nodes (Figure 3.19F). Likewise, the severity of floral defects as measured by the mean number of whorls affected by floral defects across all flowers decreased from  $2.3 \pm 0.4$  at the first reproductive node to no defects by reproductive node 7 (Figure 3.19G).



**Figure 3.18.** Wild-type floral morphology.

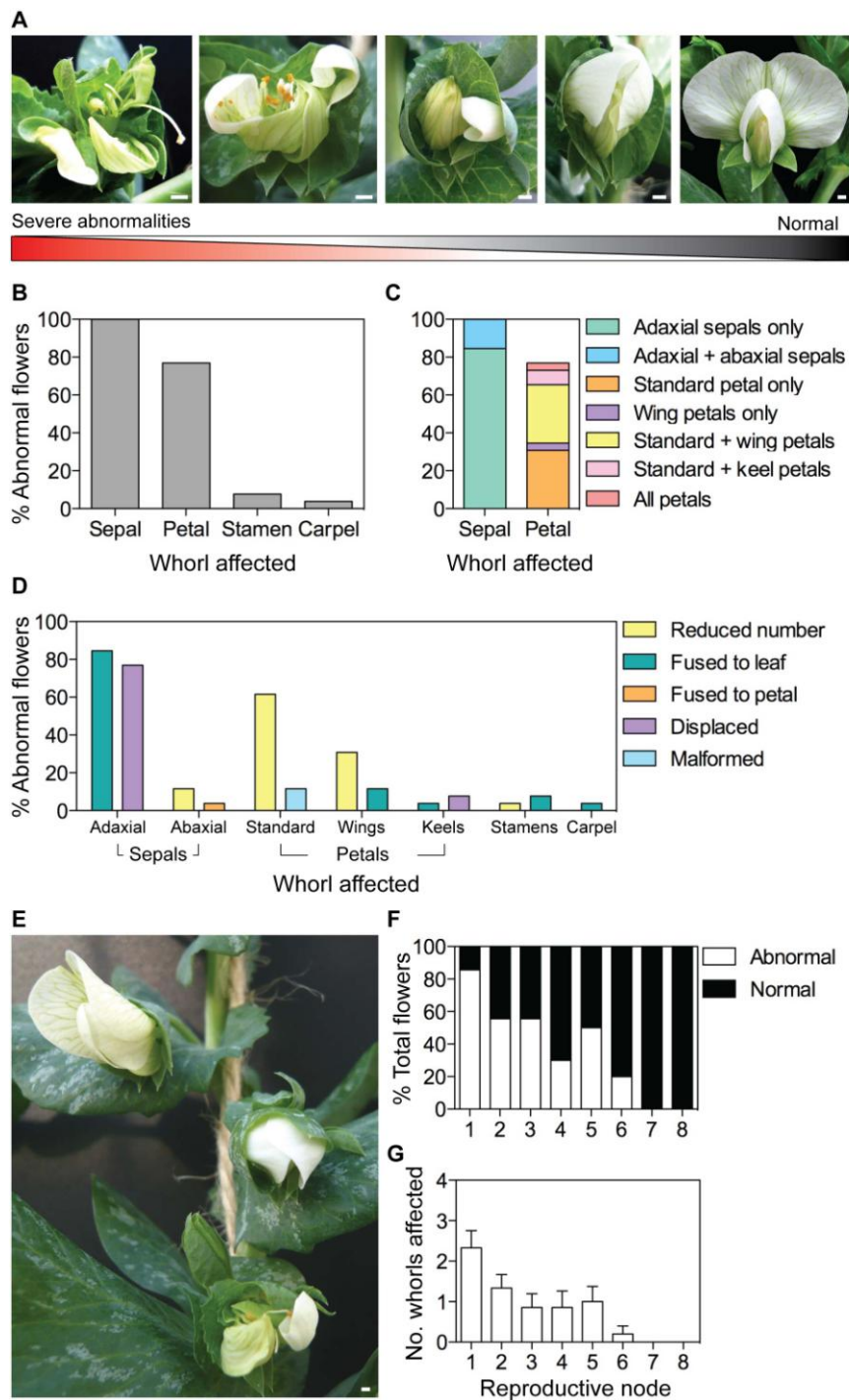
(A) Top view of wild-type pea flower with the two adaxial sepals visible.

(B) Front view of wild-type pea flower with the three abaxial sepals, standard petal, two wing petals and two fused keel petals (enclosing stamens and carpel) visible.

(C) Side view of wild-type pea flower showing the basal fusion of sepals.

(D) Dissected wild-type pea flower showing components: five sepals (Sl; fused at the base); five petals - one standard (Sd), two wings (W) and two keel petals (K); 10 stamens (Sn; fused at the base); a single central carpel (C).

Scale bars represent 5mm.



**Figure 3.19.** Floral defects in the *veg2-2* mutant under LD conditions.

(A) Examples of flowers exhibiting the range in severity of floral morphology defects seen in *veg2-2* mutant plants. Flowers range from those defective in all whorls (left), to flowers with wild-type morphology (right).

(B-D) Percentage of abnormal flowers (B) defective in each of the four floral whorls, (C) defective in sepal and petal whorls subdivided into sepal/petal type, and (D) exhibiting specific types of floral defects subdivided into floral organ type.

(E) Photo of *veg2-2* mutant exhibiting decreasing floral defects with increased reproductive node.

(F) Percentage of total flowers with defects at each reproductive node on *veg2-2* mutant plants.

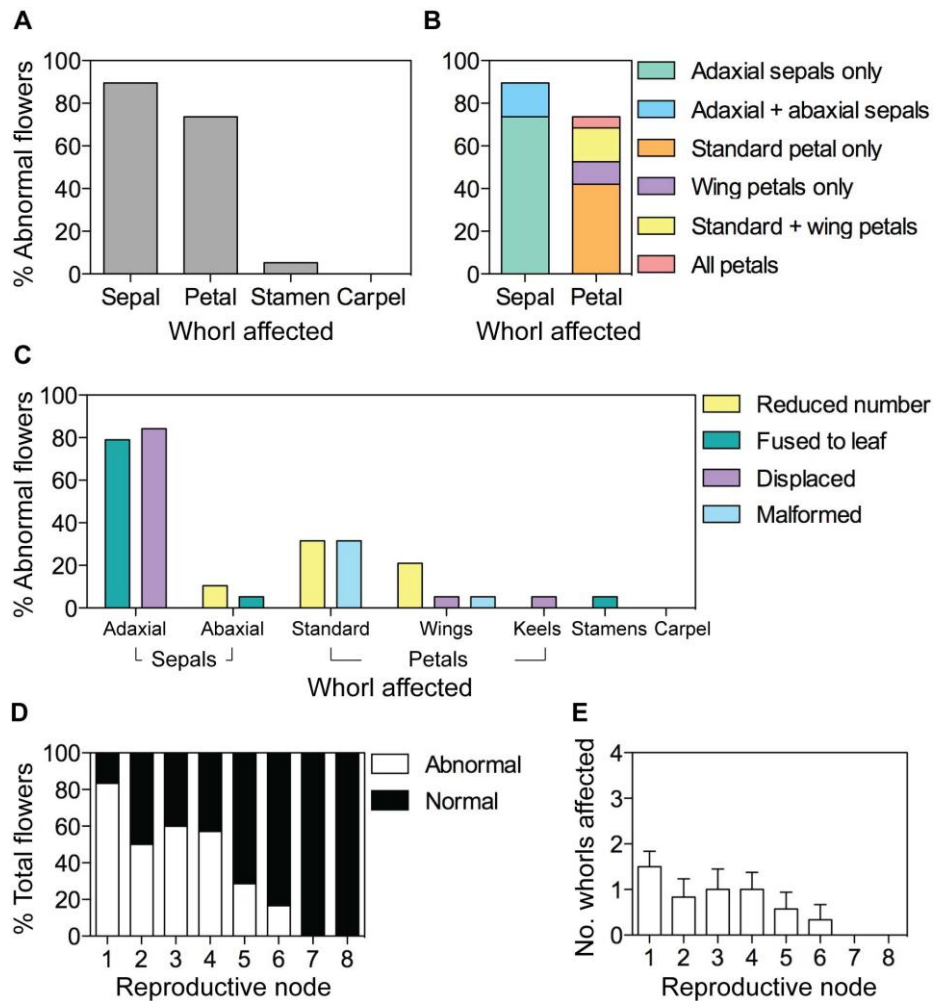
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**Figure 3.19. (continued) (G)** Mean number of whorls affected by floral defects at each reproductive node on *veg2-2* mutant plants. Values represent mean  $\pm$  standard error.

Figure is based on characterisation of all flowers borne at reproductive nodes on the main stem of 7 *veg2-2* plants (50 flowers total). For **(B-D)**  $n = 26$  abnormal flowers, for **(F-G)**  $n = 2-8$  flowers per reproductive node. Scale bars in photos indicate 1mm.

A similar pattern of floral defects was seen under SD conditions. As in LD conditions, most defects occurred in sepal (89%) and petal (74%) whorls, and defects were rarely observed in stamen or carpel whorls (percentage of all abnormal flowers indicated; Figure 3.20A). Within the sepal whorl, adaxial sepals were again most commonly affected, either alone (82%) or in combination with abaxial sepals (18%; percentage of flowers with sepal defects indicated; Figure 3.20B). Within the petal whorl, the standard petal was most commonly affected, either alone (57%), in combination with wing petals (21%), or in combination with both wing and keel petals (7%; percentage of flowers with petal defects indicated; Figure 3.20B). Displacement or reduction in the number of floral organs was commonly seen in sepal and petal whorls, and fusion to leaf tissue was seen in sepal and stamen whorls (Figure 3.20C). Both the occurrence and severity of floral defects again decreased acropetally, with 83% of flowers exhibiting defects with an average of  $1.5 \pm 0.3$  floral whorls affected at the NFI, but all flowers were normal at higher reproductive nodes (Figure 3.20D-E).





**Figure 3.20.** Floral defects in the *veg2-2* mutant under SD conditions.

(A-C) Percentage of abnormal flowers (A) defective in each of the four floral whorls, (B) defective in sepal and petal whorls subdivided into sepal/petal type, and (C) exhibiting specific types of floral defects subdivided into floral organ type.

(D) Percentage of total flowers with defects at each reproductive node on *veg2-2* mutant plants.

(E) Mean number of whorls affected by floral defects at each reproductive node on *veg2-2* mutant plants. Values represent mean  $\pm$  standard error.

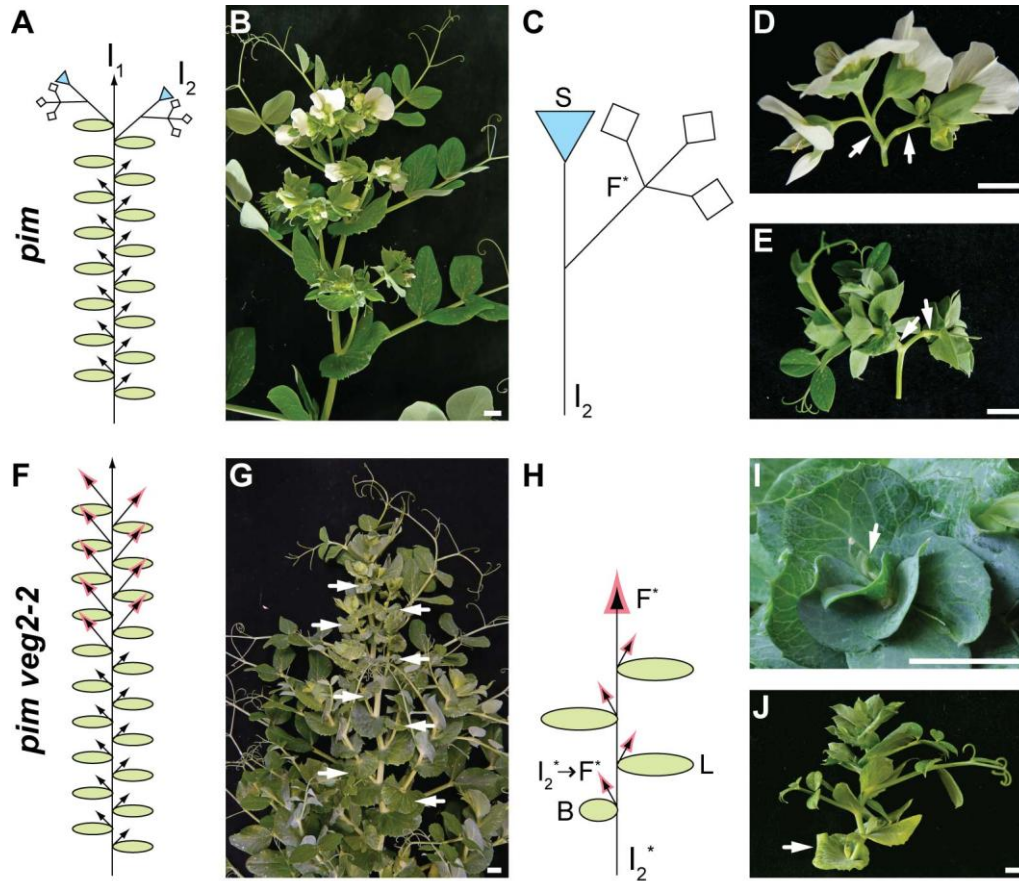
Figure is based on characterisation of all flowers borne at reproductive nodes on the main stem of 8 *veg2-2* plants (43 total flowers). For (A-C)  $n = 19$  abnormal flowers, for (D-E)  $n = 1-7$  flowers per reproductive node.

### 3.3.5.2 *The pim-2 veg2-2 double mutant phenotype*

Expression results presented previously in this chapter indicated a role for *VEG2* in controlling the expression of the key floral meristem identity gene *PIM* (Figures 3.14 and 3.15; see also Chapter 5). This suggests that at least part of the role of *VEG2* in floral development is mediated by *PIM*. To determine if *VEG2* has any further role in floral development beyond regulation of *PIM*, the phenotype of the *pim-2 veg2-2* double mutant was characterised.

The *pim* single mutant phenotype has been described previously (Singer et al., 1999; Taylor et al., 2002). The *pim* mutant fails to correctly specify floral meristems, and in place of single flowers, *pim* plants bear structures that range from leafy branches (only present occasionally and generally restricted to early reproductive nodes) to triads of abnormal flowers (Figure 3.21A-E; Singer et al., 1999; Taylor et al., 2002). The *pim* mutant correctly specifies the identity of  $I_1$  and  $I_2$  meristems, and does so at the correct time, with no significant difference between wild-type and *pim* NFI (Figure 3.22Q;  $p = 0.975$ ).

In contrast to the *pim* single mutant, the *pim veg2-2* double mutant showed extensive branching at aerial nodes and floral structures were restricted to upper nodes of some primary or secondary branches and/or the main stem (Figure 3.21F-J). There were no recognisable flowers present as discrete, axillary units on *pim veg2-2* double mutant plants. Instead, several compacted nodes of a branch or the main stem adopted partial floral identity, replacing the traditional floral whorls (Figures 3.21F-J and 3.22). These nodes either bore axillary floral organs (Figure 3.22B-D, G, I and J) or the leaves at these nodes were fused to floral organs (Figure 3.22E, F, and H). The floral organs included stamens (Figure 3.22B-G, I and J), carpels (Figure 3.22H and K), petals, or chimeric structures comprising fusions between these organs or between floral organs and leaf tissue (Figure 3.22C-D). The leaves subtending or fused to these floral organs were sometimes incomplete, comprising one or both stipules (Figure 3.22D-H) but lacking the rachis, leaflets and tendrils of a full pea compound leaf (Figure 3.22A). In most cases, the branch or main stem became determinate as it acquired floral identity (Figures 3.21I and 3.22K). However, in some cases, an indeterminate apex was retained after one or more nodes bearing axillary floral organs were produced and this apex continued to produce normal branch/stem tissue (Figure 3.22I-J).



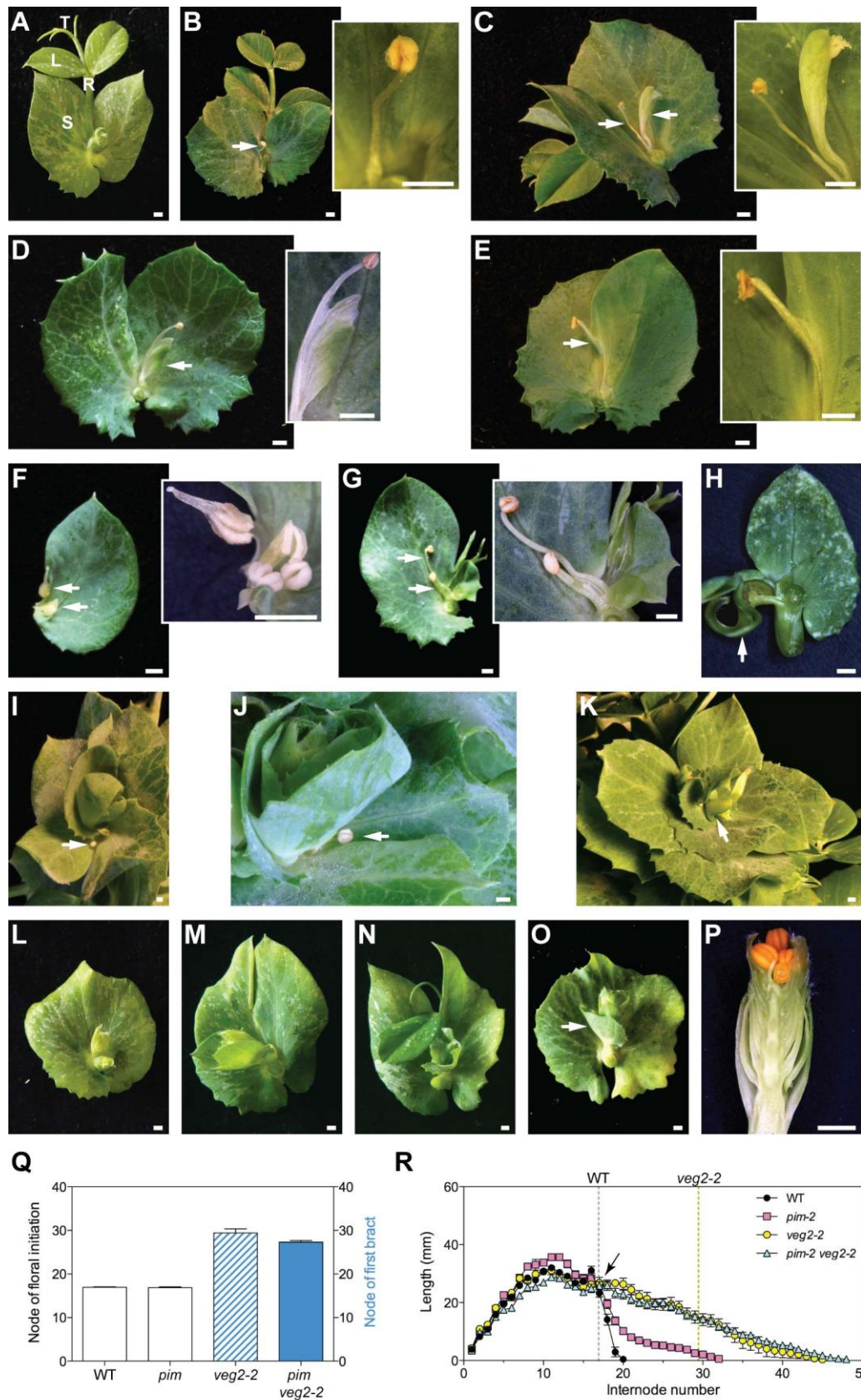
**Figure 3.21.** *pim-2 veg2-2* mutant phenotype in comparison with *pim-2* single mutant phenotype.

(A-E) The *pim* single mutant. (A) Diagram and (B) photo of reproductive nodes on the  $I_1$  stem of the *pim* mutant. (C) Diagram and (D-E) photos of the  $I_2$  of the *pim* mutant with normal  $I_2$  structure but proliferating structures ranging from (D) triads of flowers to (E) leafy branches borne in place of single flowers. Arrows indicate where the pedicel of a single flower would be in wild-type.

(F-J) The *pim veg2-2* double mutant. (F) Diagram and (G) photo of the  $I_1$  stem of the *pim veg2-2* double mutant. Arrows in photo highlight bracts on branches that are similar to bracts seen subtending flowers on ' $I_2$ ' structures in *veg2-2* mutants. (H) Diagram and (J) photo of a branch borne on the  $I_1$  of the *pim veg2-2* double mutant, which appears to acquire floral identity. Coloured arrows in (H) indicate leafy shoots that gradually acquire partial floral identity. The arrow in (J) indicates the presence of a bract at the first branch node, which is similar to bracts subtending flowers in the *veg2-2* single mutant. (I) The top of a branch that appears to terminate in floral organs. The arrow in (I) indicates a stamen with chimeric petal and leaf tissue, borne as an axillary structure at an upper branch node.

Asterisks in diagrams indicate abnormal nature of structures. Scale bars in photos indicate 1cm.





**Figure 3.22.** Reproductive structures in the *pim-2 veg2-2* double mutant.

(A) A normal pea compound leaf comprising two stipules (S), either side of a rachis (R) bearing leaflets (L) and tendrils (T), dissected from a middle node of a secondary branch from a *pim veg2-2* double mutant plant. (continued next page)

**Figure 3.22. (continued) (B-H)** Full compound leaves, or reduced/malformed leaves, dissected from upper nodes of secondary branches with partial floral identity, displaying axillary or fused floral organs, from *pim veg2-2* double mutant plants. **(B)** Leaf bearing an axillary stamen (arrow; inset at higher magnification). **(C)** Leaf bearing an axillary stamen and stamen-leaf chimeric structure (arrows; inset at higher magnification). **(D)** Reduced leaf lacking rachis, bearing an axillary stamen-petal-leaf chimeric structure (inset at higher magnification). **(E)** Back of reduced leaf with fused stamen (arrow; inset at higher magnification) in place of a rachis. **(F)** Reduced leaf comprising a single stipule with fused anthers (inset at higher magnification) in place of a rachis and second stipule. **(G)** Leaf with one normal stipule and reduced rachis, leaflets and second stipule bearing two axillary stamens (inset at higher magnification). **(H)** Reduced leaf comprising a single stipule with carpel (arrow) fused to a single leaflet in place of a rachis.

**(I-J)** Upper nodes of branches, each with single axillary stamens (arrows), but retaining indeterminate apices, from *pim veg2-2* double mutant plants.

**(K)** Main stem of a *pim veg2-2* double mutant plant, which has acquired partial floral identity and appears to terminate in floral organs including a stamen and carpel (arrow).

**(L)** Bract from first node of a branch from a *pim veg2-2* double mutant plant, resembling two stipules fully fused to single point.

**(M)** and **(N)** bracts from the second node of separate branches from *pim veg2-2* double mutant plants, each comprising two partially fused stipules and lacking a rachis. In **(N)** there is a leaflet and tendril fused directly to the stipules.

**(O)** Bract from first node of a branch from a *pim veg2-2* double mutant plant with developing axillary bud also bearing a similar bract at the first node (arrow).

**(P)** Axillary bud from **(O)** dissected to reveal stamens present at upper branch nodes.

**(Q)** NFI (left axis) in wild-type (WT; NGB5839), *pim* and *veg2-2* mutants, and earliest node of bract at first axillary branch node (blue, right y-axis) in *veg2-2* mutants (equal to NFI as first bract is located on first  $I_2$ ) and *pim veg2-2* double mutant plants.  $n = 14-23$  plants per genotype.

**(R)** Ontogenetic variation in internode length for wild-type (NGB5839), *pim*, *veg2-2* and *pim veg2-2* double mutant plants. Broken lines indicate internode between the first and second reproductive node in wild-type (grey; not significantly different from *pim* NFI), and *veg2-2* mutant plants (yellow).  $n = 11-14$  plants per genotype.

Scale bars in photos indicate 1mm. Values in graphs represent mean  $\pm$  standard error.

Bracts, or modified leaves, are commonly associated with inflorescence structures in other species, including *Arabidopsis* and *Antirrhinum* (e.g. Coen et al., 1990; Schultz and Haughn, 1991), but are fully repressed on  $I_2$  structures in wild-type pea (Figure 3.16C-D). Bracts were present at the first one or two nodes of each aerial axillary branch in *pim veg2-2* double mutant plants (Figure 3.21G, H and J; Figure 3.22L-O). These bracts comprised serrated leaf tissue and resembled wild-type stipules fully or partially fused at the tip, but lacked the rachis of a normal pea compound leaf (Figure 3.22A). In *veg2-2*, similar bracts are first present subtending a flower on the ' $I_2$ ' structure at the NFI (Figures 3.17 and 3.22Q), and are present on every subsequent ' $I_2$ ' structure, but are not seen on  $I_1$  stems. As these bracts are characteristic of *veg2-2*  $I_2$  structures, bracts seen in *pim veg2-2* plants could indicate that branches have acquired partial  $I_2$  identity. In accordance with this hypothesis,

bracts were first observed on axillary branches in the *pim veg2-2* double mutant at a similar node to the *veg2-2* single mutant NFI (mean  $\pm$  SE: *veg2-2* =  $29.4 \pm 0.9$ , *pim veg2-2* =  $27.3 \pm 0.4$ ,  $p = 0.193$ ; Figure 3.22Q), and were present on branches at every subsequent stem node. If *veg2-2* and *pim* single mutant phenotypes were simply additive, it could be expected that these bracts would subtend a proliferating floral structure. Instead, these bracts subtended axillary secondary branches that also had a distinctive bract at the first one or two nodes (Figure 3.22O) and acquired partial floral identity at higher secondary branch nodes (Figure 3.22P). Ontogenetic variation in internode length for the *pim veg2-2* double mutant followed the same pattern as that of the *veg2-2* mutant, which showed a slower rate of decrease in internode length relative to wild-type and *pim* mutant plants (Figure 3.22R). This suggests that there may be a delay in the V/I<sub>1</sub> transition in the *pim veg2-2* double mutant, similar to the *veg2-2* single mutant (Figures 3.2-3.3).

### 3.4 Discussion

Three loci have been identified as having critical roles in flowering in pea, as mutation to any of these loci can result in a non-flowering phenotype. The roles of *FTa1/GIGAS* and *VEG1* have been examined in previous studies (e.g. Reid and Murfet, 1984; Beveridge and Murfet, 1996; Hecht et al., 2011; Berbel et al., 2012). *VEG2* is the last of these three loci to be characterised in detail. In this chapter, the specific roles of the *VEG2* locus during each stage of inflorescence development have been investigated through a series of experiments examining the non-flowering *veg2-1* mutant and the late-flowering *veg2-2* mutant.

#### 3.4.1 The roles of *VEG2* and *GIGAS/FTa1* during the *V/I<sub>1</sub>* transition

The *V/I<sub>1</sub>* transition is the first transition involved in pea inflorescence development, thus determining if this transition occurs in non-flowering mutants is an important feature of mutant phenotype. Previously, expression of *DET* and ontogenetic variation in internode length have been used to infer the occurrence, and relative timing of the *V/I<sub>1</sub>* transition (Reid and Murfet, 1984; Berbel et al., 2012). Prior to this study, characterisation of one or both of these traits has indicated that this transition occurs at the same time as wild-type in *veg1* and *gigas*, but may be delayed in the *veg2-2* mutant in LD conditions (Sussmilch, 2008; Hecht et al., 2011; Berbel et al., 2012). Under SD conditions, previous characterisation of internode length has suggested that the *V/I<sub>1</sub>* transition occurs at the same time as wild-type in *veg1*, but not in the late-flowering *gigas-1* mutant (Reid and Murfet, 1984; Murfet, 1989b). It had not previously been investigated whether the non-flowering *veg2-1* mutant undergoes the *V/I<sub>1</sub>* transition at the same time as wild-type (as seen in *veg1* and *gigas* mutants under LDs), late, or even at all. In this study, three different lines of evidence suggest that the *V/I<sub>1</sub>* transition may be delayed in the *veg2* mutants under LD and SD conditions, and *gigas* mutants under SD conditions, relative to wild-type and *veg1*.

The first of these is expression of the *I<sub>1</sub>* marker gene *DET*. In this study, expression of *DET* was investigated by qRT-PCR in dissected apex samples (Figures 3.2 and 3.5). A limitation to this technique is that the 2mm<sup>2</sup> dissected apices used as samples include other tissues of the shoot apex in addition to the SAM, including developing leaf primordia and stem tissue, which dilute the level of *DET* expression

detected, thereby reducing sensitivity. *in situ* hybridisation could be used as an alternative approach, but was not used for this study based on advice that this is technically difficult due to the narrow range of *DET* expression and low signal level (A. Berbel and F. Madueño, personal communication). Reduced sensitivity in qRT-PCR is less of an issue if *DET* expression can be investigated across a developmental series, wherein early induction of *DET* is followed by clear upregulation in *DET* expression (e.g. Figure 3.5E; see also Chapter 5). Unfortunately, the non-flowering *veg2-1* mutant can only be obtained from segregating populations with an expected ratio of only 1 mutant from 6 plants. The size of the segregating population required was too prohibitive with glasshouse space limitations to allow investigation of *DET* expression in *veg2-1* across a developmental series in this study. Instead, two time-points were chosen to coincide with the expected peaks of *DET* expression in wild-type and *veg2-2* (Sussmilch, 2008; see also Chapter 5), to allow inference of I<sub>1</sub> meristem identity. Under LD conditions, *gigas* and *veg1* mutants showed upregulation of *DET* to levels equal to or higher than wild-type at the first time-point, after the wild-type V/I<sub>1</sub> transition (Figure 3.2B-C). This result was in accordance with previous findings, and supports the conclusion that the V/I<sub>1</sub> transition occurs in *veg1* and *gigas* at a similar time to wild-type plants (Hecht et al., 2011; Berbel et al., 2012). In contrast, upregulation of *DET* in either of the *veg2* mutants was not seen until the second time-point, after the V/I<sub>1</sub> transition had occurred in the *veg2-2* mutant (Figure 3.2D-E). The low level of *DET* expression seen at the first time-point, suggests that induction of *DET* may have been beginning in the *veg2* mutants at the time of sampling, but this was still later than in wild-type, where *DET* expression was already established. Under SD conditions, *DET* expression was not detected in the *veg2-1* mutant soon after the time of the V/I<sub>1</sub> transition in wild-type, and *DET* upregulation was found to be delayed in the *veg2-2* and *gigas* mutants (Figure 3.5). Insufficient numbers of *veg2-1* mutants among segregants prevented testing of *DET* expression at a second, later time-point, to determine if *DET* is eventually upregulated. As photoperiod does not alter the basic characteristics of either *veg2-1* or *veg2-2* it seems likely that *DET* would be eventually upregulated in *veg2-1* under SD conditions, similar to LD conditions (Figure 3.2), but this should be confirmed in a future experiment. These expression results do not allow elucidation of the exact time of the V/I<sub>1</sub> transition in the *veg2-1* mutant. However, the upregulation of *DET*

detected in the apex of the *veg2-1* mutant under LD conditions, reveals that *veg2-1* can eventually acquire  $I_1$  meristem identity (Figure 3.2). In addition, the delayed upregulation of *DET* in *veg2* (LD and SD) and *gigas* (SD only), relative to wild-type and *veg1*, suggests that there may be delayed acquisition of  $I_1$  identity in these mutants.

The second line of evidence is ontogenetic variation in internode length. Under LD conditions, both *veg2* mutants exhibited a pattern of internode length that was distinct from that of *gigas*, *veg1* and wild-type, (Figure 3.3). Under SD conditions, the pattern of internode length was altered in *veg2-1*, *veg2-2* and *gigas* mutants, relative to wild-type and the *veg1* mutant (Figure 3.4). Although the decrease in internode length could not be used to infer the exact timing of the V/ $I_1$  transition, the observation that this trait is normally associated with this transition suggests that an altered pattern of ontogenetic variation in internode length may indicate altered timing of the V/ $I_1$  transition. Using this interpretation, results of ontogenetic variation in internode length from this study suggest that the timing of the V/ $I_1$  transition is altered in the *veg2* mutants under LD conditions, and in the *veg2* and *gigas* mutants under SD conditions (Figures 3.3 and 3.4).

Lastly, delayed flowering time in the late-flowering *veg2-2* and *gigas* mutants also suggests that the V/ $I_1$  transition may be delayed in *veg2-2* under LD and SD, and in *gigas* under SD only (Figure 3.7). As *veg2-1* is the more severe of the two *veg2* alleles (Murfet, 1992; see also Chapter 4), it could be expected that any delay in the V/ $I_1$  transition in *veg2-2* would be reflected by an equal or greater delay in the *veg2-1* mutant.

It is possible that *VEG2* and *GIGAS/FTa1* act after the V/ $I_1$  transition but regulate timing of *DET* induction, subsequent timing of  $I_2$  meristem specification, and the hormones that affect internode length. However, as expression of *DET*, production of axillary  $I_2$  structures and decrease in internode length are the best measures of  $I_1$  identity that we currently have, the conclusion that best fits the evidence is that the V/ $I_1$  transition is delayed in the *veg2* (LD and SD) and *gigas* (SD only) mutants, relative to wild-type and *veg1*. Based on this interpretation, the results suggest that *VEG2* has an important role during the V/ $I_1$  transition in wild-type plants, and thus that *VEG2* might encode a protein that is central to the V/ $I_1$  transition process. Previously, a pea *FD* homolog was proposed as a possible candidate for

*VEG2*, based on mapping position (Weller, 2007; Sussmilch, 2008). In eudicot and monocot species, FD proteins have been found to facilitate the action of FT-based florigen signals to trigger the upregulation of flowering genes (see Chapter 1). A role for *VEG2* during the V/I<sub>1</sub> transition in pea, as indicated by the results of this chapter, supports further investigation of this pea *FD* homolog as a functional candidate for the *VEG2* locus.

Findings from this chapter have also provided a valuable insight into the role of *FTa1/GIGAS* during pea inflorescence development. For almost two decades, the *GIGAS* locus has been investigated for its inferred role in production of the pea floral stimulus, based on grafting studies wherein grafting to a wild-type graft stock with leaves allowed non-flowering *gigas* scions to flower normally under LD conditions (Murfet, 1992; Taylor and Murfet, 1994; Beveridge and Murfet, 1996). More recently, identification of *GIGAS* as *FTa1* and detailed analysis of *FTa1* expression patterns have revealed that *FTa1* expression is not induced within the time-frame of the physiological commitment to flowering under LD conditions (Hecht et al., 2011). Instead, *FTa1* is induced later, in parallel with the induction of inflorescence identity genes, indicating that *FTa1* may have a role in determining inflorescence identity (Hecht et al., 2011). An additional *FT* gene, *FTb2*, was suggested as an alternative candidate for the primary regulator of flowering in response to LD photoperiods (Hecht et al., 2011). Results from this study suggest that the *gigas* mutant undergoes the V/I<sub>1</sub> transition at the same time as wild-type under LD conditions (Figures 3.2-3.3), in accordance with previous *DET* expression results (Hecht et al., 2011). This indicates that *FTa1/GIGAS* is not essential for the timing of the V/I<sub>1</sub> transition under LD conditions, but does not preclude the possibility that *FTa1/GIGAS* could act redundantly with other *FT* genes (e.g. *FTb2*) to promote this transition. *FTb2* is expressed normally in the *gigas* mutant in LD (Hecht et al., 2011), and if *FTb2* is indeed an alternative florigen, this could account for the normal occurrence of the V/I<sub>1</sub> transition in the *gigas* mutant under this photoperiod (Figures 3.2-3.3). Instead, results from this study and previous studies suggest that *gigas* is an I<sub>2</sub> specification mutant, which fails to upregulate *VEG1* under LD conditions (Figure 3.14A; Berbel et al., 2012). The results of grafting studies, combined with expression studies that show expression of *FTa1/GIGAS* in both leaves and apex, suggest that *FTa1* protein can be transported as a mobile stimulus from leaf to apex, or act locally within the

apex to fulfil its roles (Murfet, 1992; Taylor and Murfet, 1994; Beveridge and Murfet, 1996; Hecht et al., 2011). Under SD conditions, expression of meristem marker genes and measurements of internode length, combined with phenotype, indicate that there is a delay in the V/I<sub>1</sub> transition but that specification of I<sub>2</sub> and floral meristem identity genes occurs normally in the *gigas* mutant (Figures 3.4-3.5 and 3.15A-C). These results suggest that *FTa1/GIGAS* does have an important role in the V/I<sub>1</sub> transition but is not critical for I<sub>2</sub> and subsequent floral meristem specification under SD photoperiods. This has not previously been recognised in the literature, despite clues present in published data and observations (Murfet, 1989b; Beveridge and Murfet, 1996; Hecht et al., 2011). This indicates that an alternative mechanism is in place under SD conditions, which is different from the mechanism that functions under LD conditions.

The eventual occurrence of the V/I<sub>1</sub> transition in all three of the non-flowering mutants currently known in pea suggests that the transition to I<sub>1</sub> meristem identity can be activated by many alternative routes within the flowering pathway, to occur even when key genes are blocked. In species with simple inflorescence forms, including *Arabidopsis*, I<sub>1</sub> meristem specification is the only step before floral meristem specification. Based on current knowledge, no mutation to any single flowering gene in *Arabidopsis* can completely prevent flowering. Even the genes central to the floral transition in *Arabidopsis*, are not critical for it to occur, with *ft* and *fd* null alleles each resulting in a late-flowering phenotype (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991). Genetic redundancy (e.g. as seen between *FT/TSF*, *FD/FDP*) and multiple independent pathways (e.g. *FT/FD*, *LFY*, *miR156/SPL*) ensure that flowering eventually occurs in *Arabidopsis* (Abe et al., 2005; Yamaguchi et al., 2005; Wang et al., 2009; Jaeger et al., 2013). It is likely that there are similar systems functioning in pea that ensure the V/I<sub>1</sub> transition occurs, but these remain to be investigated.



### ***3.4.2 The link between ontogenetic variation in internode length and the V/I<sub>1</sub> transition***

In wild-type pea, ontogenetic variation in internode length can be largely explained by fluctuation in biologically active gibberellins, including gibberellin A<sub>1</sub> (GA<sub>1</sub>), which decrease after floral initiation in the apical portions of the plant under LD photoperiods (Ross and Reid, 1992; Ross et al., 1992; Zhu and Davies, 1997). Gibberellins promote internode elongation by stimulating cell division and/or cell elongation and destabilising growth-inhibiting DELLA proteins (including LA and CRY in pea; Weston et al., 2008). There is also evidence that the major auxin indole-3-acetic acid (IAA) has a role in internode elongation (e.g. Law and Davies, 1990; Behringer and Davies, 1992; McKay et al., 1994). IAA appears to act by moving from the apex into elongating internodes to promote GA<sub>1</sub> biosynthesis and accumulation, and to inhibit GA<sub>1</sub> deactivation (Ross et al., 2000; Ross et al., 2003; Weston et al., 2009). Accordingly, a reduced level of GA<sub>1</sub> is thought to be a major cause of the reduced length of aerial internodes in the *veg1* and *gigas* mutants under LD conditions, with reduced IAA levels also identified (Beveridge et al., 2001). Other hormones that act to control shoot growth include abscisic acid (ABA) and ethylene, which inhibit stem elongation, and brassinosteroids and strigolactones, which promote stem elongation (e.g. Hasson and Poljakoff-Mayber, 1983; Ross and Reid, 1986; Nomura et al., 1997; de Saint Germain et al., 2013).

From the results of this experiment, combined with the results of previous studies, it appears that the pattern of ontogenetic variation in internode length can be affected by mutations that alter the timing of the V/I<sub>1</sub> transition (Figures 3.2-3.5; Reid and Murfet, 1984). This indicates that there is feedback between the flowering pathway and the hormonal system controlling stem length, downstream of the genes controlling the V/I<sub>1</sub> transition. This feedback must be independent of the subsequent development of flowers or pods, as it also occurs in non-flowering mutants (Figures 3.3-3.5). After the floral transition, GA and IAA levels may have a role in regulating nutrient partitioning, increasing the sink strength of reproductive structures (Lockhart and Gottschall, 1961; Jahnke et al., 1989; Zhu and Davies, 1997). Thus one possible explanation is that a mechanism controlling hormone levels is linked to the V/I<sub>1</sub> transition, which normally alters hormone levels in order to maximise reproductive success through flower/fruit/seed development. At this point it is not clear why

*veg2-1* and *veg2-2* mutants exhibit different patterns of ontogenetic variation in internode length, why internode length decreases before the first flowering node in late-flowering *veg2-2* and *gigas* mutants, or why *veg1* mutants exhibit a higher peak in internode length than wild-type siblings (Figures 3.3 and 3.4), but these would be interesting research questions to be addressed in a future study.

### **3.4.3 Branching in the late- and non-flowering mutants**

The timing of lateral outgrowth in late- and non-flowering mutants and wild-type plants investigated in this study (Figures 3.10-3.11), did not reflect apparent differences in the timing of the V/I<sub>1</sub> transition between genotypes (Figures 3.2-3.5). Two stages of lateral outgrowth were considered in this study: the early outgrowth of an axillary bud to a length of 5mm or longer (development of an ‘enlarged aerial bud’) and the later development of an ‘aerial branch’ with one or more nodes with fully expanded leaves, at node 8 or above on the main stem. Comparison of the timing of development of the first enlarged aerial bud and aerial branch in terms of plant age and node of first development did not show any consistent differences between genotypes, under either LD or SD conditions (Figures 3.10-3.11). Instead, development of laterals in all mutants appeared to coincide with the timing of reproductive development in wild-type plants (Figure 3.7-3.11). Branch outgrowth is not in itself a characteristic of I<sub>1</sub> identity and there is no evidence that the timing of lateral outgrowth is linked to the timing of the V/I<sub>1</sub> transition in mutants in which this transition is delayed. Therefore the results of this experiment did not support the hypothesis that timing of branch outgrowth is linked to timing of the V/I<sub>1</sub> transition in inflorescence mutants.

It has previously been proposed that branch outgrowth can be suppressed by developing pods or seeds (e.g. Lockhart and Gottschall, 1961; Malik and Berrie, 1975). In this study, the extent of branch outgrowth was increased in non-flowering mutants and deflowered wild-type plants, in which no pods were present, and in late-flowering mutants, in which the development of pods was delayed, relative to corresponding intact wild-type plants (Figure 3.12). Results of a recent study which identified sugar demand, not auxin, as the initial regulator of apical dominance in pea, provide a possible explanation for this. Mason et al. (2014) found that supplementing intact plants with exogenous sucrose results in rapid axillary bud

release and hypothesised that the sugar demand of the shoot tip is critical for maintaining apical dominance in intact plants. By the same token, it is possible that the high sugar demand of developing fruit inhibits bud outgrowth in intact, flowering plants by limiting sugar availability to axillary buds. Deflowered wild-type plants and late-/non-flowering mutants would have lower sugar demands than intact wild-type plants during early stages of flowering and fruit development, which could increase sugar availability to axillary buds, allowing increased bud outgrowth.

The results of this study support a model in which a higher propensity to branch is temporally linked to the flowering signal in wild-type plants, coinciding with early stages of wild-type reproductive development, when not repressed by developing pods or seeds. This increased propensity for branching may be due to a developmental signal or increased availability of sugars. Timing of lateral outgrowth appears to be decoupled from the V/I<sub>1</sub> transition in the *veg2* mutants, suggesting that lateral outgrowth may be induced upstream or independently of this transition.

Similar to flowering, branching in pea seems to be controlled by photoperiod, as plants grown under SD conditions have a distinctive basal-branching phenotype (e.g. Beveridge et al., 2003). Mutation to components of the photoperiod pathway (light receptors and circadian clock) affect lateral outgrowth, with increased branching at basal and aerial nodes in *phytochrome A* (*phyA*) mutants grown under LD conditions, reduced basal branching in the *dne* mutant (pea ortholog of *Arabidopsis* *EARLY FLOWERING 4*) under SD conditions, and increased basal branching in *late1* mutants (pea ortholog of *Arabidopsis* *GIGANTEA*) under LD conditions (Weller et al., 1997; Hecht et al., 2007; Liew et al., 2009). *FT* genes have been recognised as the major output of the photoperiod pathway in a range of species (e.g. Izawa et al., 2002; Wigge et al., 2005; Hecht et al., 2011). In *Arabidopsis*, *FT* and *TSF* have been found to have a role in elongation of axillary buds through interaction with *BRANCHED1* (*BRC1*), a negative regulator of differentiation in axillary buds that acts downstream of the *MORE AXILLARY GROWTH* (*MAX*) genes involved in strigolactone biosynthesis or signalling (Aguilar-Martínez et al., 2007; Challis et al., 2013; Hiraoka et al., 2013; Niwa et al., 2013). In *Medicago*, a close relative of pea, quantitative trait loci (QTL) for branch development, branch elongation and flowering were correlated and mapped to a similar chromosomal location, suggesting common genetic regulation, with *CO* and *FT* homologs

identified as putative candidate genes (Lagunes Espinoza et al., 2012). A pea homolog of *BRC1* has been isolated and found to act downstream of strigolactones to inhibit branch outgrowth in pea in a similar manner to that described for *Arabidopsis* (Braun et al., 2012). It is plausible that a member of the *FT* gene family could have a role in regulating branch outgrowth in pea via a conserved mechanism of interaction with *BRC1*, providing the link between branching and flowering. This remains an exciting area for future research.

The lateral branches in non-flowering pea mutants have previously been described as inflorescences that lack flowers, and these mutants have been interpreted to be homeotic mutants with branches replacing  $I_2$  structures (Murfet and Reid, 1993; Wiltshire et al., 1994; Reid et al., 1996). This is based on the observation that axillary  $I_2$  structures are not present in these mutants, but branches develop at aerial nodes. However, two main observations made in this study are inconsistent with the description of branches as homeotic substitutions for  $I_2$  structures in the non-flowering mutants. Firstly, as discussed above, the timing of lateral outgrowth does not appear to reflect the timing of the V/ $I_1$  transition. Secondly, branches in the non-flowering mutants are not limited to nodes corresponding to those that bear  $I_2$  structures in wild-type plants (Figures 3.8 and 3.9). In wild-type plants, the first  $I_2$  is borne at the NFI, every subsequent node bears an  $I_2$  structure, and  $I_2$  structures do not arise later, directly from the main stem at nodes beneath the NFI (Figure 3.13C-D). In contrast, lateral outgrowth can begin at multiple nodes simultaneously, and outgrowth radiates to both higher and lower nodes with time (Figures 3.8-3.9). Taking these observations into account, the presence of branches in the non-flowering mutants does not appear to be an example of homeosis. Instead, it would appear that shoot branching is controlled by a separate system which is normally inhibited in wild-type plants, but is not inhibited in late- and non-flowering mutants.

#### ***3.4.4 Apical senescence in the late- and non-flowering mutants***

When senescence occurs in pea, the primary SAM ceases to generate new stem nodes, becomes chlorotic and dies. In this study, the total number of nodes on the main stem was measured at multiple time-points during development and at plant harvest. The production of new stem nodes ceased earlier in intact wild-type plants, than in deflowered wild-type plants and in late- and non-flowering mutants (Figure 3.13A-B). As growth rate was comparable between genotypes at early time-points (Figure 3.13A-B), this indicated that the relative timing of plant senescence was delayed in the deflowered plants and late- and non-flowering mutants, relative to corresponding intact wild-type plants (Figure 3.13A-B).

The influence of developing seeds on promoting plant senescence has been well documented in pea (e.g. Lockhart and Gottschall, 1961; Malik and Berrie, 1975; Proebsting et al., 1976; Gianfagna and Davies, 1981). This phenomenon is explained by the reallocation of nutrients from vegetative sources to reproductive sinks, likely via the action of hormones including gibberellin and auxin (Lockhart and Gottschall, 1961; Kelly and Davies, 1988; Jahnke et al., 1989; Zhu and Davies, 1997). A resulting global shift in hormonal and/or nutrient balance in the plant during reproductive development is believed to be the mechanism behind monocarpic senescence (e.g. Davies and Gan, 2012; Thomas, 2013). Thus the delay in plant senescence seen in the late- and non-flowering mutants and deflowered wild-type plants is likely the result of the absence or delayed occurrence of pod development in these plants. The transformation in wild-type plant appearance that results from removal of all flowers after anthesis is quite remarkable and highlights the influence of pod development on vegetative growth. The combination of increased branching, delayed senescence and production of many small internodes gives the plants a drooping apical appearance (Figure 3.8F), that at least partially phenocopies the non-flowering mutants (e.g. Figure 3.8I).

### 3.4.5 The role of *VEG2* in specifying and maintaining *I*<sub>2</sub> meristem identity

The absence of visible *I*<sub>2</sub> structures coupled with the lack of apical expression of *VEG1* as a marker for *I*<sub>2</sub> identity in the *veg2-1* mutant, indicates that the specification of *I*<sub>2</sub> meristems is blocked in this mutant (Figures 3.14J-L and 3.15J-L). This same step is blocked in *veg1* mutants under all conditions, and *gigas* mutants under LD conditions (Figures 3.2-3.5, 3.14 and 3.15). Previously, a model was proposed in which *VEG2* and *PsFTa1* act upstream of *VEG1*, and activate *VEG1* expression for specification of *I*<sub>2</sub> meristems, offering an elegant explanation for the similarity in mutant phenotypes and shared lack of expression of *VEG1* in *gigas*, *veg2-1* and *veg1* mutants under LD conditions (Figure 3.14; Berbel et al., 2012). Given the characterisation of *PsFTa1* as an *FT* homolog (Hecht et al., 2011), the identification of *VEG1* as a MADS-box gene of the *API/SQUA/FUL* lineage (Berbel et al., 2012), and the investigation of a pea *FD*-like gene as a candidate for the *VEG2* locus (Weller, 2007; Sussemilch, 2008; see also Chapter 4), this model is largely consistent with knowledge from other species, where *FT* homologs are known to interact with *FD* homologs to upregulate the expression of MADS-box genes (see Chapter 1). However, it is noteworthy that a role in specification of meristem identity, uncoupled from induction of the floral transition, as is apparent for *FTa1* in LD conditions, has not so far been documented for *FT* homologs in other species. This is reminiscent of the separation of the two roles of *Arabidopsis TFL1*, delaying flowering time and maintaining SAM indeterminacy, between the pea homologs *LF* and *DET* (Foucher et al., 2003). Alternatively, it is possible that *FTa1* has a role during the V/*I*<sub>1</sub> transition that is redundant with one or more other *FT* genes under LD conditions. This possibility could be investigated in a future study using mutants containing null mutant alleles for multiple *FT* genes.

In the compound inflorescence of pea, it seems that the presence of the *I*<sub>2</sub> as an additional inflorescence branch level, relative to the simple raceme of *Arabidopsis*, introduces a critical point or bottleneck within the pea flowering pathway, where specification of *I*<sub>2</sub> meristems must occur before floral meristems can be produced (Figures 3.14-3.15), at least in the presence of *DET* (Berbel et al., 2012). Although many papilionoid legume species have a similar inflorescence form to pea, including *Medicago*, soybean, lentil and common bean, it is not yet clear if any of these species have a similar requirement for correct *I*<sub>2</sub> meristem specification. In *Medicago*, no

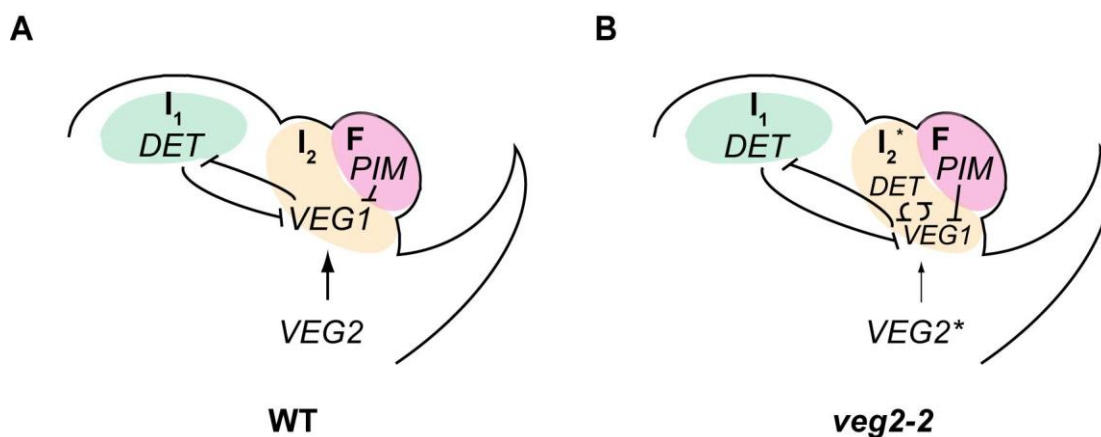
mutant phenotypes for homologs of *FD* or *VEG1* have been reported as yet, but the *Medicago fta1* null mutant (*PsFTa1/GIGAS* ortholog) is merely late-flowering under both LD and SD conditions (Laurie et al., 2011). The difference between the *Medicago fta1* and *gigas* mutant phenotypes seems more likely to reflect a difference in distribution of roles between members of the pea and *Medicago FT* families, rather than a reduced importance for  $I_2$  specification in *Medicago*, but this should be investigated further.

As the *veg2-2* mutant does eventually flower, this mutant offers valuable insight into the roles of *VEG2* beyond the critical point of initial  $I_2$  specification, which is blocked in the non-flowering *veg2-1* mutant. In *veg2-2* there are axillary structures borne from the main stem that in turn bear axillary flowers and thus meet the key criterion of an  $I_2$  structure (Figure 3.16I-L). However, these structures could be described as having only partial  $I_2$  identity, as leaf formation is not fully suppressed allowing development of bracts subtending these flowers, and an indeterminate apex is retained (Figures 3.16I-L, 3.17). After one or two nodes, this partial  $I_2$  identity is lost and the structure reverts to an  $I_1$ , which resembles the main stem, expresses *DET*, and continues to produce nodes with full compound leaves and axillary buds, or flower-bearing  $I_3$  structures, that reiterate the pattern (Figure 3.16I-N). This phenotype reveals a clear role for *VEG2* in both the initial specification and the maintenance of  $I_2$  identity.

It seems likely that both initial specification of  $I_2$  meristems and maintenance of  $I_2$  identity until the termination of the  $I_2$  in a stub, is controlled by continued expression of a specific gene or genes with three key roles: (i) maintaining  $I_2$  determinacy by excluding expression of the  $I_1$  meristem gene *DET*, (ii) allowing expression of floral genes in axillary primordia for development of flowers, and (iii) repressing leaf development. *VEG1* has been identified as a gene critical for  $I_2$  meristem specification and a target of *VEG2* (Figures 3.14-3.15; Berbel et al., 2012). In addition, *VEG1* is believed to repress *DET* expression in the  $I_2$  meristem thereby allowing expression of the floral meristem gene *PIM* in axillary primordia (Berbel et al., 2012). In turn, *DET* is believed to repress *VEG1*, in order to limit *VEG1* expression to axillary structures on the main stem (Berbel et al., 2012). Without *DET* function, *VEG1* is expressed in the SAM of the *det* mutant resulting in termination of the main stem in an ectopic  $I_2$  (Singer et al., 1990; Berbel et al., 2012). This is

comparable to the negative feedback loop that operates between *TFL1* and *AP1* in *Arabidopsis*, to maintain SAM indeterminacy and allow development of determinate, axillary flowers (Ratcliffe et al., 1999; Kaufmann et al., 2010).

Based on this model for pea, it follows that if expression of *VEG1* was insufficiently upregulated in an  $I_2$  structure, ectopic expression of *DET* may occur, further reducing *VEG1* expression and allowing increased *DET* expression. As *VEG1* is a target of *VEG2* (direct or indirect; Figures 3.14-3.15), it is possible that the *veg2-2*  $I_2$  phenotype could be the result of insufficient expression of *VEG1* and consequent negative interaction between *VEG1* and *DET*, with the dominant gene determining identity of the structure (Figure 3.23). In this model for  $I_2$  development in the *veg2-2* mutant, initially there would be enough *VEG1* to give partial  $I_2$  identity and allow production of one or two axillary flowers (Figures 3.14-3.16). But this level of *VEG1* would be insufficient to completely repress *DET* (Figure 3.16M), which would repress *VEG1* expression, become dominant after a few nodes, give the structure  $I_1$  identity (Figure 3.16N) and exclude any further expression of *VEG1* to axillary  $I_3$  structures. This model could be further tested by close examination of expression of *VEG1* and *DET* throughout  $I_2$  development in the *veg2-2* mutant. Interestingly, a recent study has shown that *FT* has an important role in maintaining inflorescence identity in *Arabidopsis* (Liu et al., 2014). Given the pea *FTa1* gene is required in conjunction with *VEG2* for upregulation of *VEG1* under LD conditions (Figure 3.14; Berbel et al., 2012), it is possible that *VEG2* normally acts with *FTa1* to maintain  $I_2$  identity in pea, similar to the role of *FT* in maintaining inflorescence identity in *Arabidopsis*.



**Figure 3.23.** Model for gene interactions determining  $I_2$  meristem identity. (continued on next page)



**Figure 3.23. (continued)** Diagrams represent developing  $I_2$  structures in (A) wild-type and (B) *veg2-2* mutant plant apices. The inferred roles for genes are indicating as promoting (→) or repressing (→|) the function of other genes. Meristems are as follows: primary inflorescence ( $I_1$ ), secondary inflorescence ( $I_2$ ), and floral (F). Asterisks for the *veg2-2* mutant indicate defects in the *VEG2* gene and in  $I_2$  identity. Wild-type model adapted from Berbel et al. (2012).

### 3.4.6 The role of *VEG2* in floral development

The *veg2-2* mutant phenotype also reveals that *VEG2* has a role in floral development, which was explored in this study through characterisation of floral morphology under LD and SD conditions. Some severely abnormal flowers with all whorls affected were observed in the *veg2-2* mutant, but sepal and petal whorls were most commonly affected (Figures 3.19A-B and 3.20A). This indicates that *VEG2* has a role in correct development of all floral whorls within the flower, especially sepal and petal whorls. Within the sepal and petal whorls, the two adaxial sepals and the standard petal (borne in an adaxial position within the petal whorl) were most commonly affected (Figures 3.19C and 3.20B). Pea floral organ primordia develop unidirectionally beginning on the abaxial side of the flower (Tucker, 1989; Ferrandiz et al., 1999). The frequency of defects affecting the adaxial sepals and standard petal in the *veg2-2* mutant may indicate either positional or temporal importance of *VEG2* during initiation of floral organ primordia. Although the nature of floral defects varied between whorls and between flowers, fusion to leaf/bract tissue was seen to affect each floral whorl, and a reduction in organ number was observed in sepal, petal and stamen whorls (Figures 3.19D and 3.20C). Floral defects decreased acropetally and flowers at later reproductive nodes of *veg2-2* mutant plants were completely normal (Figures 3.19E-G and 3.20D-E). This indicates that *VEG2* is especially important for correct floral development at early reproductive nodes, but due to either slow accumulation of downstream targets through partial function of *VEG2* in the *veg2-2* mutant, or activation via alternative age-related gene pathways, the importance of *VEG2* decreases with plant age. Similar patterns of floral defects were seen in both LD and SD conditions (Figures 3.19-3.20), indicating that the role of *VEG2* in floral development is not dependent on photoperiod.

According to the ABCE model in *Arabidopsis*, floral organ identity is determined by expression patterns of three classes of genes: A (*API*, *AP2*), B (*AP3*, *PI*) and C (*AG*), with an additional E-class (*SEP1*, *SEP2*, *SEP3*, *SEP4*) necessary for formation of higher order complexes (e.g. Coen and Meyerowitz, 1991; Theissen and Saedler, 2001). Complexes of ABCE genes are capable of changing leaves into floral organs (Honma and Goto, 2001). Accordingly, floral organs are replaced with leaves in all floral whorls of the triple mutants *ap2 ap3 ag* and *ap2 pi ag* that have mutant alleles for genes in each of the three ABC classes (Bowman et al., 1991), and in the quadruple mutant *sep1 sep2 sep3 sep4* where function of all E-class genes is impaired (Ditta et al., 2004). The genes involved in floral organ specification are predominantly type II MIKC group MADS-box genes (Parenicova et al., 2003). While there are considerable differences in the characteristics of floral organ initiation between pea and *Arabidopsis* (Tucker, 1989; Smyth et al., 1990; Ferrandiz et al., 1999), genes corresponding to key A-, B-, C- and E-class genes have been isolated in pea and the ABCE model remains a good starting point for understanding the intricacies of the system controlling pea flower development (e.g. Taylor et al., 2002; Berbel et al., 2005; Hecht et al., 2005).

Sepal and petal whorls were most commonly affected in abnormal flowers of the *veg2-2* mutant (Figures 3.19B and 3.20A). Based on this it is tempting to suggest that *VEG2* may be important for regulation of A-class genes using the traditional ABC model for floral development, where these genes specify identity in sepal and petal whorls (Coen and Meyerowitz, 1991). Of course it is not quite that simple, as using the traditional model, carpels would be expected in the first (outer) whorl and stamens in the second whorl (Coen and Meyerowitz, 1991), which is not the case in the *veg2-2* mutant. In addition, the roles of A-class genes have since been redefined with the role in sepal identity now thought to reflect a role in floral meristem identity, and the importance of A-class genes for petal development questioned (e.g. Litt, 2007; Causier et al., 2010). Nonetheless, *PIM*, the pea homolog of the *Arabidopsis* A-class gene *API*, is misregulated in the *veg2-2* mutant, and there are some similarities between the *veg2-2* floral defects and those seen in mutants for A-class genes characterised in pea and *Arabidopsis* (Figures 3.14-3.15; Irish and Sussex, 1990; Taylor et al., 2002; see also Chapter 5). *PIM* is still expressed in the *veg2-2* mutant, thus *veg2-2* floral morphology would not be expected to be as

severely affected as mutants that completely lack *PIM/AP1* function. Like *veg2-2*, mutant alleles for *PIM* exhibit replacement of the first floral whorl (sepals) with leafy bracts, second and third whorl organs are missing or mosaic, and severity of floral morphology defects decreases acropetally (Singer et al., 1999; Taylor et al., 2002). In *Arabidopsis*, presence of leaf/bract tissue in the first whorl and reduced number of organs in the second whorl, were also observed for single mutants of the A-class genes *AP1* and *AP2* (Bowman et al., 1989; Irish and Sussex, 1990). However, unlike *veg2-2*, both mutants showed an acropetal increase in severity of floral defects (Kunst et al., 1989; Bowman et al., 1993). The ectopic flowers within flowers seen in *pim*, *ap1* and *ap2* mutants are not seen in the *veg2-2* mutant (Irish and Sussex, 1990; Bowman et al., 1991; Taylor et al., 2002). However, for *Arabidopsis ap1*, at least, this phenotype is separable from floral organ morphology, with weak *ap1* mutants exhibiting floral organ identity defects but no ectopic flowers under certain growth conditions (Bowman et al., 1993).

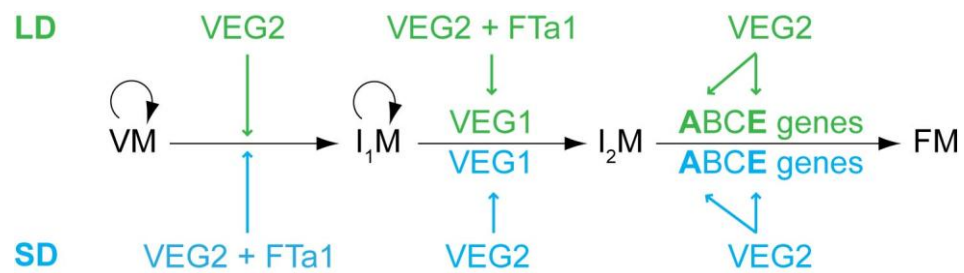
The presence of leaf tissue noted in each whorl of flowers from the single *veg2-2* mutant is reminiscent of (but not as severe as) the acquisition of leaf identity in all floral whorls of *Arabidopsis* mutants with defects in genes of each ABC class or all E-class genes, indicating failure to fully specify floral meristem identity (Figures 3.19D and 3.20C; Bowman et al., 1991; Ditta et al., 2004). In addition, floral morphology improves acropetally in the E-class *sep1 sep2 sep3 sep4* quadruple mutant, as is seen in the single *veg2-2* mutant (Figures 3.19E-G and 3.20D-E). Two *SEP* homologs have been identified in pea (Buchner and Boutin, 1998; Hecht et al., 2005) and preliminary studies indicated *SEPI* is transcriptionally downstream of *VEG2* (Sussmilch, 2008; see Chapter 5). To confirm if *VEG2* has a role in floral development beyond regulation of *PIM*, the phenotype of the *pim-2 veg2-2* double mutant was characterised. In the *pim veg2-2* double mutant, floral morphology was found to be further affected than in the *pim* single mutant (Figures 3.21-3.22; Taylor et al., 2002). This indicates that *PIM* is not the only gene involved in floral development that is targeted by *VEG2*.

The phenotype of the *pim-2 veg2-2* double mutant also shows an interesting genetic interaction between *VEG2* and *PIM*. If the interaction between *VEG2* and *PIM* was simply additive, it could be expected that one to two nodes of *pim*-like proliferating flowers would be borne on a *veg2-2*-like  $I_2$  structure that reverted to an  $I_1$  after several floral nodes. Instead, *pim veg2-2* double mutants had aerial branches that only resembled  $I_2$ s due to the bracts at the first one or two nodes, which were similar to the bracts subtending flowers in the *veg2-2* mutant  $I_2$  (Figures 3.16, 3.17, 3.21 and 3.22). Upper nodes of branches or the main stem of *pim veg2-2* mutant plants acquired floral identity with the leaf nodes themselves appearing similar to floral whorls with fused or axillary floral organs, and leaf morphology at these nodes often compromised (Figures 3.21-3.22). Axillary structures subtended by these bracts did not resemble any structure borne in place of flowers in the *pim* mutant (Taylor et al., 2002). Instead branches were borne that reiterated the pattern of a bract at the first few branch nodes and acquisition of floral identity at upper branch nodes (Figures 3.21-3.22). Even when flowers in the *pim* mutant are replaced by leafy branches, these branches do not acquire floral identity (Taylor et al., 2002; personal observation). The phenotype of the *pim veg2-2* mutant is reminiscent of the *Arabidopsis lfy* mutant, which exhibits conversion of flowers to inflorescence branches subtended by bracts, with branches occasionally acquiring floral identity and terminating in floral organs (Schultz and Haughn, 1991; Huala and Sussex, 1992; Souer et al., 1998). This acquisition of floral identity in the *Arabidopsis lfy* mutant was explained by expression of ABC genes including *AP1*, *AP2* and *AG* (Huala and Sussex, 1992; Bowman et al., 1993). Results of a preliminary study suggested that expression of *UNI*, the pea *LFY* ortholog, may be misregulated in the *veg2-2* mutant (Sussmilch, 2008; see also Chapter 5), so it is plausible that the *pim veg2-2* double mutant phenotype could be a result of misregulation of *UNI*, and slow upregulation of other floral genes as the plant ages.

In summary, it seems likely that *VEG2* acts to ensure correct floral development in wild-type plants through regulation (either direct or indirect) of multiple floral organ identity genes, particularly A- and E-class genes. It appears that these target genes are either important during early stages of flowering, or accumulate or become active by alternative means as the plant ages.

### 3.4.7 Chapter conclusions

Based on the results presented in this chapter, *VEG2* appears to be important for specification of each of the three meristems involved in pea inflorescence development (Figure 3.24). The results indicate that *VEG2* is necessary for the correct timing of the V/I<sub>1</sub> transition under both LD and SD conditions (Figures 3.2-3.5), critical for both initial specification and maintenance of I<sub>2</sub> identity (Figures 3.14-16), and important for specification of floral meristems (Figures 3.14-15) and development of flowers at early floral nodes (Figures 3.19-3.22). These findings and suggested mechanisms are summarised in the model presented in Figure 3.24. Characterisation of the *VEG2* locus at a molecular level is the next step necessary for further investigation of the roles and interactions of the *VEG2* locus.



**Figure 3.24.** A model summarising the roles of *VEG2* during each of the meristem transitions involved in pea inflorescence development, and suggested mechanisms, based on the results of Chapter 3.

Genes and gene pathways regulating each step under inductive LD (green) and non-inductive SD (blue) photoperiods, are shown. Coloured arrows indicate a promotive influence. Meristems and meristem transitions are shown in black. Meristems are as follows: vegetative meristem (VM), primary inflorescence meristem (I<sub>1</sub>M), secondary inflorescence meristem (I<sub>2</sub>M), and floral meristem (FM). Straight black arrows indicate meristem transitions and products, while circular black arrows indicate meristem indeterminacy.



# CHAPTER 4: Molecular characterisation of the *VEG2* locus

## 4.1 Introduction

### 4.1.1 A forward genetics approach

Prior to the molecular era, a genetic approach was adopted to investigate the influence of natural variation and induced mutations on flowering behaviour in pea. In more recent years, the challenge has been to characterise flowering loci at a molecular level, as successful identification of the genes involved can allow further investigation of gene action. Ultimately, this can improve understanding of the gene networks controlling flowering and inflorescence development in pea, and allow comparison with other species. In the previous chapter, the phenotypes of two mutant alleles for the *VEG2* locus were described: the more severe non-flowering *veg2-1*, and the weaker late-flowering *veg2-2*. Characterisation of the gene affected in the *veg2* mutants is the next step in determining the mechanisms of *VEG2* function during pea inflorescence development.

At the time this study commenced, there were relatively few transcript and genomic sequence resources available for pea. However, the close synteny between pea and *Medicago* has previously enabled the use of publicly available *Medicago* sequence resources for successful identification of candidate genes for pea mutant loci using a comparative mapping strategy (e.g. Hecht et al., 2007; Liew et al., 2009; Weller et al., 2012). Early reports suggested that *VEG2* was located on pea linkage group I (Murfet and Reid, 1993), and subsequent linkage analysis indicated that *VEG2* was located towards the base of this linkage group, above the marker loci *FENR1*, *AF* and *I* (Sussmilch, 2008). This corresponds to a region towards the base of *Medicago* chromosome 5 (Aubert et al., 2006; Bordat et al., 2011), which contains a *Medicago* homolog of *FD* (Medtr5g022780 in Mt3.5; Sussmilch, 2008). Preliminary results from phylogenetic analysis and investigation of microsynteny between the genomic regions flanking *FD* in *Arabidopsis* and the *FD* gene identified in *Medicago*, provided evidence of a common evolutionary origin (Sussmilch, 2008).

### 4.1.2 *FD* genes in other species

In *Arabidopsis*, two closely related bZIP transcription factors, *FD* and *FDP*, act in the apex through interaction with various CETS proteins, including the floral promoters *FT* and *TSF*, and the floral repressor *TFL1*, to regulate the expression of floral integrator and floral meristem identity genes (Abe et al., 2005; Wigge et al., 2005; Jang et al., 2009; Hanano and Goto, 2011). The *Arabidopsis* bZIP family of transcription factors has been subdivided into groups according to structure, with *FD* and *FDP* placed into group A (Jakoby et al., 2002). *fd* and *fdp* mutants are both late-flowering, and flowering is further delayed in the *fd fdp* double mutant, indicating that the *FT/FD* pathway is important for promoting inflorescence meristem identity, but not critical for flowering to occur (Koornneef et al., 1991; Jaeger et al., 2013). Similar to the *Arabidopsis fd* mutant, *OsFDI* RNAi transgenic rice plants have a simple late-flowering phenotype (Koornneef et al., 1991; Taoka et al., 2011). In contrast, *dlf1*, a mutant for a maize *FD* homolog, has defects in tassel and ear morphology, in addition to delayed flowering time, suggesting that *DLF1* has an extended role in maize inflorescence development (Muszynski et al., 2006). A recent study identified additional *FD* genes from a large range of eudicot and monocot species, but did not identify any *FD* genes in gymnosperm or moss species, suggesting that *FD* genes are specific to angiosperms (Tsuji et al., 2013b).

Relatively little is known about *FD* gene(s) in legume species. In soybean one *FD*-like gene has been identified (Glyma01g36810 in v1.1) and suggested to be the only soybean homolog of *AtFD* and *FDP* (Jung et al., 2012). This finding seems somewhat surprising given the tetraploid origin of the soybean genome (Shoemaker et al., 1996; Choi et al., 2004), and the fact that nearly 75% of genes have been found to be present in multiple copies (Schmutz et al., 2010). More specifically, the soybean bZIP transcription factor family is more than double the size of that of *Arabidopsis* (Schmutz et al., 2010). A recent characterisation of angiosperm *FD* genes conducted by Tsuji et al. (2013b) included two new soybean *FD*-like genes (Glyma04g02420 and Glyma06g02470), but did not include the gene identified by Jung et al. (2012). In *Medicago*, in addition to Medtr5g022780, a second gene has also been identified as *FD*-like (Medtr7g088090), and named as *MtFD* accordingly (Pierre et al., 2008; Stanton-Geddes et al., 2013). SNPs within and surrounding this gene were found to be associated with flowering time in a genome-wide association



study (Stanton-Geddes et al., 2013). However, no phylogenetic analysis has been conducted and doubt has since been raised over whether this is truly an *FD* homolog (Yeoh et al., 2013).

#### ***4.1.3 Isolation and preliminary investigation of an FD-like gene from pea***

In a preliminary study, a portion of the *Medicago FD* gene identified on chromosome 5 (Medtr5g022780) was used as a probe to isolate partial sequence for the corresponding gene in pea (Sussmilch, 2008). This partial pea sequence was extended with RACE PCR to obtain full-length *FD* coding sequence and mapped to the same region as the *VEG2* locus on the bottom half of linkage group I (Sussmilch, 2008). Initial investigation of this gene as a candidate for *VEG2*, revealed a missense mutation in the late-flowering *veg2-2* mutant (Sussmilch, 2008). In addition, certain regions of this *FD* gene could not be amplified by PCR from *veg2-1* mutant genomic template, indicating deletion of at least part of the coding sequence (Sussmilch, 2008). However, the boundaries of this putative deletion were not identified in this preliminary study, and segregation of the *veg2-2* SNP with mutant phenotype was not confirmed.

#### ***4.1.4 Chapter aim(s)***

The main aim for this chapter was to complete the molecular characterisation of the *VEG2* locus. Firstly, to identify *FD*-like genes, searches of available legume sequence resources, and phylogenetic analyses were conducted to characterise the legume *FD* gene family in detail for the first time. To investigate microsynteny, the genes surrounding identified legume *FD* genes and *Arabidopsis FD* were examined. Next, to investigate the extent of the putative deletion in the *veg2-1* mutant, the putative flanking genes for the pea *FD* gene were isolated and their presence in the *veg2-1* mutant was determined. Finally, the previously identified *veg2-2* SNP was further investigated in terms of (a) co-segregation with the *veg2-2* mutant phenotype and (b) the level of conservation of the affected amino acid between diverse *FD* proteins and other members of the bZIP family of transcription factors.

## 4.2 Materials and methods

This section contains specific details of materials and methods for studies included in this chapter. General materials and methods also relevant to this chapter are described in Chapter 2. Primer details are given in Appendix 1. Details of online resources are given in Chapter 2 (Table 2.4) and details of sequences and alignments are given in Appendix 2. For all molecular work involving *veg2-1*, the mutant allele in the original Kaliski background was used with Kaliski as wild-type (see Section 2.1 for details of plant materials).

### 4.2.1 Identification of *FD* genes

*Medicago* bZIP genes were identified in tBLASTn searches of the *Medicago* genome in the ‘Pseudomolecules’ database and BLASTp searches of *Medicago* proteins in the ‘IMGAG Proteins’ database using predicted protein sequence for *AtFD*, *AtFDP* and *Medicago* Medtr5g022780 and the identified pea *FD* gene as queries. Group A bZIP genes were distinguished among the identified *Medicago* bZIP transcription factors, by preliminary phylogenetic analyses with the entire *Arabidopsis* bZIP family, using predicted amino acid sequence for the bZIP domain.

*FD*-like genes were identified in other legume species in tBLASTn searches in genome resources for soybean, *Lotus* and common bean. BLAST searches were initially conducted using predicted protein sequence for *AtFD*, *AtFDP* and the *FD* genes identified in *Medicago* and pea as query sequences. Subsequently, these searches were expanded to include all identified legume FD proteins as queries, to confirm that no more legume *FD*-like genes were present in available resources. *FD* genes from other angiosperm species were identified in BLAST searches of available expressed and genomic sequence resources using identified FD proteins as query sequence. In cases where identified genes were not annotated, or where alignments suggested that the annotation provided in online genome databases was incorrect, protein sequence was inferred from transcript sequence(s) and from genome sequence based on alignments between species. Most instances of incorrect annotation involved inclusion of intron sequence within the highly conserved bZIP domain, or incorrect amino acid sequence for exons 2 and 3.

In attempts to identify a second *FD* gene in pea, PCR was performed on pea gDNA template from wild-type line NGB5839 under low stringency conditions using primers designed directly from *MtFDb* (Medtr8g075130) in combination with degenerate primers designed in accordance with the Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) strategy (Rose et al., 1998). Degenerate primers were designed prior to this study with the aid of CODEHOP software (<http://blocks.fhcrc.org/codehop.html>) to target the conserved Motif A (Primer FD-1D) and the bZIP domain (Primers FD-2D and FD-3D). Primers designed directly from *MtFDb* were placed to target the bZIP domain (MtFDP-1F, MtFDP-1R) and the Ser-Ala-Pro motif (MtFDP-2R; Appendix 1).

#### 4.2.2 Microsynteny

The genes flanking *FD* genes were identified in *Arabidopsis*, *Medicago*, soybean and common bean, using GBrowse genome annotation viewers. Gene identity was determined for each putative flanking gene, using reciprocal tBLASTn searches of predicted protein sequence against *Arabidopsis*, *Medicago*, soybean and common bean genomes.

#### 4.2.3 Determining the *veg2-1* deletion boundaries

##### 4.2.3.1 Attempts to isolate *FDa* in the *veg2-1* mutant

To test for the presence of *FDa* coding sequence in the *veg2-1* mutant, various combinations of primer pairs within the 5' UTR, coding sequence and 3' UTR of *FDa* were tested on wild-type and *veg2-1* gDNA (Table 4.1). These PCRs failed to yield PCR product from the *veg2-1* mutant, but successfully yielded product from the wild-type template included as a positive control in each PCR (Table 4.1). *veg2-1* gDNA quality was confirmed in positive control PCRs for the unrelated flowering genes *DET* (TFL1a-1F+TFL1a-Rev03) and *LATE1* (GI-L11F+GI-L11R), using the same *veg2-1* template. The gel photo for a representative PCR covering full-length *FDa* coding sequence (primer pair: PsFD-7F and PsFD-6R) is shown in the Results Section 4.3.3. A downstream portion of DNA, 1.4 kb after the *FDa* stop codon, was found to be present in the *veg2-1* mutant using the primer pair PsFD-9F and PsFD-12R (Appendix 1). All PCRs were conducted using standard PCR conditions.

**Table 4.1.** Details of primer pairs used to confirm deletion of all coding sequence of *FDa* in the *veg2-1* mutant. All primer pairs yielded PCR product from wild-type (Kaliski) gDNA included as a positive control template in each PCR, but failed to yield any PCR product from *veg2-1* gDNA. *veg2-1* gDNA yielded PCR product for positive control reactions performed with TFL1a-1F+TFL1a-Rev03 and GI-L11F+GI-L11R. Abbreviations are as follows: coding sequence (CDS), full-length (FL), untranslated region (UTR). Primer details are given in Appendix 1.

Forward primer	Primer location	Reverse primer	Primer location	Wild-type product size (bp)	Region of <i>FDa</i> CDS included
PsFD-1F	5' UTR	PsFD-1R	Exon 1	496	Partial exon 1
PsFD-1F	5' UTR	PsFD-3R	Exon 2	904	Exon 1, partial exon 2
PsFD-1F	5' UTR	PsFD-5R	3' UTR	1232	FL
PsFD-1F	5' UTR	PsFD-7R	3' UTR	1608	FL
PsFD-7F	5'UTR/Exon 1	PsFD-6R	3' UTR	1236	FL
PsFD-5F	Exon 2	PsFD-6R	3' UTR	537	Exon 3, partial exon 2

#### 4.2.3.2 *Isolation of putative 5' flanking gene (LA RELATED PROTEIN 1C) for FDa*

*Medicago LA RELATED PROTEIN 1C* (*MtLARP1C*; Medtr5g022790 in Mt3.5) was identified as the 5' flanking gene for the *Medicago FD* homolog (*MtFDa*; Medtr5g022780 in Mt3.5; see Section 4.2.2). At the commencement of this study, *MtLARP1C* was incorrectly annotated in available online resources as three genes (Medtr5g022740, Medtr5g022750 and Medtr5g022760 in Mt3.0). A 208bp portion of the pea gene was isolated using primers (MtHyp-F and MtHyp-R) designed from *Medicago* sequence (Medtr5g022750 in Mt3.0; TC127352 at DFCI TGI) under low stringency PCR conditions. This portion was originally thought to correspond to the coding sequence for the central gene of these three genes (Medtr5g022750; Mt3.0), but was later found to correspond to the 3' UTR of *MtLARP1C*. However, even updated annotation for *MtLARP1C* (Mt3.5) appears to be incorrect (see Appendix 2). When pea TSA sequences became available (Franssen et al., 2011; Kaur et al., 2012), pea sequences for this gene were identified in BLASTn searches against the GenBank TSA Sequence Database (GenBank accessions: JR963915; JI924790, JI919144, JI949799). Full-length coding sequence was isolated using primers designed from pea TSA sequence (PsWiHe-1F designed from JR963915) and the original pea fragment (PsHyp-1R) under standard PCR conditions. tBLASTx searches were conducted using the region of the *Medicago* genome between the coding sequences of *MtFDa* (Medtr5g022780 in Mt3.5) and the 5' flanking gene (Medtr5g022790 in Mt3.5) as a query against *Arabidopsis* (TAIR10 transcripts) and *Medicago* (IMGAG CDS Mt3.5) resources to confirm that this

region did not contain any genes that were not annotated, but no extra genes were identified.

#### 4.2.3.3 Isolation of putative 3' flanking gene (*RING finger*) for *FDa*

*RING finger* (Medtr5g022790 in Mt3.5), a gene that encodes a RING finger protein, was identified as the 3' flanking gene for *MtFDa* (see Section 4.2.2). Initially, a 430bp portion within exon 1 of the corresponding pea gene was isolated from Kaliski using primers designed from *Medicago* sequence (Medtr5g022720 in Mt3.0; MtZnFin-F and MtZnFin-R), under low stringency PCR conditions. PCR product was sequenced and used for design of pea primers (PsZnFin-2F and PsZnFin-2R). Full-length coding sequence and surrounding intergenic sequence was amplified using primer pairs PsZnFin-2R + PsDUF-1F (situated in adjacent gene *DUF343*; see below Section 4.2.3.4), and PsFD-GSP2F + PsZnFin-2F (see below Section 4.2.3.6). Primers designed within the 5' and 3' UTR from these sequences (PsZnFin-5F and PsZnFin-3R) were used under standard conditions to isolate full-length coding sequence from pea. tBLASTx searches were conducted using the region of the *Medicago* genome between the coding sequences of *MtFDa* (Medtr5g022780 in Mt3.5) and the 3' flanking gene (Medtr5g022790 in Mt3.5) as a query against TAIR (TAIR10 transcripts) and *Medicago* (Mt3.5 IMGAG CDS) resources to confirm that this region did not contain any genes that were not annotated, but no extra genes were identified.

#### 4.2.3.4 Isolation of *PsDUF343*

Initially, a 240bp portion of the pea *DUF343* gene (including exon 3 and a section of 3' UTR) was isolated using primers designed from *Medicago* sequence (Medtr5g022710 in Mt3.0; MtDUF-2F and MtDUF-R), under low stringency conditions. PCR product was sequenced and used for design of pea primers. Later, BLASTn searches against the GenBank TSA Sequence Database were used to identify a pea TSA sequence to give full coding sequence for *PsDUF343* (GenBank accession: JI907170).

#### 4.2.3.5 Mapping of *FDa* and surrounding genes

Markers were scored on existing DNA from 92 F<sub>2</sub> progeny of a cross between lines NGB5839 and JI1794 that was generated previously by V. Hecht, S. Ridge, J. Vander Schoor and J. Weller. Specific details of molecular markers are given in Appendix 1 (Table A1.4). Linkage analysis was conducted as outlined in Chapter 2 (Section 2.11).

#### 4.2.3.6 Attempts to isolate regions between *FDa* and putative flanking genes

The full-length coding sequence for *FDa* obtained previously (Sussmilch, 2008) was extended at the 3' end by genome walking using separate wild-type gDNA libraries digested with DraI, EcoRV or HpaI and nested gene-specific primers (PsFD-GSP1F and PsFD-GSP2F). However, this approach yielded only 141bp of new 3' sequence. Next, the region between *FDa* and *RING finger* (the putative 3' flanking gene) was amplified using primer pair PsFD-GSP2F (placed in 3' UTR of *PsFDa*) and PsZnFin-2F (placed in Exon 1 of *RING finger*; see above Section 4.2.3.3) with Long Amp<sup>®</sup> Taq DNA Polymerase (New England Biolabs; Ipswich, MA, USA) according to the manufacturer's specifications. This yielded a PCR product larger than 10kb which was purified and sequenced in from either end, in two rounds, using the standard DNA sequencing services at Macrogen Inc. (Seoul, Korea). The first sequencing round used the original primer pair (PsFD-GSP2F and PsZnFin-2F), and the second used new nested primers designed after the first round (PsFD-8F and PsZnFin-3R). In this manner, sequence was obtained for the regions 1.2 kb after the stop codon of the putative 3' flanking gene and 1.7 kb after the stop codon of *FDa*. A primer pair designed from this sequence (PsFD-9F and PsFD-12R), starting 1.4 kb beyond the stop codon of *FDa*, was tested and found to successfully amplify product from the *veg2-1* mutant, so no further rounds of sequencing were needed to determine the 3' *veg2-1* deletion boundary.

At the 5' end, full-length coding sequence for *FDa* was also extended first by genome walking using separate wild-type gDNA libraries digested with DraI, SspI, EcoRV, PvuII or SwaI and two sets of nested gene-specific primers (PsFD9R and PsFD-2R; PsFD-GSP1R and PsFD-GSP2R). However, this approach yielded only 176bp of new wild-type 5' sequence. To determine the 5' *veg2-1* deletion boundary, multiple genome walking attempts were made to extend sequence upstream from the

portion downstream of *FDa* present in the *veg2-1* mutant, using separate *veg2-1* mutant gDNA libraries digested with PvuII, HaeIII, NruI, SmaI or XmnI and PsFD-14R, PsFD-13R, and PsFD-11R as gene-specific primers, but these were all unsuccessful. Genome walking attempts to extend sequence downstream from *LARP1C* (the putative 5' *FDa* flanking gene) using separate wild-type gDNA libraries digested with AluI, HaeIII, NruI, SmaI and *veg2-1* mutant gDNA libraries digested with PvuII, HaeIII, NruI, SmaI or XmnI, and gene-specific primers PsHyp-GSP1F, PsHyp-GSP2F, PsHypGSP-3F and PsHyp-GSP4F yielded only 428bp of new sequence.

Attempts to isolate the region between *FDa* and *LARP1C* (the putative 5' flanking gene) were not successful. Details of specific PCRs using wild-type and *veg2-1* mutant gDNA template are outlined in Tables 4.2 and 4.3, respectively. These involved some secondary PCRs conducted using nested primer pairs on primary PCR product to specifically target the PCR product of interest, and used a range of different DNA polymerases including MangoTaq™ DNA polymerase (Bioline, Alexandria, NSW, Australia), Phusion® DNA polymerase (Finnzymes, Espoo, Finland), Long Amp® Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA), BIO-X-ACT™ Long DNA Polymerase (Bioline, Alexandria, NSW, Australia), Advantage® Genomic LA Polymerase Mix (Clontech, Mountain View, CA, USA) and GoTaq® Long PCR Master Mix (Promega, Madison, WI, USA), according to each manufacturer's specifications. All efforts were made to clone and sequence any suitable bands obtained from these PCRs but no PCR yielded product for the region between *FDa* and *LARP1C*, as determined by colony PCR and/or sequencing.

**Table 4.2.** Details of PCRs conducted on Kaliski (wild-type) gDNA during attempts to isolate the region between *FDa* and the putative 5' flanking gene (*LARP1C*). Primer details are given in Appendix 1.

	Template	Primer pair	Primer placement	DNA polymerase
1	KAL gDNA	PsHyp-1F PsDUF-1F	3' UTR of <i>LARP1C</i> Exon 3 of <i>DUF343</i>	Phusion <sup>®</sup> DNA polymerase
2	KAL gDNA	PsHyp-1F PsFD-2R	3' UTR of <i>LARP1C</i> Exon 1 of <i>FDa</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
3	KAL gDNA	PsHyp-1F PsFD-2R	3' UTR of <i>LARP1C</i> Exon 1 of <i>FDa</i>	BIO-X-ACT <sup>™</sup> Long DNA Polymerase
4	KAL gDNA	PsHyp-1F PsDUF-1F	3' UTR of <i>LARP1C</i> Exon 3 of <i>DUF343</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
5	KAL gDNA	PsHyp-1F PsDUF-1F	3' UTR of <i>LARP1C</i> Exon 3 of <i>DUF343</i>	BIO-X-ACT <sup>™</sup> Long DNA Polymerase
6	PCR product from 5	PsHyp-1F PsFD-1R	3' UTR of <i>LARP1C</i> Exon 1 of <i>FDa</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
7	KAL gDNA	PsHyp-1F PsDUF-1F	3' UTR of <i>LARP1C</i> Exon 3 of <i>DUF343</i>	Advantage Genomic LA Polymerase Mix
8	KAL gDNA	PsHyp-1F PsFD-1R	3' UTR of <i>LARP1C</i> Exon 1 of <i>FDa</i>	Advantage Genomic LA Polymerase Mix
9	PCR product from 8	PsHyp-1F PsFD-2R	3' UTR of <i>LARP1C</i> Exon 1 of <i>FDa</i>	Advantage Genomic LA Polymerase Mix
10	KAL gDNA	PsHyp-GSP1F PsFD-14R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>FDa</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
11	PCR product from 10	PsHyp-GSP2F PsFD-13R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>FDa</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
12	KAL gDNA	PsHyp-GSP2F PsFD-13R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>FDa</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
13	KAL gDNA	PsHyp-GSP3F PsFD-14R	Downstream (3') of <i>LARP1C</i> Downstream (3') of <i>FDa</i>	GoTaq <sup>®</sup> Long PCR Master Mix
14	PCR product from 13	PsHyp-GSP4F PsFD-13R	Downstream (3') of <i>LARP1C</i> Downstream (3') of <i>FDa</i>	GoTaq <sup>®</sup> Long PCR Master Mix



**Table 4.3.** Details of PCRs conducted on *veg2-1* mutant gDNA (on Kaliski background) during attempts to isolate the region between *Fda* and the putative 5' flanking gene (*LARP1C*). Primer details are given in Appendix 1.

	Template	Primer pair	Primer placement	DNA polymerase
1	<i>veg2-1</i> gDNA	PsHyp-1F PsDUF-1F	3' UTR of <i>LARP1C</i> Exon 3 of <i>DUF343</i>	Phusion <sup>®</sup> DNA polymerase
2	<i>veg2-1</i> gDNA	PsHyp-1F PsDUF-1F	3' UTR of <i>LARP1C</i> Exon 3 of <i>DUF343</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
3	<i>veg2-1</i> gDNA	PsHyp-1F PsDUF-1F	3' UTR of <i>LARP1C</i> Exon 3 of <i>DUF343</i>	BIO-X-ACT <sup>™</sup> Long DNA Polymerase
4	<i>veg2-1</i> gDNA	PsHyp-GSP1F PsFD-11R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>Fda</i>	MangoTaq <sup>™</sup> DNA Polymerase
5	<i>veg2-1</i> gDNA	PsHyp-GSP2F PsFD-11R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>Fda</i>	MangoTaq <sup>™</sup> DNA Polymerase
6	<i>veg2-1</i> gDNA	PsHyp-GSP1F PsFD-12R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>Fda</i>	MangoTaq <sup>™</sup> DNA Polymerase
7	<i>veg2-1</i> gDNA	PsHyp-GSP2F PsFD-12R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>Fda</i>	MangoTaq <sup>™</sup> DNA Polymerase
8	<i>veg2-1</i> gDNA	PsHyp-GSP1F PsFD-14R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>Fda</i> ; present in <i>veg2-1</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
9	PCR product from 8	PsHyp-GSP2F PsFD-13R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>Fda</i> ; present in <i>veg2-1</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
10	<i>veg2-1</i> gDNA	PsHyp-GSP2F PsFD-13R	3' UTR of <i>LARP1C</i> Downstream (3') of <i>Fda</i> ; present in <i>veg2-1</i>	Long Amp <sup>®</sup> Taq DNA Polymerase
11	<i>veg2-1</i> gDNA	PsHyp-GSP3F PsFD-14R	Downstream (3') of <i>LARP1C</i> Downstream (3') of <i>Fda</i> ; present in <i>veg2-1</i>	GoTaq <sup>®</sup> Long PCR Master Mix
12	PCR product from 11	PsHyp-GSP4F PsFD-13R	Downstream (3') of <i>LARP1C</i> Downstream (3') of <i>Fda</i> ; present in <i>veg2-1</i>	GoTaq <sup>®</sup> Long PCR Master Mix

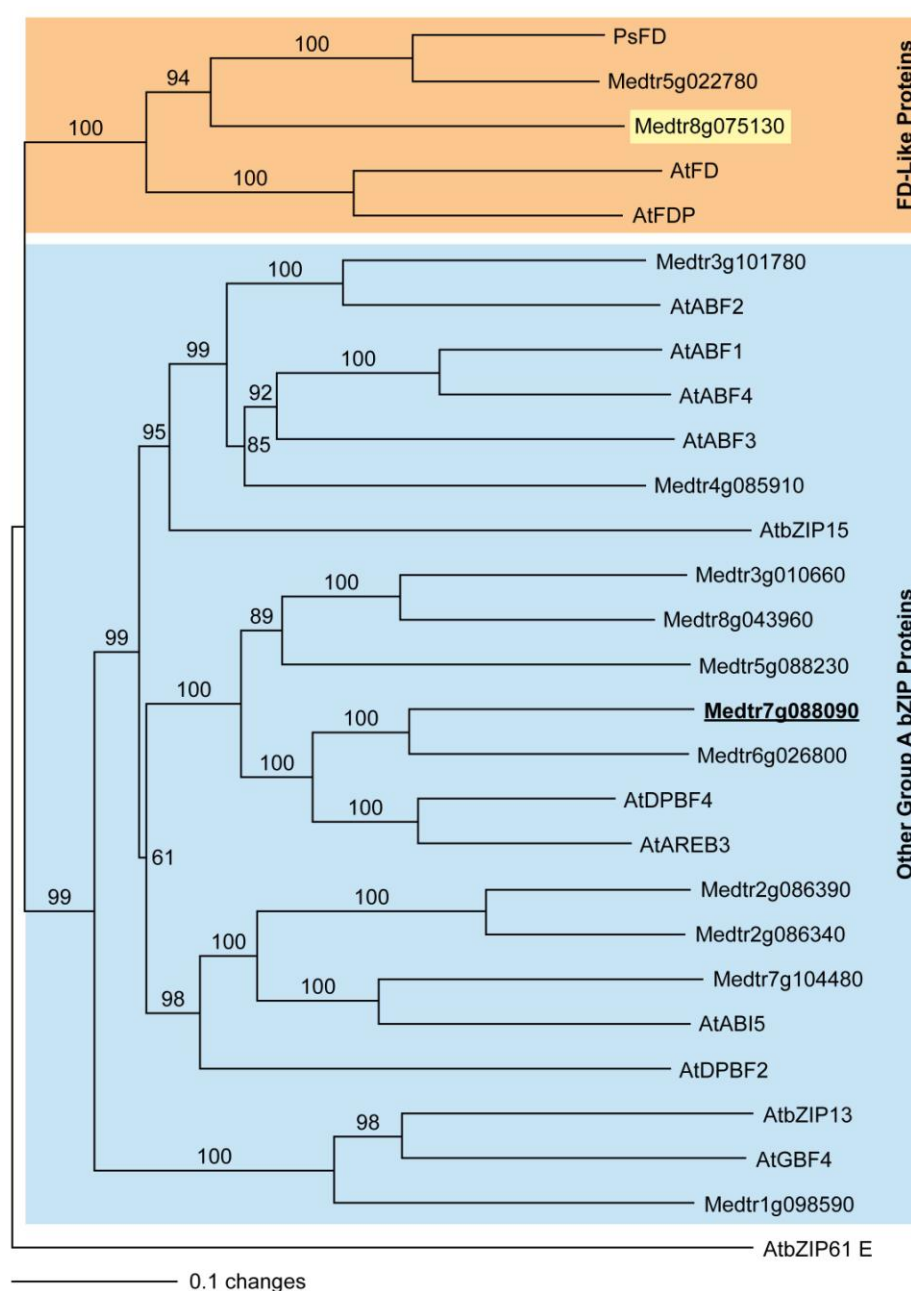
#### 4.2.4 Testing co-segregation of the G536A SNP with the *veg2-2* mutation

A CAPS marker for primer pair PsFD-4F+PsFD-5R using BspHI (New England Biolabs, Inc., Ipswich, MA, USA) according to the manufacturer's instructions, which digests *veg2-2* mutant but not wild-type PCR product, was used to score presence of the G536A SNP in 114 F<sub>2</sub> progeny of a cross between the *veg2-2* mutant (NGB5839 background) and Solara. *veg2-2* mutant phenotype was determined on the basis of flowering time and I<sub>2</sub> morphology after all plants had flowered.

## 4.3 Results

### 4.3.1 Characterisation of the legume *FD* gene family

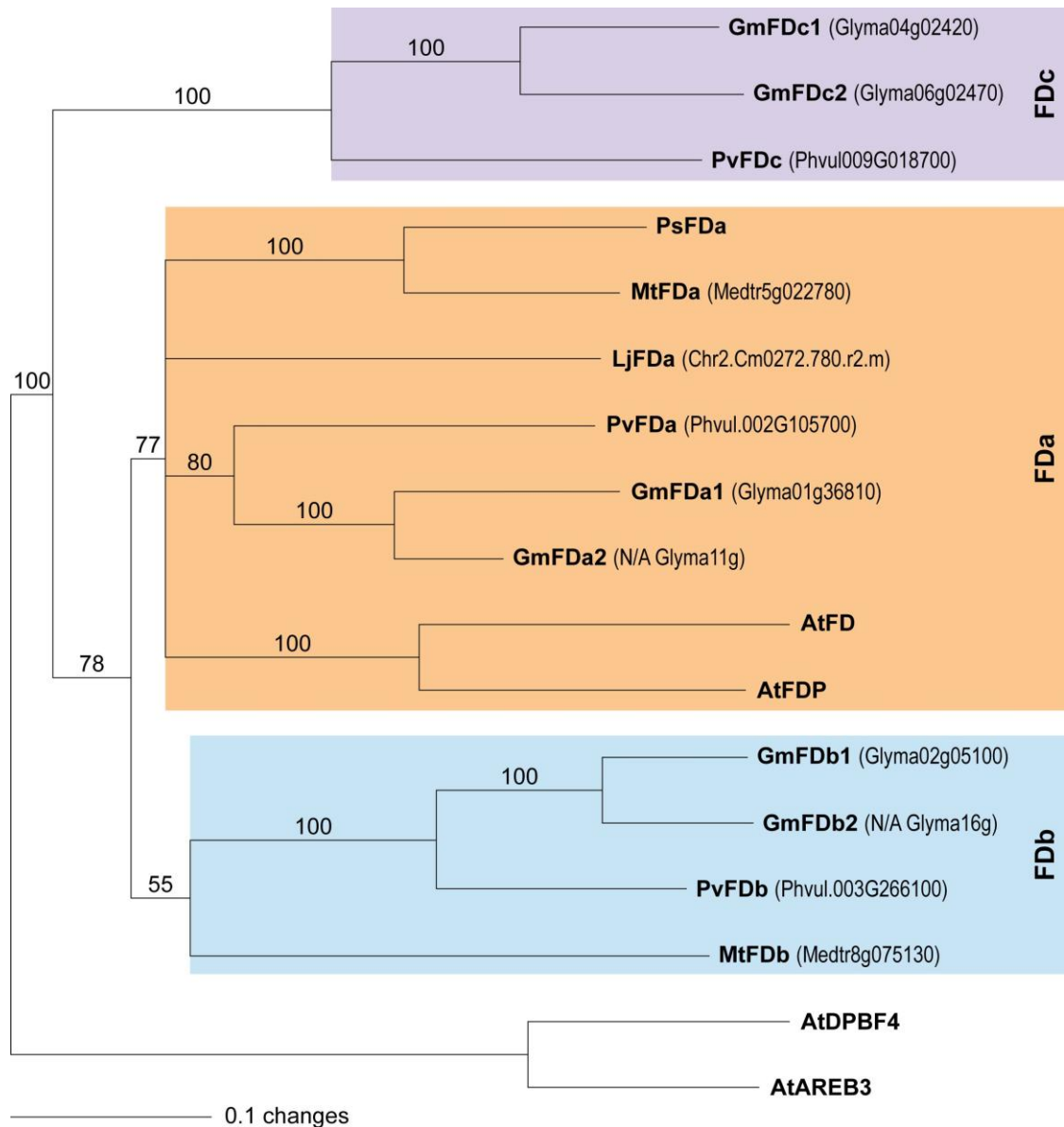
Previously, a *Medicago FD* gene (Medtr5g022780) was identified in reciprocal blast searches between *Arabidopsis* and *Medicago* genomes and full length sequence for the corresponding pea *FD* gene was isolated (Sussmilch, 2008). In this study, BLAST searches of *Medicago* genome resources were conducted to identify other *Medicago* bZIP transcription factors, and ultimately to determine whether other *FD* genes might be present in the *Medicago* genome. Phylogenetic analysis did in fact reveal a second *Medicago FD* gene (Medtr8g075130; Figure 4.1). Another *Medicago* gene that had previously been referred to as *MtFD* (Medtr7g088090; Pierre et al., 2008; Stanton-Geddes et al., 2013), was found instead to be more closely related to other *Arabidopsis* group A bZIP protein members, *DPBF4* (*AtbZIP12/EEL*) and *AREB3*, than to *FD* and *FDP* (Figure 4.1).



**Figure 4.1.** Phylogenetic neighbour-joining tree of group A *Arabidopsis* and *Medicago* bZIP proteins.

The phylogram was constructed from full-length predicted protein sequence for all *Arabidopsis* group A bZIP transcription factors (Jakoby et al., 2002; Correa et al., 2008), *Medicago* group A bZIP genes identified in this study (Mt3.5), and the previously identified pea *FD* gene (*PsFD*; Sussmilch, 2008), and rooted to the Group E bZIP AtbZIP61 (Jakoby et al., 2002; Correa et al., 2008). A second *Medicago* FD (Medtr8g075130) is highlighted in addition to the one previously identified (Medtr5g022780; Sussmilch, 2008). Medtr7g088090 (underlined) was previously named MtFD (Pierre et al., 2008; Stanton-Geddes et al., 2013), but is shown here to be more closely related to AtDPBF4 and AtAREB3, than to AtFD and AtFDP. Bootstrap values obtained from 1000 trees are indicated as a percentage above or next to each branch. Sequence and alignment details are given in Appendix 2.

In order to characterise the legume *FD* gene family, reciprocal tBLASTn searches were performed in soybean, *Lotus* and common bean genomes, with protein sequence for identified *FD* genes as queries (Figure 4.2; Table 4.4). Phylogenetic analysis revealed three distinct subclades of legume *FD* genes: *FDa*, *FDb* and *FDc* (Figure 4.2). The *FDa* subclade clustered with *Arabidopsis FD* and *FDP*, and included the previously isolated pea *FD* gene and corresponding *Medicago* gene (Medtr5g022780; Sussmilch, 2008), and a previously identified soybean gene (Glyma01g36810; Jung et al., 2012). These genes were renamed *PsFDa*, *MtFDa* and *GmFDa1* respectively (Figure 4.2; Table 4.4). A single *FDa* gene was identified in each of *Lotus* and common bean, and a second *FDa* gene was identified in soybean (Figure 4.2; Table 4.4). The second *FD* gene identified in *Medicago* (Medtr8g075130; Figure 4.1) fell into the *FDb* subclade and was renamed *MtFDb* accordingly (Figure 4.2; Table 4.4). *FDb* genes were also identified in soybean and common bean (Figure 4.2; Table 4.4), but no *FDb* genes were identified in *Lotus* or pea sequence databases, and attempts to isolate a putative pea *FDb* gene using degenerate and *Medicago* primers were unsuccessful. Two soybean *FD*-like genes (Glyma04g02420 and Glyma06g02470) that were included in a recent study of the *FD* gene family (Tsuji et al., 2013b), were found to fall into a third subclade of *FD* genes, *FDc*, which also included a gene from common bean (Figure 4.2; Table 4.4). No *FDc* genes were identified in thorough searches of *Lotus* or *Medicago* genomes, suggesting that *FDc* genes may be specific to the Phaseoleae tribe of papilionoid legumes.



**Figure 4.2.** Phylogenetic neighbour-joining tree of the legume FD family.

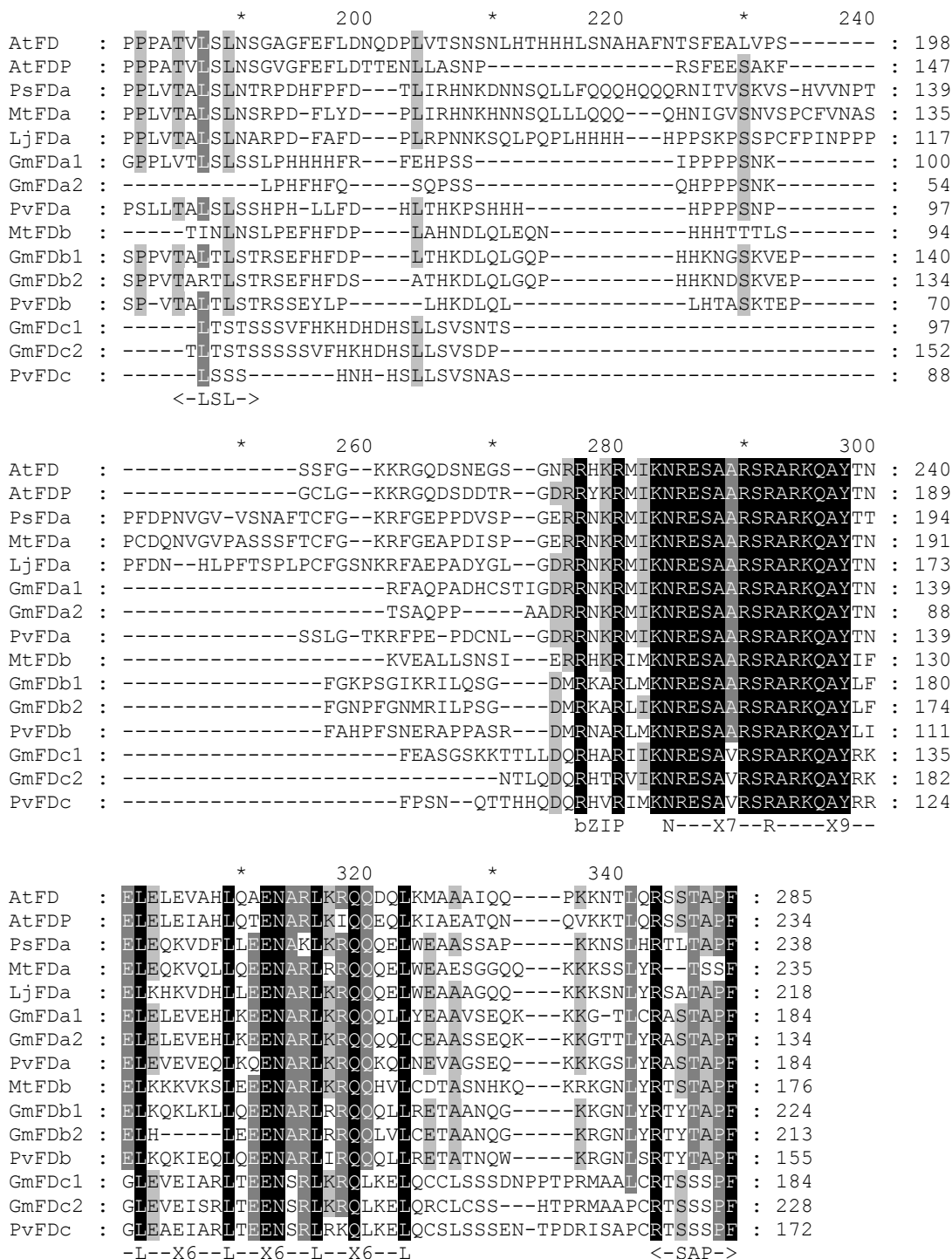
The phylogram was constructed from full-length predicted protein sequence for all *FD* genes from *Arabidopsis thaliana* (At), common bean (Pv), *Lotus japonicus* (Lj), *Medicago truncatula* (Mt), pea (Ps), and soybean (Gm), identified previously (Abe et al., 2005; Wigge et al., 2005; Sussmilch, 2008; Jung et al., 2012; Tsuji et al., 2013b), or during the course of this study (see Table 4.4). The phylogram is rooted to *AtDPBF4* and *AtAREB3*, which are other *Arabidopsis* group A bZIP transcription factors (Jakoby et al., 2002; Correa et al., 2008). Members of the legume FD family have been named (or renamed) in accordance with the three subclades visible in this phylogram (FDa, FDb and FDc). Locus names are given where genes were annotated in common bean (v1.0), *Lotus* (build 2.5), *Medicago* (Mt3.5), and soybean (v1.1) genomes. For the two predicted soybean proteins that are not annotated on the soybean genome (*GmFDa2* and *GmFDb2*), the soybean chromosome is indicated (see also Table 4.4). Bootstrap values obtained from 10,000 trees are indicated as a percentage above each branch. Sequence and alignment details are given in Appendix 2.

**Table 4.4.** Legume *FD* genes identified in available online sequence resources.

*FD* genes were identified in *Medicago* (Mt3.5), *Lotus* (build 2.5), soybean (v1.1), and common bean (v1.0) genome resources. Genes were named or renamed according to the subclades identified by phylogenetic analysis (see Figure 4.2). Previous gene names are indicated where applicable. Asterisks indicate incorrect annotation in online resources, as determined by alignments with expressed sequences and between legume species.

Species	Name	Locus ID	Chromosomal location	Reference
<i>Medicago truncatula</i>	<i>MtFDa</i>	Medtr5g022780*	Chr5: 8758327-8759707	Sussmilch (2008)
	<i>MtFDb</i>	Medtr8g075130*	Chr8: 20225953-20227169	This study
<i>Lotus japonicus</i>	<i>LjFDa</i>	Chr2.Cm0272.780.r2.m	Chr2: 26530026-26530886	This study
Soybean ( <i>Glycine max</i> )	<i>GmFDa1</i>	Glyma01g36810*	Chr1: 49235180-49236798	Jung et al. (2012)
	<i>GmFDa2</i>	Not annotated Proposed Glyma11g08490	Chr11: 5997867-5996321	This study
	<i>GmFDb1</i>	Glyma02g05100*	Chr2: 4139368-4140828	This study
	<i>GmFDb2</i>	Not annotated Proposed Glyma16g23200	Chr16: 26917990-26913770	This study
	<i>GmFDc1</i> (prev. <i>GmFD1</i> )	Glyma04g02420	Chr4: 1680703-1683111	Tsuji et al. (2013b)
	<i>GmFDc2</i> (prev. <i>GmFD2</i> )	Glyma06g02470	Chr6: 1639376-1641643	Tsuji et al. (2013b)
Common bean ( <i>Phaseolus vulgaris</i> )	<i>PvFDa</i>	Phvul.002G105700*	Chr2: 21293640-21294548	This study
	<i>PvFDb</i>	Phvul.003G266100	Chr3: 49289696-49290831	This study
	<i>PvFDc</i>	Phvul009G018700	Chr9: 3423224-3427367	This study

		*	20	*	40	*	60					
AtFD	:	-----	MLSSAKHQ	RNRHLS	SATNKNQ	TLTKV	SSISSSSP	SSSSS	: 38			
AtFDP	:	-----	MLSSAKHN	-----	-----	KINNHS	AFSIS	SSSSS	: 23			
PsFDa	:	-----	-----	-----	-----	-----	-----	-----	: -			
MtFDa	:	-----	-----	-----	-----	-----	-----	-----	: -			
LjFDa	:	-----	-----	-----	-----	-----	-----	-----	: -			
GmFDa1	:	-----	-----	-----	-----	-----	-----	-----	: -			
GmFDa2	:	-----	-----	-----	-----	-----	-----	-----	: -			
PvFDa	:	-----	-----	-----	-----	-----	-----	-----	: -			
MtFDb	:	-----	-----	-----	-----	-----	-----	MSH	: 3			
GmFDb1	:	-----	-----	MLSS	SSTST	TTTTTTT	CHKRNN	LNHKALSP	: 29			
GmFDb2	:	-----	-----	MLSS	-----	TSTTT	SCHSRNN	LNHKALSP	: 24			
PvFDb	:	-----	-----	-----	-----	-----	-----	-----	: -			
GmFDc1	:	-----	-----	-----	-----	MASWP	-----	PKPTEIFC-VH	: 15			
GmFDc2	:	MGR	RYPGGGLIVWGHE	PGTSL	STLNVEI	PLSLAK	VEKKEEGEAWPRGHPNLQRYFVRLS		: 60			
PvFDc	:	-----	-----	-----	-----	MASSP	CDCWTHLS	QSLSSS	: 19			
		*	80	*	100	*	120					
AtFD	:	SSSTSSSS	PLPSQDSQAQ	KRSLVT	MEEVW	ND--	INLAS	IHHLNRHSPHPQH	NHEPRFRGQ	: 96		
AtFDP	:	SLSTSSS	-----	LGHNKSQ	VTMEEV	WKE--	INLGS	LHYHRQLN	-----	: 69		
PsFDa	:	-----	-----	MEELW	KD--	INMSS	LNEQN	TRR-----	PMIMST	: 26		
MtFDa	:	-----	-----	MEEVW	KD--	INLSS	LNDQN	TR-----	PMIMST	: 25		
LjFDa	:	-----	-----	MEEVW	KD--	INLAS	LNDHNT	-----	PS	: 21		
GmFDa1	:	-----	-----	MEEVW	KD--	INLAT	LNEQ	STI-----	-----	: 19		
GmFDa2	:	-----	-----	MEEVW	KD--	IN	-----	-----	-----	: 9		
PvFDa	:	-----	-----	MEEVW	KD--	INLAT	LNDQ	VSS-----	-----	: 19		
MtFDb	:	QPLQE	QTPQQQHHLRPNK	PKNTM	EDVW	KD--	INLPS	LTNHMSN	-----	: 46		
GmFDb1	:	TTTKP	SHF	SHQTP	ISISS	SSNSK	AMEDV	WEGIN	INLTS	LNDHNTNT	: 75	
GmFDb2	:	STTKP	SHF	SHLTP	RSSH	ISTNN	KDMED	VWEG	INLTS	LSDHNTNTN	: 69	
PvFDb	:	-----	-----	MEDVW	NG--	INSTA	LSEHN	TTH-----	-----	: 20		
GmFDc1	:	AREK	AMASSP	CDWPH	LSP	SS--	IEHV	WND--	IKLDS	LSNSPVDIDF	: 59	
GmFDc2	:	VREK	AMASSP	CDWPH	SSSSSS	SSSIE	HVW	ND--	IKLAS	LSNSPVDLDL	: 106	
PvFDc	:	SSSP	SSSS	LQTL	PPPP	SSSS--	EHV	WND--	IKLPS	LSNSPVDFN	: 61	
										<---Motif A--->		
		*	140	*	160	*	180					
AtFD	:	NHHNQ	NPNSIFQ	DEIKG	SLNQEP	-----	APTSQ	TG	SAPNGD	STTVTVLY	-----	: 145
AtFDP	:	NPNN	-----	SIFQ	DEFLN	MPLNQ	PP-----	PPP	SSSTI	VTALY	G---	: 109
PsFDa	:	RDS	STFGG	VILQ	DEFLA	RPLN	INPPK	NIDH	HYSSN	NNSSSV	ASDQN--	: 83
MtFDa	:	RNST	FGG	VILQ	DEFLT	RPLT	LDPT	KSLD--	YSSN	NNSSSV	ASDQNNN	: 82
LjFDa	:	TTH	STFGG	AIFQ	DEFLG	GHF	PPN-----	TTV	SSSS	LSAS	PQS---	: 64
GmFDa1	:	STR	PNVEG	VMFQ	DEFLA	RPFT	TID-----	PPN	TLLS	SAS	SETAA	: 67
GmFDa2	:	-----	VFQD	-----	LTTID	-----	SPN	---	IILS	SAS	SETG	: 34
PvFDa	:	-TH	SNLGG	VILQ	DEFLA	RPFT	IDP-----	PNAT	LSS	QTT--	SSLYGP	: 62
MtFDb	:	-----	TVSSP	SLMT	PSSL	LHS-----	-----	-----	-----	-----	-----	: 61
GmFDb1	:	-----	SKGAK	FQDE	FLSR	PFTN	FS-----	-----	-----	-----	TIASA	: 100
GmFDb2	:	-----	TSKGA	NFQDE	FLSS	PFTN	FS-----	-----	-----	-----	TIAS	: 94
PvFDb	:	-----	ISKGA	KFQDE	FLAG	PFN-----	-----	-----	-----	-----	P	: 37
GmFDc1	:	--	NNN	---	HSVSD	SSFLN	-----	-----	-----	-----	-----	: 72
GmFDc2	:	--	NNNN	HSV	SVSS	FLNQ	PLSTFL	-----	-----	-----	-----	: 127
PvFDc	:	-----	PSSSH	SSL	LS	-----	-----	-----	-----	-----	-----	: 71

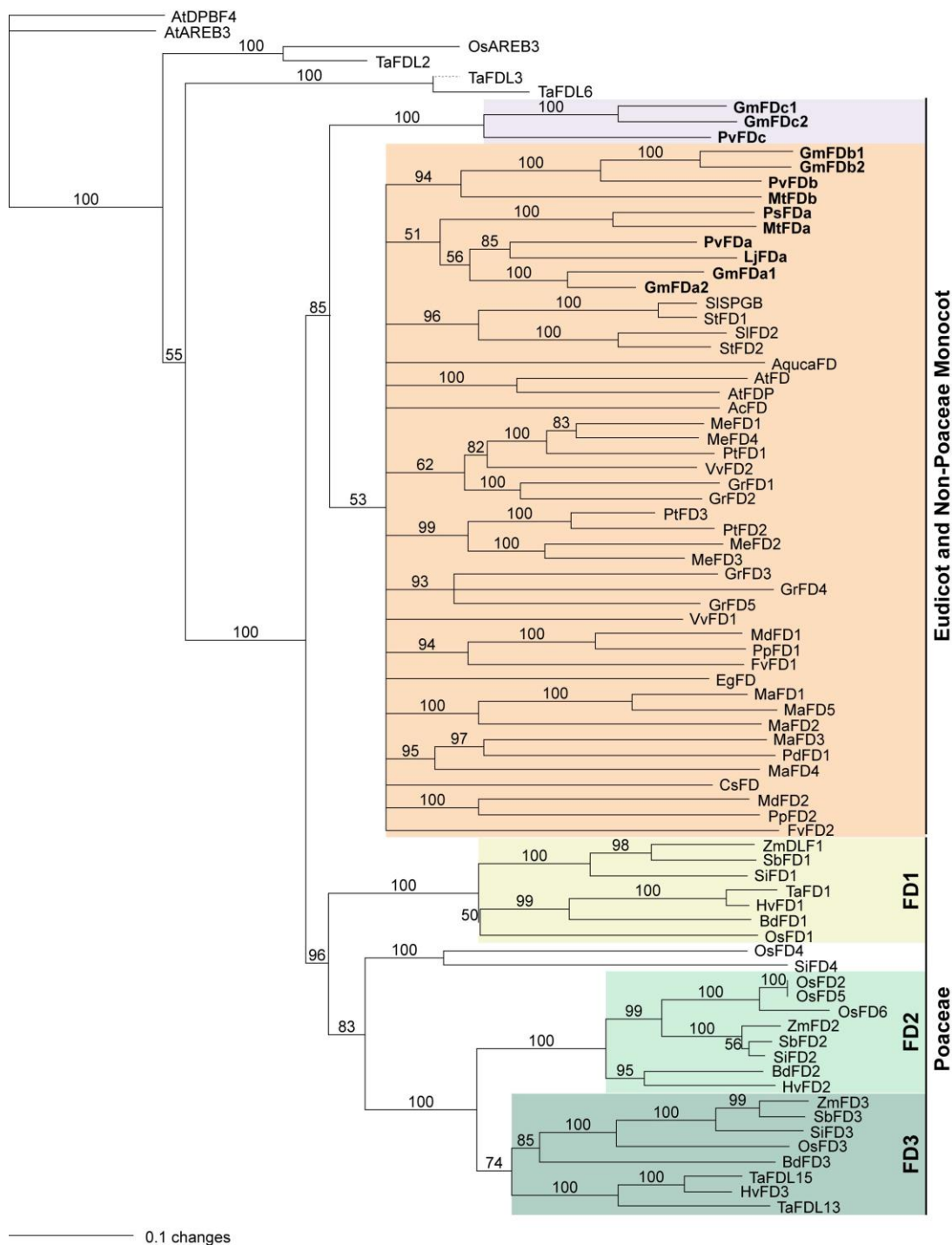


**Figure 4.3.** Conserved motifs in legume FD proteins.

Locations are indicated for Motif A and the LSL motif (Tsuji et al., 2013b), the bZIP domain with invariant N-x<sub>7</sub>-R/K motif (within the basic region) and leucine zipper (Jakoby et al., 2002), and the Ser-Ala-Pro (SAP) motif (Taoka et al., 2011). The alignment was created with full-length predicted protein sequences aligned with ClustalX (Thompson et al., 1997) and manually adjusted with GeneDoc (Nicholas and Nicholas, 1997). Shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Species abbreviations are as follows: *Arabidopsis thaliana* (At), common bean (Pv), *Lotus japonicus* (Lj), *Medicago truncatula* (Mt), pea (Ps), and soybean (Gm). Sequence details are given in Appendix 2.



Among the three legume *FD* subclades, *FDc* showed the most divergence from *Arabidopsis FD* and *FDP* (Figures 4.2 and 4.3). Indeed, BLASTp searches using predicted protein sequence for the legume *FDc* genes against *Arabidopsis* proteins, indicated equal similarity with *Arabidopsis FD/FDP* and a different group A bZIP transcription factor, *AREB3*. This raised the question of whether the legume *FDc* genes were true *FD* homologs. In order to understand the relationships between identified legume *FD* genes and those in other angiosperm species, phylogenetic analysis was conducted with angiosperm *FD* genes characterised previously, or identified in this study from sequence database searches. Figure 4.4 shows that the legume *FDa* and *FDb* subclades were included within a large clade of *FD* genes from non-Poaceae monocot species (banana and date palm) and diverse eudicot species. The legume *FDc* subclade fell outside this core eudicot and non-Poaceae monocot *FD* clade (Figure 4.4). Nonetheless, the *FDc* genes still showed higher similarity to *FD* genes than to the next most closely related bZIP genes, including *AtAREB3* and related genes (Figure 4.4). As previously reported (Tsuji et al., 2013b), *FD* genes from the grass family formed a separate clade with three subclades: Poaceae *FD1*, *FD2* and *FD3* (Figure 4.4). Curiously, three *FD*-like genes previously described in wheat (*TaFDL2*, *TaFDL3* and *TaFDL6*; Li and Dubcovsky, 2008), showed greater similarity to *AREB3* genes than to Poaceae *FD* genes (Figure 4.4). This was confirmed by BLAST searches against *Arabidopsis*, which identified *AtAREB3* as the *Arabidopsis* gene most similar to each of these genes.



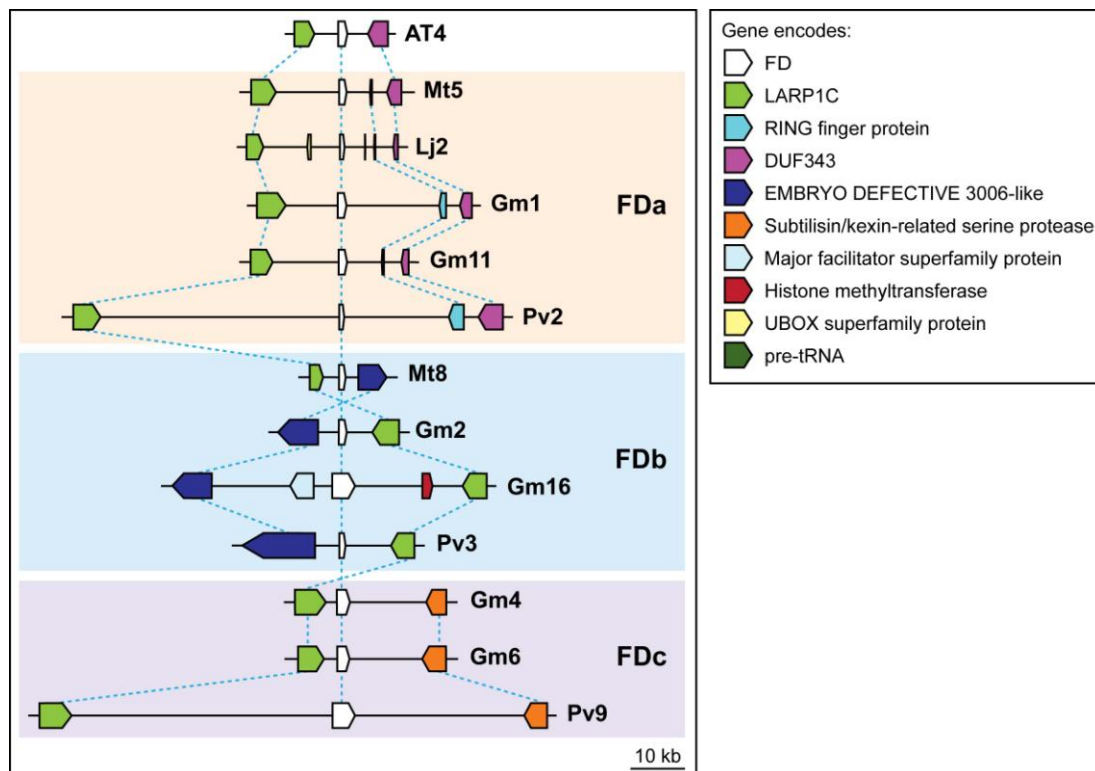
**Figure 4.4.** Phylogenetic neighbour-joining tree of FD transcription factors from diverse monocot and eudicot species.

The phylogram was constructed from full-length predicted protein sequence for *FD* genes identified previously (Pnueli et al., 2001; Abe et al., 2005; Wigge et al., 2005; Muszynski et al., 2006; Sussmilch, 2008; Taoka et al., 2011; Tsuji et al., 2013b; Varkonyi-Gasic et al., 2013), or during this study. Species are as follows: apple (*Md*), *Aquilegia coerulea* (*Aquca*), *Arabidopsis thaliana* (*At*), banana (*Ma*), barley (*Hv*), *Brachypodium distachyon* (*Bd*), cassava (*Me*), common bean (*Pv*), cotton (*Gr*), cucumber (*Cs*), date palm (*Pd*), *Eucalyptus grandis* (*Eg*), foxtail millet (*Si*), grape (*Vv*), kiwifruit (*Ac*), *Lotus japonicus* (*Lj*), *Medicago* (*Mt*), maize (*Zm*), pea (*Ps*), peach (*Pp*), poplar (*Pt*) potato (*St*), rice (*Os*), *Sorghum bicolor* (*Sb*), soybean (*Gm*), strawberry (*Fv*), tomato (*Sl*), wheat (*Ta*). Bootstrap values obtained from 10,000 trees are indicated as a percentage above each branch. Legume *FD* transcription factors are shown in bold. Sequence and alignment details are given in Appendix 2.

Online sequence and transcriptome resources were also examined for data on expression of legume *FD* genes. Expression data was present for *MtFDa*, *MtFDb*, *GmFDa1*, *GmFDb1*, *GmFDc1*, *GmFDc2* and *LjFDa* in gene expression atlases for *Medicago*, soybean and *Lotus*, respectively, but there was no clear pattern of expression for *FD* subclades between species (Appendix 3). In addition, several genes were represented in online databases of expressed sequences, including *GmFDb1* (GenBank dbEST accession FK012008), *GmFDc1* (GenBank dbEST accession BM892830; DFCI TGI accession TC443698), *GmFDc2* (GenBank dbEST accessions FK025137, BU081844, BE020009 and HO028603; DFCI TGI accession TC435144). This indicates that members of each subclade of legume *FD* genes are expressed and not merely pseudogenes.

#### 4.3.2 Microsynteny

The genomic regions surrounding *AtFD* in *Arabidopsis* and *MtFDa* in *Medicago*, were previously found to exhibit microsynteny (Sussmilch, 2008). In this study, this analysis was extended to other sequenced legume genomes. The gene immediately upstream (5') of *FD* in *Arabidopsis*, *LARP1C*, was also present near all legume *FD* genes examined (Figure 4.5; Table 4.5), consistent with a probable common origin of *AtFD* and all three legume *FD* subclades. The gene present immediately downstream (3') of *AtFD*, *DUF343*, was conserved near legume *FDa* genes, but not *FDb* or *FDc* genes, and an additional gene, *RING finger*, which encodes a RING finger protein, was also present downstream (3') of all legume *FDa* genes investigated (Figure 4.5; Table 4.5). An *EMBRYO DEFECTIVE 3006*-like gene was present near every legume *FDb* gene, whereas a gene encoding a subtilisin/kexin-related serine protease was present downstream of every legume *FDc* gene (Figure 4.5; Table 4.5). In the Phaseoleae, there appears to have been a rearrangement altering the orientation of each *FDb* gene, relative to flanking genes, when compared with *MtFDb*, *FDa* and *FDc* genes (Figure 4.5). No microsynteny was apparent between the regions containing *AtFDP* and legume *FD* genes.



















































**Figure 4.5.** Microsynteny between genomic regions containing *FD* and flanking genes in *Arabidopsis* and legume species.

The genes flanking *FD* in *Arabidopsis*, and legume *FDa*, *FDb*, and *FDc* genes (white) in *Medicago* (Mt), *Lotus* (Lj) common bean (Pv) and soybean (Gm) are shown. Genes are represented as boxes with point showing putative direction of transcription. Gene details are given in Table 4.5. Scale is based on available genome builds for each species (see Table 4.5). Chromosome number is indicated for each species. Between species, corresponding genes are connected by dashed lines. The genes encoding a UBOX superfamily protein and pre-tRNA, which cannot easily be identified by colour coding due to small size, are present as the 5' and 3' flanking genes, respectively, for *LjFDa*.

**Table 4.5.** *FD* and flanking genes in *Arabidopsis* and legume species.

This table should be viewed in conjunction with Figure 4.5. Details in this table are based on available genome builds for *Arabidopsis* (TAIR10), common bean (v1.0), *Lotus* (build 2.5), *Medicago* (Mt3.5), and soybean (v1.1). Single asterisks indicate incorrect annotation in online resources, as determined by alignments with expressed sequences and between legume species, which was corrected for the purposes of this table and Figure 4.4 (see Appendix 2).

Species	Chromosome	Symbol	Gene encodes	Locus ID	Chromosomal location
<i>Arabidopsis thaliana</i>	At4		LARP1C	AT4G35890	16997139-17000833
			FD	AT4G35900	17004595-17006287
			DUF343	AT4G35905	17007028-17008154
<i>Medicago truncatula</i>	Mt5		LARP1C	Medtr5g022790*	8775734-8771155
			FDa	Medtr5g022780*	8758327-8759707
			RING finger protein	Medtr5g022770*	8754049-8754848
			DUF343	Medtr5g022760	8749173-8751848
	Mt8		LARP1C	Medtr8g075120	20220967-20223420
			FDb	Medtr8g075130*	20225953-20227169
			EMBRYO DEFECTIVE 3006-like	Medtr8g075140	20228872-20234066
<i>Lotus japonicus</i>	Lj2		LARP1C	Chr2.Cm0272.760.r2.m	26514037-26517142
			UBOX superfamily protein	Chr2.Cm0272.770.r2.m	26525206-26524565
			FDa	Chr2.Cm0272.780.r2.m	26530026-26530886
			pre-tRNA	Chr2.Cm0272.790.r2.m	26534410-26534481
			RING finger protein	Chr2.Cm0272.800.r2.m	26535356-26535105
			DUF343	Chr2.Cm0272.810.r2.m	26538534-26537696
Common bean ( <i>Phaseolus vulgaris</i> )	Pv2		LARP1C	Phvul.002G105500	21244748-21249771
			FDa	Phvul.002G105700*	21293640-21294548
			RING finger protein	Phvul.002G105800	21312952-21315712
			DUF343	Phvul.002G105900	21317735-21322244
	Pv3		LARP1C	Phvul.003G266200	49298693-49303027
			FDb	Phvul.003G266100	49289696-49290831
			EMBRYO DEFECTIVE 3006-like	Phvul.003G266000	49272428-49285826
	Pv9		LARP1C	Phvul.009G018800	3474640-3480559
			FDc	Phvul.009G018700	3423224-3427367
			Subtilisin/kexin-related serine protease	Phvul.009G018600	3474640-3480559

Soybean ( <i>Glycine max</i> )	Gm1		LARP1C	Glyma01g36801	49220680-49225998
			FDa1	Glyma01g36810*	49235180-49236798
			RING finger protein	Glyma01g36820	49253298-49254542
			DUF343	Glyma01g36830	49256522-49258723
	Gm2		LARP1C	Glyma02g05090	4130098-4135052
			FDb1	Glyma02g05100*	4139368-4140828
			EMBRYO DEFECTIVE 3006-like	Glyma02g05110	4144145-4151509
	Gm4		LARP1C	Glyma04g02405	1673134-1678845
			FDc1	Glyma04g02420	1680703-1683111
			Subtilisin/kexin-related serine protease	Glyma04g02431	1696836-1699749
	Gm6		LARP1C	Glyma06g02460	1632699-1637471
			FDc2	Glyma06g02470	1639376-1641643
			Subtilisin/kexin-related serine protease	Glyma06g02481	1654709-1659109
	Gm11		LARP1C	Glyma11g08500*	6008627-6013845
			FDa2	Not annotated Proposed Glyma11g08490	5997867-5996321
			RING finger protein	Glyma11g08480*	5990036-5991373
			DUF343	Glyma11g08470	5986226-5987561
	Gm16		LARP1C	Glyma16g23170	26890147-26894598
			Histone methyltransferase	Glyma16g23191	26899881-26901749
			FDb2	Not annotated Proposed Glyma16g23200	26917990-26913770
			Major facilitator superfamily protein	Glyma16g23210	26920731-26925063
			EMBRYO DEFECTIVE 3006-like	Glyma16g23220	26939014-26946253

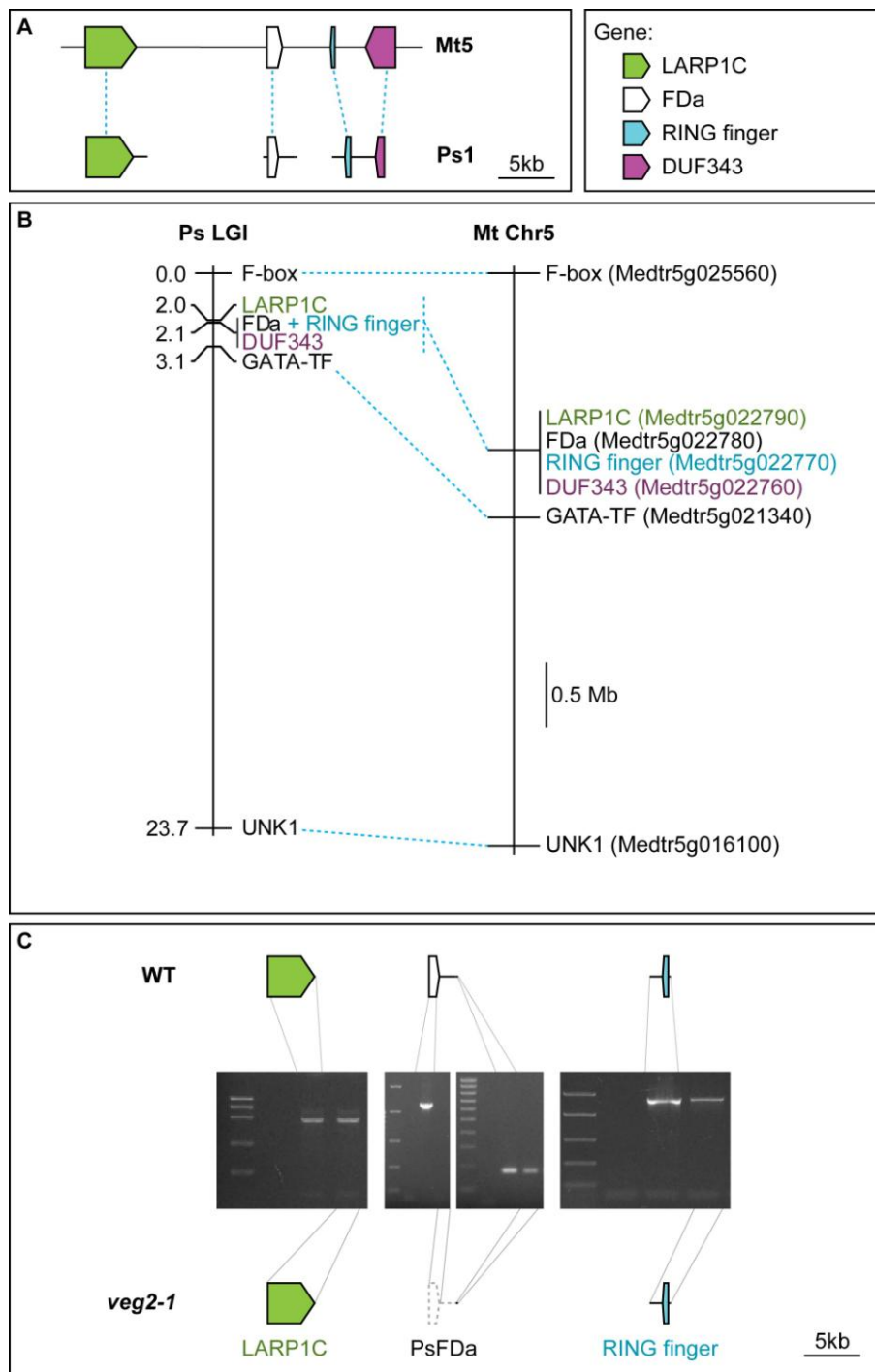
### 4.3.3 The *veg2-1* mutation

Previous linkage analyses indicated that *FDa* is located on the lower half of pea linkage group I, in a similar region to the *VEG2* locus and preliminary investigation of *FDa* as a candidate for the *VEG2* locus, suggested that at least part of the *FDa* coding sequence was deleted in the non-flowering *veg2-1* mutant (Sussmilch, 2008). This deletion was further investigated in this study, with the aim of characterising the boundaries of the deletion.

Primers within or spanning the *FDa* coding sequence yielded no PCR product in the *veg2-1* mutant despite successful amplification from wild-type template (Figure 4.6C; see also Section 4.2.3.1). This indicates that the entire coding sequence of *FDa* is likely to be absent in the *veg2-1* mutant. Consistent with this conclusion, primers designed to isolate full coding sequence for *FDa* were used effectively as a PCR-based marker to identify 37 *veg2-1* mutants from a segregating population comprising 210 plants (see Chapter 3).

This gave rise to the question of whether the deletion was limited to *FDa*, or whether neighbouring genes could also be affected, and contribute to mutant phenotype. The flanking genes for *FDa* were predicted to be conserved between *Medicago* and pea, as the same flanking genes were conserved in soybean and common bean (Figure 4.5; Table 4.5), which represent a tribe of papilionoid legumes (Phaseoleae) that is evolutionarily distant from that of *Medicago* (Trifolieae; Choi et al., 2004). Full-length coding sequences for these genes were isolated from a wild-type pea line (cultivar Kaliski) by standard PCR with primers designed from either pea TSA sequences or the corresponding *Medicago* genes, followed by genome walking (Figure 4.6A). These genes were mapped in an F<sub>2</sub> population of a wide cross between lines NGB5839 and JI1794, and found to be closely linked to *FDa* (Figure 4.6B), confirming that the relative location of these genes is also conserved in pea. For both putative flanking genes, full coding sequence was isolated from the *veg2-1* mutant, which suggests that the deletion in the *veg2-1* mutant does not affect the genes adjacent to *FDa* (Figure 4.6C). Primers located downstream of *FDa* yielded product in the *veg2-1* mutant, revealing that the 3' deletion boundary is less than 1.4kb downstream of the *FDa* stop codon, and more than 10kb after the stop codon of the putative 3' flanking gene (*RING finger*), which is coded by the reverse strand (Figure 4.6C). To address the possibility that additional genes could be present

between *FDa* and the putative 5' flanking gene in pea (*LARP1C*), attempts were made to isolate this region in wild-type and in the *veg2-1* mutant, but these attempts were unsuccessful. While it is not possible to be certain of the gene arrangement in pea without isolation of the pea contig containing *FDa* and flanking genes, the argument from synteny is strong, and suggests that the deletion in the *veg2-1* mutant is limited to *FDa*.



**Figure 4.6.** Characterisation of the *veg2-1* mutation. (continued next page)



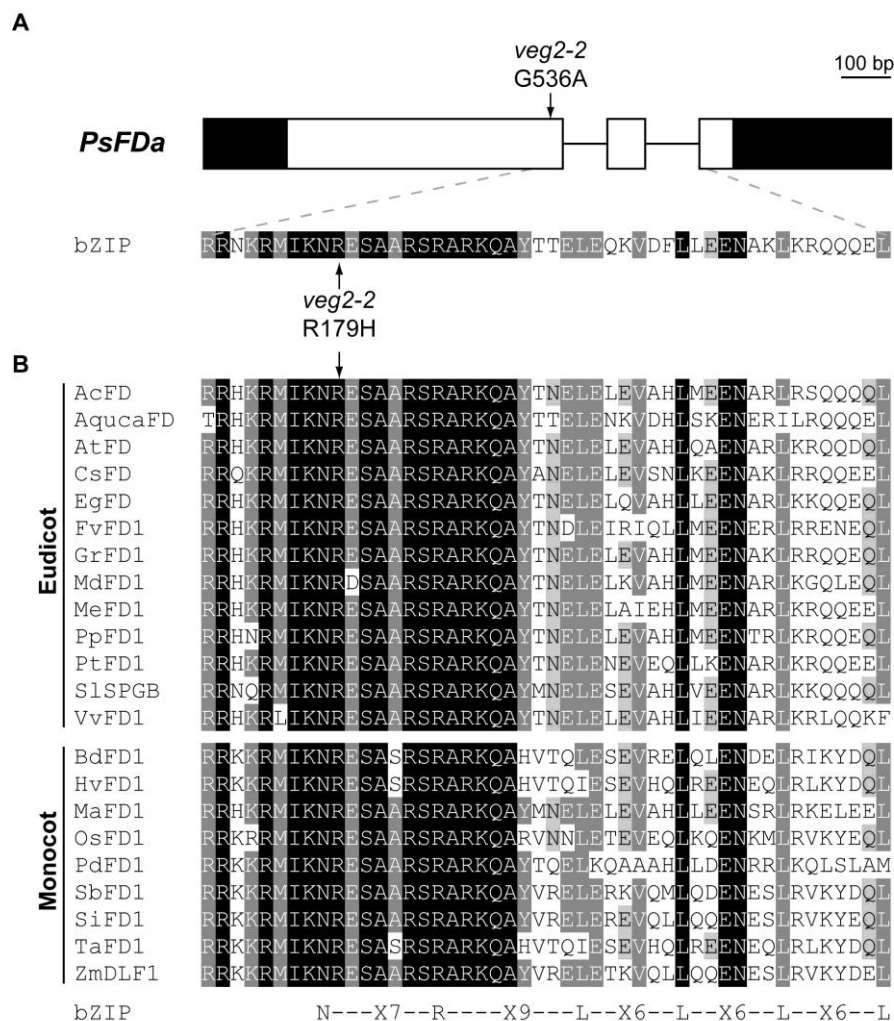
**Figure 4.6. (continued)** (A) Diagram illustrating the genes flanking *MtFDa* and corresponding genes (connected by dashed lines) isolated from pea during this study. Chromosome number is indicated for each species. Genes are represented as boxes with point showing putative direction of transcription. Intergenic sequence is shown as a black line connecting genes. As not all intergenic regions were sequenced from pea, pea genes are placed according to *Medicago* genomic sequence.

(B) Comparative map between pea and *Medicago* showing *FDa* and surrounding genes. Linkage map for pea (left) shows the relative map positions of *FDa* and putative flanking genes: *LARPIC* (putative 5' flanking gene), *RING finger* (putative 3' flanking gene) and *DUF343*. Three nearby marker loci are also included (see Appendix 1, Table A1.4 for details). Molecular markers were scored in the F<sub>2</sub> population of a cross between lines NGB5839 and JI1794, containing 92 individuals. Numbers represent distances (cM) estimated from segregation data using JoinMap software (v4; Kyazma B.V., Wageningen, Netherlands). Relative locations of corresponding genes (linked by dashed lines) on the physical map of *Medicago* chromosome 5 (right; based on Mt3.5) are shown.

(C) PCR using primers to isolate full-length coding sequence for *FDa*, the putative 5' flanking gene *LARPIC*, and the putative 3' flanking gene *RING finger* was performed on wild-type (Kaliski) and *veg2-1* mutant template. Presence and absence of PCR bands is represented diagrammatically as presence or absence of genes in wild-type and the *veg2-1* mutant. For each gel, lanes containing a DNA ladder and PCR product for a no template negative control, wild-type (Kaliski) positive control and *veg2-1* mutant templates are shown (from left to right). Primer pairs are as follows (gels from left to right: PsWiHe-1F+PsHyp-1R (full-length coding sequence); PsFD-7F+PsFD-6R (full-length coding sequence); PsFD-9F+PsFD-12R (portion of 3' UTR); PsZnFin-5F+PsZnFin-3R (full-length coding sequence; see Appendix 1). Ladders are as follows (gels from left to right): Bioline EasyLadder II, Bioline EasyLadder I, Bioline HyperLadder IV, Bioline EasyLadder I.

#### 4.3.4 The *veg2-2* mutation

Prior sequencing of *FDa* in the *veg2-2* mutant revealed a SNP in *FDa* (Sussmilch, 2008), which was investigated further in this study. Homozygosity for the G536A SNP was found to co-segregate perfectly with the *veg2-2* phenotype in a segregating population of 114 F<sub>2</sub> progeny containing 34 *veg2-2* mutants. This SNP affects an arginine (R179H) within the DNA-binding, basic region of the bZIP domain (Figure 4.7A). An arginine is highly conserved at this position in FD proteins from diverse angiosperm species including both eudicot and monocot species (Figure 4.7B). Furthermore, this arginine was found to be conserved at this position in 95% of all *Arabidopsis* bZIP family proteins (Figure 4.7C), which comprise 13 divergent groups with very little sequence similarity outside of the bZIP domain (Correa et al., 2008).



**Figure 4.7.** Conserved nature of the amino acid affected by the *veg2-2* SNP.

(A) Diagram of the pea *FDa* gene showing the nature of the mutation in *veg2-2* and location within the bZIP domain region. Exons are shown as boxes, with the isolated untranslated regions shown in black. The predicted amino acid sequence for the bZIP domain region is from the alignment shown in (B) and is shaded accordingly (see below).

(B) Alignment of predicted protein sequence for the *FD* bZIP domain showing conservation of the arginine altered in the *veg2-2* mutant between *FD* homologs from diverse eudicot species (Ac, kiwifruit; Aquca, *Aquilegia caerulea*; At, *Arabidopsis*; Cs, cucumber; Eg, *Eucalyptus grandis*; Fv, strawberry; Gr, cotton; Md, apple; Me, cassava; Pp, peach; Pt, *Populus*; Sl, tomato; Vv, grape) and monocot species (Bd, *Brachypodium*; Hv, barley; Ma, banana; Os, rice; Pd, date palm; Sb, *Sorghum*; Si, foxtail millet; Ta, wheat; Zm, maize).

(C, on next page) Alignment of the basic region of all *Arabidopsis* bZIP family proteins, illustrating conservation of arginine altered in the pea *veg2-2* mutant (arrow). Letters in parenthesis indicate group of bZIP protein as defined by Correa (2008).

The consensus bZIP motif given by Jakoby et al. (2002) is indicated in (B) and (C).

Shading in alignments indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Sequence details are given in Appendix 2.

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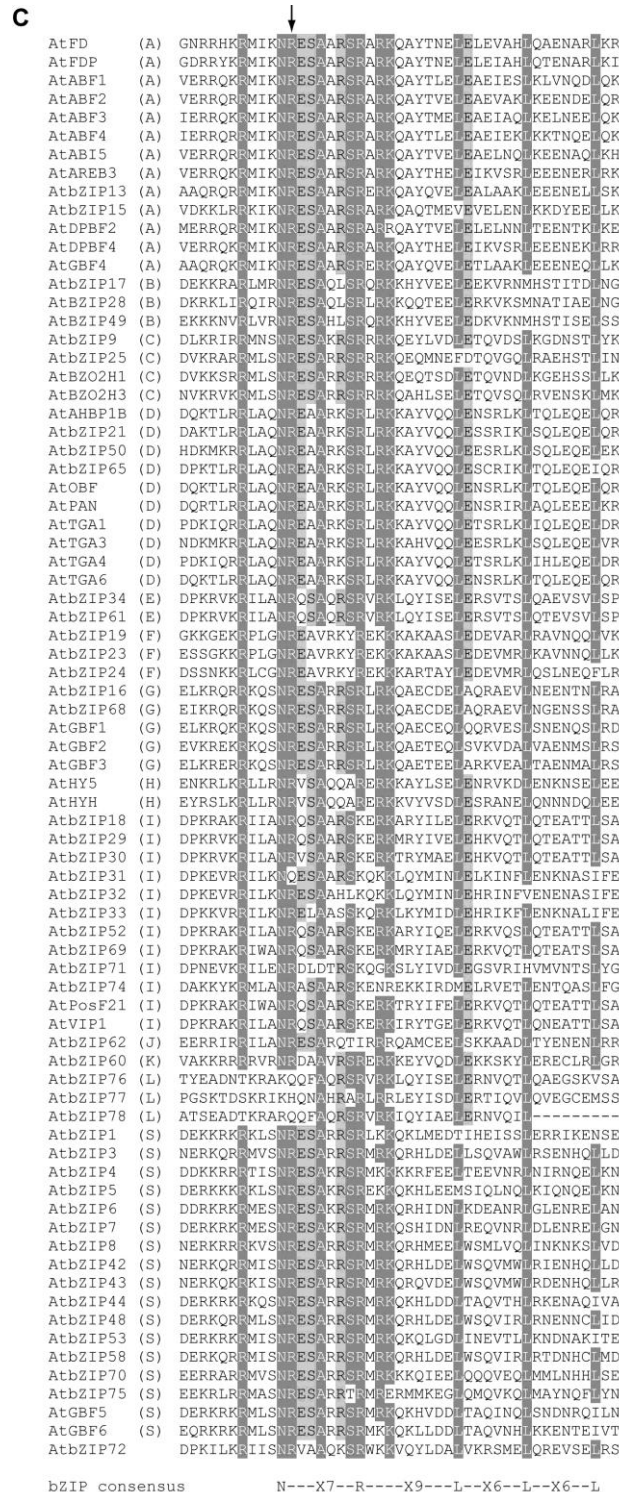


Figure 4.7. (continued)

## 4.4 Discussion

### 4.4.1 The legume *FD* gene family

Prior to this study, relatively little was known about the legume *FD* gene family. One *FD* gene had been isolated in pea, one identified in *Medicago*, and three identified in soybean (Sussmilch, 2008; Jung et al., 2012; Tsuji et al., 2013b). In this study, additional *FD* genes were identified in *Medicago*, *Lotus*, common bean and soybean (Table 4.4). Three distinct legume subclades, *FDa*, *FDb* and *FDC*, were identified by phylogenetic analysis (Figure 4.2). When compared with *FD* genes from diverse angiosperm species, *FDa* and *FDb* subclades fell within a large clade of *FD* genes from other eudicot species and from non-Poaceae monocot species (Figure 4.4). Resolution between groups of *FD* genes within this clade was relatively poor, which makes it difficult to discern the relationships between legume *FDa* and *FDb* genes with other subclades of eudicot *FD* genes (Figure 4.4). *FDC* genes formed a separate sister group, but showed higher similarity to *FD* genes than to *AREB3* homologs based on protein sequence (Figure 4.4).

Expression data was found in online resources for *MtFDa*, *MtFDb*, *GmFDa1*, *GmFDb1*, *GmFDC1*, *GmFDC2* and *LjFDa* (see Appendix 3), which suggests that members of all three subclades of legume *FD* genes can be expressed and may be functional. This expression data should be treated with some caution and confirmed by targeted analysis, as probe sets in both *Medicago* and soybean gene expression atlases appear to be based on incorrectly annotated coding sequences for these genes which includes intron sequence within the highly conserved bZIP domain. However, the representation of *GmFDb1*, *GmFDC1* and *GmFDC2* in online resources of expressed sequences (GenBank dbEST and/or DFCI TGI) provides supporting evidence that at least one *FDb* gene and both *FDC* genes are expressed in soybean.

No *FDb* genes could be identified in pea or *Lotus* using resources available at the time of the study. Given the presence of *FDb* genes in *Medicago*, soybean and common bean (Figure 4.2; Table 4.4), and taxonomic positions of *Lotus* and *Pisum* (Choi et al., 2004), it seems likely that *FDb* genes should also be present in pea and *Lotus*, although the possibility of gene loss in one or both species cannot be excluded. Current efforts to sequence the pea genome by an international consortium led by INRA-Dijon (France) and the University of Saskatchewan (Saskatoon,

Canada) may facilitate identification of an *FDb* gene in pea in the future. It is difficult to speculate on what role an *FDb* gene may fulfil, if this gene is indeed present in pea and expressed. Functional redundancy with *FDa* seems unlikely based on the severity of the *veg2-1* null mutant phenotype (see Chapter 3). This remains an area for future investigation. As *FDc* genes were only identified in soybean and common bean (Figure 4.2; Table 4.4), it seems likely that *FDc* genes may be specific to the Phaseoleae tribe of papilionoid legumes.

The legume FD family exhibits considerable sequence divergence, and regions of similarity are largely limited to the previously defined conserved motifs: Motif A, the LSL motif, the bZIP domain and the SAP motif (Figure 4.3). Tsuji et al. (2013b) recently noted that the LSL motif [T(A/V)LSLNS] is well conserved between monocot and eudicot *FD* groups containing members that activate the *API/FUL* clade of MADS-box genes for the promotion of flowering, and suggested that the presence of the LSL motif may serve as an indicator for this capability. If true, this would suggest that legume *FDb* and *FDc* genes may have divergent functions in plant development, compared to *FDa* genes, as the LSL motif is well conserved in legume *FDa* genes, but not in legume *FDb* and *FDc* genes (Figure 4.3). However, this somewhat speculative theory would clearly need to be tested directly, especially as the functional significance of the LSL motif has not yet been examined. *FDa* and *FDb* subclades could be investigated in *Medicago*, as mutant collections for reverse genetics and techniques for transgenic manipulation are relatively advanced in this species (e.g. Trieu et al., 2000; Tadege et al., 2008). Alternatively, all three subclades are represented by a single gene in common bean (Figure 4.2), but resources are still in the early stages of development for this system, relative to *Medicago*.

The SAP motif [R-X-X-(pS/pT)-A-P-F], as defined by Taoka et al. (2011), is well conserved among legume FD proteins with the exception of MtFDa (Figure 4.3). This motif is required for binding with 14-3-3 proteins, which facilitate interaction between FD and FT homologs (Taoka et al., 2011; Tsuji et al., 2013b). It would be interesting to determine empirically if the variation in SAP motif in MtFDa is real, and if so, whether it affects the ability of MtFDa to interact with 14-3-3 proteins and FT homologs.

There are also differences among legume FD proteins in the basic region of the bZIP domain, which binds to target promoter sequences (Agre et al., 1989; Hurst, 1995). In particular, at position 289 in the alignment shown in Figure 4.3, a valine is present in the FDc proteins in place of an arginine that is conserved between *Arabidopsis* and other legume FD proteins at this position. It is possible that differences in this region of the bZIP domain could alter DNA-binding properties and thus the target genes regulated by these transcription factors. There is some precedent for this idea in rice, where OsFD1 and OsFD2 contain several amino acid differences in the basic region of the bZIP domain, and OsFD1 is able to bind to *API/FUL* homolog *OsMADS15*, but OsFD2 lacks this capability (Tsuji et al., 2013b; see sequence alignment for Figure 4.4 in Appendix 2). An area of future interest will be to determine the direct targets of legume FD proteins and whether these differ between legume FD subclades.

Phylogenetic analysis showed that Medtr7g088090, previously referred to as *MtFD* (Pierre et al., 2008; Stanton-Geddes et al., 2013), is more closely related to *Arabidopsis* genes *AREB3* (*DPBF3/AtbZIP66*) and *DPBF4* (*AtbZIP12/EEL*) than to *FD* or *FDP* (Figure 4.1). In *Arabidopsis*, *AREB3* acts in the ABA signalling network (Kline et al., 2010), while *DPBF4* is involved in fine-tuning gene expression during seed maturation and also acts to inhibit aspartate kinase enzymes in the aspartate-derived biosynthesis pathway (Bensmihen et al., 2002; Ufaz et al., 2011). The finding that Medtr7g088090 is instead an *AREB3/DPBF4* homolog is in agreement with a previous study that raised doubt about the homology between Medtr7g088090 and *FD*, based on BLASTp search results (Yeoh et al., 2013). This highlights the need for reciprocal blasting and appropriate phylogenetic analysis prior to naming of putative homologs to avoid perpetuating confusion through the literature (see Samach, 2013). Even though Medtr7g088090 is not a true *FD* homolog, it has been linked to flowering time variation in a genome-wide association study (Stanton-Geddes et al., 2013), suggesting a possible role related to flowering. However, a cluster of *FT* genes is also present in this region on *Medicago* chromosome 7 (Laurie et al., 2011), which seem more likely as candidates for variation in flowering time. Medtr7g088090 is not the only *AREB3*-like gene to be linked to the flowering pathway, as wheat genes *TaFDL2*, *TaFDL3* and *TaFDL6* show higher similarity with *AREB3/DPBF4* genes than with Poaceae *FD* genes (Figure 4.4; Tsuji et al., 2013b).

TaFDL2 and TaFDL6 proteins are capable of interacting with a wheat FT homolog and TaFDL2 can bind to the promoter of the wheat *API/FUL* homolog *VERNALIZATION1* (*VRN1*), leading to the proposal that it may be the functional homolog of *FD* in wheat (Li and Dubcovsky, 2008). However, direct analysis of *TaFDL2* or *TaFDL6* function has not yet been reported, and will clearly be needed to test this proposition. These results from wheat are perhaps not surprising in view of protein sequence comparisons, which show that the DNA-binding basic region of the bZIP domain and 14-3-3 binding SAP motif are well conserved between AREB3 and FD homologs (see sequence alignment for Figure 4.4 in Appendix 2). This suggests that genes in both groups could potentially act in similar complexes to upregulate target genes. In gymnosperms, there do not appear to be any *FD* homologs present (Tsuji et al., 2013b), but there are gymnosperm *FT/TFL1*-like genes that function as repressors similar to *TFL1*, and control annual growth cycles (Karlgrén et al., 2011; Karlgrén et al., 2013). The mechanism of action for *FT/TFL1*-like genes in gymnosperms has not yet been determined, but in the absence of *FD* genes, other related bZIP transcription factors are clear candidates for acting in complexes with gymnosperm *FT/TFL1*-like proteins. Although these observations do not provide direct evidence that other group A bZIP transcription factors can have *FD*-like function, they do provide some incentive for further investigation of this possibility.

#### 4.4.2 Microsynteny

Previous knowledge of microsynteny between the regions surrounding *AtFD* and *MtFDa* (Sussmilch, 2008), was expanded by investigation of the regions surrounding all legume *FD* genes identified in this study. The gene present immediately upstream (5') of *FD* in *Arabidopsis* (*LARP1C*; AT4G35890), was found to be conserved close to all legume *FD* genes investigated (Figure 4.5; Table 4.5). In conjunction with the results of phylogenetic analyses, this microsynteny provides evidence that these genes share a common evolutionary origin (Figures 4.2, 4.4 and 4.5; Table 4.5). No microsynteny was found between the genomic regions containing *AtFDP* and legume *FD* genes. Combined with the results of phylogenetic analyses, this suggests that the duplication that gave rise to *FD* and *FDP* in *Arabidopsis* occurred separately to the duplications that gave rise to the three legume *FD* subclades (Figures 4.2 and 4.4). *DUF343*, which was present immediately

downstream (3') of *AtFD*, was conserved near *FDa* genes, but not *FDb* or *FDc* genes in the legume species investigated (Figure 4.5; Table 4.5). Instead an *EMBRYO DEFECTIVE 3006*-like gene was present close to every legume *FDb* gene investigated, whereas a gene encoding a subtilisin/kexin-related serine protease was conserved close to every legume *FDc* gene (Figure 4.5; Table 4.5). Combined with the results of phylogenetic analysis, this could indicate that legume *FDa* genes and *AtFD* are the closest representatives of the ancestral *FD* gene in legumes and *Arabidopsis*, respectively (Figures 4.1, 4.2 and 4.5).

#### 4.4.3 The *veg2-1* mutation

Previous findings of linkage analyses indicated that *FDa* is located in a similar region to the *VEG2* locus, towards the base of pea linkage group I (Sussmilch, 2008). Initial investigation indicated that at least part of the *FDa* coding sequence was deleted in the non-flowering *veg2-1* mutant (Sussmilch, 2008). In this study, the entire coding sequence of *FDa* was found to be absent in the *veg2-1* mutant, which indicates that a large deletion encompassing *FDa* has occurred (Figure 4.6C). The *veg2-1* mutant was generated by fast neutron mutagenesis (I. Murfet, personal communication), which is renowned for causing large deletions (e.g. Sun et al., 1992; Bruggemann et al., 1996). To determine if the deletion in the *veg2-1* mutant is limited to *FDa* or if other flowering genes may be affected and responsible for the severity of mutant phenotype, the boundaries of the deletion in the *veg2-1* mutant were investigated further.

Two genes, *LARPIC* and *RING finger*, have conserved positions flanking *FDa* genes in *Medicago*, soybean and common bean, and these genes were also predicted to flank *PsFDa*, accordingly (Figure 4.5). In support of this, the pea homologs of these genes were found to be closely linked (Figure 4.6B). The 3' boundary for the *veg2-1* deletion was found to be less than 1.4kb after the *FDa* stop codon, and more than 10kb after the stop codon of the putative 3' flanking gene (*RING finger*; Figure 4.6C). The precise 5' boundary of the deletion could not be determined within the time frame of this study, but it does not affect the *LARPIC* coding sequence (Figure 4.6C). In fact, it is possible that the size of the intergenic region between *FDa* and *LARPIC* was too large for the PCR-based techniques employed during this study, as the corresponding region is more than 10kb in *Medicago* and genome size suggests



intergenic regions in pea could be larger (e.g. Young et al., 2003; Macas et al., 2007). It is of course theoretically possible that a downstream recognition element associated with either *LARPIC* or *RING finger* could be affected by the deletion, and expression of each gene was not examined within the time frame of this study in order to rule out this possibility. However, neither of the predicted flanking genes appear to have roles in flowering. In *Arabidopsis*, *LARPIC* has a role in leaf senescence with overexpression resulting in premature leaf senescence and the *larplc-1* null mutant exhibiting impaired ABA, salicylic acid, and methyl jasmonate induced leaf senescence (Zhang et al., 2012). Although the exact function of *RING finger* is unknown, proteins from this family are generally known for their role in ubiquitination (e.g. Lorick et al., 1999; Jackson et al., 2000). Thus it was not considered likely that any misregulation of either flanking gene would contribute to the specific flowering phenotype seen in the *veg2-1* mutant (Chapter 3).

It also remains a possibility that an additional gene might be present in pea between *LARPIC* and *FDa* that could be affected by the *veg2-1* deletion. However this also seems unlikely, given that no such gene is present in the corresponding, closely syntenic regions of the *Medicago*, soybean or common bean genomes (Figure 4.5; Choi et al., 2004). The additional possibility that such a gene, if it existed, might contribute to an *FD*-like function seems highly implausible. The availability of pea genomic sequence resources in the future would provide a basis for primer design closer to *FDa* in order to narrow down deletion boundaries and confirm that the *veg2-1* deletion is limited to *FDa*.

The phenotype of the *veg2-1* mutant is similar to that of the *veg1* mutant, which results from deletion of the pea ortholog of *AGL79*, a MADS-box gene of the same lineage as *FD* target *FUL* (Teper-Bamnolker and Samach, 2005; Berbel et al., 2012). Under LD conditions, the *gigas-2* mutant has a similar phenotype, resulting from the deletion of *FTa1*, a pea homolog of the *FD* interactor *FT* (Abe et al., 2005; Wigge et al., 2005; Hecht et al., 2011). Previously, a genetic model was proposed where *VEG2* and *FTa1/GIGAS* both activate *VEG1* expression for specification of I<sub>2</sub> meristems (Berbel et al., 2012; see Chapter 3). Identification of *FDa* as *VEG2* is consistent with this model, as *FD* proteins interact with *FT* proteins to upregulate MADS-box genes in other diverse systems (see Chapter 1).

In contrast to the non-flowering *veg2-1* mutant, null alleles of homologous genes characterised in *Arabidopsis* and maize (*fd* and *dlf1*) are merely late-flowering (Koornneef et al., 1991; Abe et al., 2005; Wigge et al., 2005; Muszynski et al., 2006). To obtain a non-flowering phenotype in *Arabidopsis*, comparable to that of pea *veg2-1*, functional *LFY* must be absent in addition to either *FD* or *FT*, as *LFY* acts redundantly with *FD* and *FT* to upregulate *API* for specification of floral meristems (Ruiz-Garcia et al., 1997; Abe et al., 2005; Wigge et al., 2005). As suggested in Chapter 3 (Section 3.4.1), it seems that an important additional control point within the pea flowering pathway is introduced by the novel role of *VEG1* in the specification of  $I_2$  meristems (Berbel et al., 2012). It is possible that the role of *FDa* in pea is enhanced relative to *FD* genes in other species due to the novel role of *VEG1*, coupled with the inability of *UNI* (the pea ortholog of *LFY*) to bypass *FDa* function for *VEG1* activation and subsequent reproductive development (see Chapter 5).

#### 4.4.4 The *veg2-2* mutation

As further support that the *VEG2* locus corresponds to *FDa*, a SNP (G536A) identified in the *veg2-2* mutant was found to co-segregate with the *veg2-2* mutant phenotype. The *veg2-2* mutant was created by fast neutron mutagenesis and although this method is renowned for resulting in deletions, it has recently been found to result more frequently in G/C to A/T nucleotide substitutions (Belfield et al., 2012), as is seen in the *veg2-2* mutant (Figure 4.7A). The amino acid affected by this missense mutation (R179H) is within the DNA-binding, basic region of the bZIP domain (Figure 4.7). At this position, arginine was found to be highly conserved in FD proteins between diverse angiosperm species (Figure 4.7B), and between the divergent *Arabidopsis* bZIP family proteins (Figure 4.7C). This suggests that this amino acid could be important for basic function of bZIP proteins.

By an unusual coincidence, exactly the same amino acid substitution that is seen in the *veg2-2* mutant has previously been described for the *dlf1-N2461A* mutant of the maize *FD* homolog *DLF1* (Muszynski et al., 2006). Using 3D modelling of the maize DLF1 protein, Muszynski et al. (2006) found that the arginine at this position comes into direct contact with the phosphate groups on target DNA. Conversion of this arginine to a histidine causes distortion of the DNA backbone, reducing strength

of binding to target DNA, and accounting for reduced DLF1 function in the *dlf1-N2461A* mutant (Muszynski et al., 2006). The R179H amino acid substitution observed in *FDa* in the *veg2-2* mutant is likely to cause reduced *FDa* function in a similar manner. The maize *dlf1-N2461A* mutant is late-flowering with inflorescence defects including abnormal tassel morphology (Muszynski et al., 2006). This phenotype is comparable to that of the *veg2-2* mutant which is late-flowering with inflorescence defects (see Chapter 3).

#### 4.4.5 Chapter conclusions

This chapter has investigated *FDa* as a candidate for the *VEG2* locus. Firstly, the legume *FD* gene family was characterised, using available online resources, and *FD* genes identified in *Medicago*, *Lotus*, soybean and common bean were found to fall into three subclades: *FDa*, *FDb* and *FDc* (Figure 4.2). The *FDa* and *FDb* subclades showed greatest similarity to other eudicot *FD* genes, based on predicted protein sequence (Figure 4.4). Microsynteny between the genomic regions containing legume *FD* genes and *AtFD* provided further evidence that these genes share a common evolutionary origin (Figure 4.4). The entire coding sequence for *FDa* was found to be deleted in the non-flowering *veg2-1* mutant, but the mutation appears to be limited to *FDa* (Figure 4.6). A SNP affecting a highly conserved amino acid within the DNA-binding, basic region of the bZIP domain, thought to reduce strength of binding to target DNA, was found to co-segregate with the late-flowering *veg2-2* mutant phenotype (Figure 4.7). Thus an independent and functionally significant mutation was found to affect *FDa* in each of the *veg2* mutant alleles, and severity of mutation corresponds well with severity of mutant phenotype. The phenotype of the *veg2-2* mutant is comparable to a mutant in maize (*dlf1-N2461A*) which contains exactly the same mutation in the maize *FD* homolog *DLF1* (Figure 4.7; Chapter 3; Muszynski et al., 2006). Furthermore, *veg2-1* mutant phenotype is similar to that of *gigas/fta1* (Chapter 3), just as *fd* mutant phenotype bears similarity to that of *ft* in *Arabidopsis* (Koornneef et al., 1991). These findings provide strong evidence that the *VEG2* locus corresponds to *FDa*. Identifying *FDa* as the gene affected in the *veg2* mutants allows further investigation of the roles of this gene by dissection of the molecular mechanisms of *FDa* action.



## CHAPTER 5: Actions and interactions of *FDa*/*VEG2*

### 5.1 Introduction

The results presented in the previous chapter indicate that the *VEG2* locus corresponds to the *FDa* gene, which is a member of the legume subclade of *FD* genes that is most closely related to *Arabidopsis FD*. The phenotypes of the *veg2* mutants reveal that *FDa* is important for each stage of pea inflorescence development (Chapter 3). This chapter will investigate the molecular mechanisms by which *FDa*/*VEG2* fulfils these roles.

#### 5.1.1 Conserved mechanisms of *FD* action in other species

In *Arabidopsis*, *FD* and its paralog *FDP* are expressed in the apex, where *FD* and *FDP* proteins interact with *CETS* proteins, including the floral promoters *FT* and *TSF* and the floral repressor *TFL1* (Abe et al., 2005; Wigge et al., 2005; Jang et al., 2009; Hanano and Goto, 2011). Together, these proteins regulate expression of genes including the floral integrator *SOC1*, and floral meristem identity genes *API1*, *FUL*, and *SEP3*, either directly or indirectly (Abe et al., 2005; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005; Searle et al., 2006; Wang et al., 2009). Interactions between *FD* and *CETS* proteins have been found to be conserved across diverse angiosperm species. In tomato, *SPGB* is expressed in the leaves and shoot apex, and *SPGB* protein can interact with both the *TFL1* homolog *SP* and the *FT* homolog *SFT* (Pnueli et al., 2001; Lifschitz et al., 2006). In maize, *DLF1* is expressed in the shoot apex and acts upstream of numerous *MADS*-box genes (Muszynski et al., 2006; Danilevskaya et al., 2008b). *DLF1* protein interacts with the *FT* homolog *ZCN8*, and the *TFL1* homolog *ZCN2* (Danilevskaya et al., 2008a; Danilevskaya et al., 2010; Meng et al., 2011). Likewise, the kiwifruit *FD* protein can interact physically with kiwifruit *FT* and *TFL1*/*CEN* homologs (Varkonyi-Gasic et al., 2013).

Recent studies in rice have provided new insight into the nature of FD/FT interactions. Binding of OsFD1/OsFD2 and Hd3a was found to be facilitated by 14-3-3 proteins, which act as bridging molecules to create a ternary florigen activation complex (Taoka et al., 2011; Tsuji et al., 2013b). In rice, this complex activates downstream targets including *OsMADS15* (an *API/FUL* homolog) to trigger floral development (Taoka et al., 2011). The SAP motif at the C-terminal end of OsFD1 is required for interaction with 14-3-3 proteins (Taoka et al., 2011). This motif has been found to be well conserved between FD homologs from a wide range of angiosperm species (Tsuji et al., 2013b), suggesting that this mechanism of interaction may be conserved. In support of this, the SAP motif at the C-terminal end of AtFD was found to be critical for interaction with FT to occur in yeast two-hybrid analysis (Abe et al., 2005). In addition, tomato 14-3-3 proteins have been found capable of interacting with TFL1 homologs from tomato, *Antirrhinum* and *Arabidopsis* (Pnueli et al., 2001).

### 5.1.2 14-3-3 proteins

14-3-3 proteins were first isolated from mammalian brain extract, and subsequently named after the fraction number on DEAE-cellulose chromatography and migration pattern after starch–gel electrophoresis (Moore and Perez, 1968). These proteins have since been found to be ubiquitous in eukaryote species (e.g. Wang and Shakes, 1996). 14-3-3 proteins can facilitate interaction between target proteins as adaptor or scaffold molecules, as in the case of rice FD and FT proteins described above (Taoka et al., 2011). However, this is not the only mechanism of action for 14-3-3 proteins (see Roberts, 2003; Obsil and Obsilova, 2011). They can also alter target protein activity by activating, inhibiting, or altering speed of turnover. Alternatively, they can act as chaperone molecules to shuttle proteins into organelles or between the nucleus and cytoplasm. 14-3-3 proteins regulate target proteins through binding to phosphorylated serine or threonine residues (e.g. Muslin et al., 1996; Yaffe et al., 1997; Olsson et al., 1998). They can bind to target proteins as homo-dimers, hetero-dimers, and possibly even as monomers (Jones et al., 1995; Sluchanko and Gusev, 2012). With diverse modes of action and a large range of target proteins, it is not surprising to find that 14-3-3 proteins are involved in many

different cellular and developmental processes (e.g. Finnie et al., 1999; Darling et al., 2005; Boer et al., 2013).

The number of 14-3-3 isoforms is greater in multicellular organisms, with only two present in the unicellular yeast *Saccharomyces cerevisiae*, whereas *Arabidopsis* has fifteen (Van Heusden et al., 1995; Rosenquist et al., 2001). There is some redundancy in function between 14-3-3 proteins, for example disruption to either of the yeast 14-3-3 proteins *BMH1* or *BMH2* in a single gene mutant does not disrupt cell function, whereas *bmh1 bmh2* double mutants are completely non-viable (Van Heusden et al., 1995). However, 14-3-3 isoforms can also show functional specificity, with variation in expression patterns, interactions, sequence (to a limited extent), and response to phosphorylation (Rosenquist et al., 2000; Aitken, 2002; Roberts and de Bruxelles, 2002; Sehnke et al., 2006). Some 14-3-3 genes have been characterised previously in legumes. The soybean 14-3-3 family comprises eighteen 14-3-3 genes, sixteen of which are transcribed (Li and Dhaubhadel, 2011). Two pea 14-3-3 proteins have also been identified in previous studies (Stanković et al., 1995; May and Soll, 2000).

### 5.1.3 Possible mechanisms of *FDa/VEG2* action

Based on the precedent for FD-FT/TFL1 interactions in other species, it is likely that *FDa* acts in conjunction with one or more of the pea FT and TFL1 proteins to regulate expression of flowering genes within the apex. The phenotypes of *FTa1/GIGAS* and *FDa/VEG2* null mutants, *gigas-2* and *veg2-1*, suggest that *FTa1* and *FDa* may act together for specification of  $I_2$  and floral meristems under LD conditions (Chapter 3). In addition, the apparent delay in the timing of the V/ $I_1$  transition in *veg2-1* and *veg2-2*, but not *gigas* under LD conditions, indicates that *FDa* can act to control the correct timing of the V/ $I_1$  transition independently of *FTa1* and suggests that *FDa* can also act with other FT proteins (Chapter 3). *LF* and *DET*, which perform the two main roles of *Arabidopsis TFL1*, delaying flowering time and maintaining SAM indeterminacy, respectively (Murfet, 1975; Singer et al., 1990; Foucher et al., 2003), are both candidates for protein interactions with *FDa*. *veg2-1* and *veg2-2* are epistatic to *det* (Reid et al., 1996), and *veg2-2* is epistatic to *lf* (Taylor, 1998; Sussmilch, 2008). These epistatic relationships indicate that both *DET* and *LF* are dependent on *FDa/VEG2* to fulfil their functions, and support a model in

which *DET* and *LF* both act via interaction with *FDa*, similar to homologous genes in other species.

Some genes that are directly or indirectly regulated by *FDa*/*VEG2* were identified earlier in this study, through altered expression in the *veg2-1* and *veg2-2* mutants (Chapter 3). The timing of *DET* expression was found to be delayed and expression of *VEG1* and *PIM* was completely absent in the *veg2-1* mutant, while expression of these three genes was found to be delayed in the *veg2-2* mutant (Chapter 3). In addition, the severity of the *pim veg2-2* double mutant phenotype suggests that *FDa* acts beyond regulation of *PIM*, to regulate other floral organ identity genes (Chapter 3). Furthermore, preliminary qRT-PCR results indicated altered expression of *UNI* (pea ortholog of *LFY*) and *SEPI* in the *veg2-2* mutant (Sussmilch, 2008), but these results required confirmation.

#### 5.1.4 Chapter aims

The main aim of this chapter was to investigate how *FDa* acts at a molecular level to promote inflorescence development in pea. This aim was subdivided into a series of experimental aims. Firstly, the wild-type expression pattern of *FDa* was investigated by qRT-PCR analysis and specific patterns of apical expression were further investigated by *in situ* hybridisation analysis. Grafting was conducted to determine if *FDa* could be transmitted over a graft union to affect scion phenotype. Next, protein interactions of *FDa* were investigated by yeast two- and three-hybrid analyses and bimolecular fluorescence complementation (BiFC). Lastly, the genes transcriptionally downstream of *FDa* were investigated by examining expression of known flowering genes in the *veg2-1* and *veg2-2* mutants.



## 5.2 Materials and methods

This section contains specific details of materials and methods for studies included in this chapter. General materials and methods also relevant to this chapter are described in Chapter 2. Details of sequences and alignments are shown in Appendix 4.

### 5.2.1 *qRT-PCR analysis*

The results of qRT-PCR analysis that are presented in Sections 5.3.1, 5.3.3.2 and 5.3.4, investigating gene expression across a developmental series, were produced from the same expression experiment. Apex and leaf tissue samples were harvested from wild-type (NGB5839) and *veg2-2* plants at seven day intervals at the same time of day until early pod development (56 days for wild-type, 77 days for *veg2-2*). Plants were grown in controlled-environment growth cabinets under 20°C and LD photoperiod (24h, fluorescent light). Each apex sample comprised the main stem apices from two plants, dissected to approximately 2mm<sup>2</sup> to remove excess stem and developing leaf tissue. Each leaf tissue sample comprised leaflets from the uppermost fully expanded leaf from two plants. Two replicates were collected for each genotype at each time-point. Tissue harvest, the processing of one replicate of apex samples, and the analysis of expression data for *FDa*, *UNI*, *PIM*, *SEP1*, *LF*, *DET* and *VEG1* for this single processed replicate was completed prior to commencement of this study (Sussmilch, 2008). During this study, the second replicate of apex samples and both replicates of leaf samples were processed, gene expression was investigated in the remaining apical replicate and analysis of other genes on both replicates was conducted. qRT-PCR experiments for the 14-3-3 genes were performed by undergraduate research students (Bronwyn Smithies, Amelia Beckett and Erika Bransky) under direct supervision.

For investigation of gene expression in the *veg2-1* mutant, qRT-PCR analysis was conducted on samples from the same LD experiment used in Chapter 3. Details for this experiment are given in Section 3.2.3.

### 5.2.2 *In situ* hybridisation

RNA *in situ* hybridization with digoxigenin-labelled probes was performed in collaboration with A. Berbel, C. Ferrándiz and F. Madueño at the Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia, Spain. Growth of plant material, sample fixation and cloning of the *FDa* probes was conducted at UTAS. Initial *in situ* experiments for troubleshooting of the *FDa* probes were conducted in Valencia with the assistance of A. Berbel. Follow-up *in situ* experiments were conducted by A. Berbel in Valencia from plans produced at UTAS after discussions with F. Madueño and C. Ferrándiz.

Pea apices from wild-type (NGB5839) and *veg2-1* mutants grown under LD conditions (8h natural light + 8h fluorescent light) were dissected to 3-5mm<sup>2</sup> and fixed in 3.7% FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) with vacuum infiltration. Tissues were stained with 0.2% EosinY and 90% ethanol and dehydrated by incubating in a series comprising 100% ethanol, 25% histoclear:75% ethanol, 50% histoclear:50% ethanol, 75% histoclear:25% ethanol, 100% histoclear. Tissues were embedded in paraffin, sectioned to 8µm, and transferred to a microscope slide (*Fisherbrand*® ProbeOn™ Plus, Fisher Scientific, USA).

A probe for *FDa* was generated using a 426bp fragment of wild-type (NGB5839) *FDa* cDNA, amplified by PCR with primer pair PsFD-5F and PsFD-6R and cloned into the pGEMT Easy vector (Promega). A second probe was also generated to target the 5' end of *FDa*, using primer pair PsFD-1F and PsFD-2R, but this probe was found to give background signal, so was not used further in this study. Details of probes used for *VEG1* and *PIM* have been described previously (Berbel et al., 2001; Berbel et al., 2012). 10µg of plasmid was linearised by restriction enzyme digestion using *Sall* (Roche; for antisense probes) or *PaeI* (Fermentas; for sense probes) and purified by ethanol precipitation. Probes were transcribed in 20µL reactions each containing 1.7µg linearised plasmid in 11µL autoclaved MilliQ water (SDW), 2µL 10x transcription reaction buffer (Roche), 2µL 10x DIG-labelling mix (Roche), 1µL RNasin, and 2µL T7 polymerase (for antisense; Roche) or SP6 polymerase (for sense; Roche), according to the manufacturer's instructions, then incubated with DNaseI. Transcription reactions were each precipitated with 1µL 10µg/µL yeast tRNA, 25µL 7.5M ammonium acetate, 36.5µL SDW, and 220µL 96% ethanol at -20°C.

Long probes requiring hydrolysis were hydrolysed at this point. Precipitated RNA was washed with 70% ethanol and resuspended in 50µL 100mM sodium bicarbonate (pH 10.2) and incubated at 60°C. Duration of incubation was determined by the formula shown below. Hydrolysed probes were precipitated with volumes of 0.1x 5% acetic acid, 0.1x 3M sodium acetate (pH 4.5) and 2.5x 100% ethanol.

$$t = \frac{(L_o - L_f)}{(K \times L_o \times L_f)}$$

Where:

t = time in minutes

L<sub>o</sub> = starting length in kb

L<sub>f</sub> = final length in kb (optimum = 0.15 kb)

K = 0.11 cuts/kb/min

The precipitated RNA was washed with 80% ethanol and resuspended in 10µL SDW. A sample of 1µL was taken from each reaction for determination of probe concentration and the remaining 9µL was stored in 91µL hybridisation buffer at -20°C. The probe sample was serially diluted in water to 1/20, 1/250, 1/2000 and 1/2500, spotted onto membrane, and fixed with UV light (70mJ/cm<sup>2</sup>). Antibody reaction and detection was conducted as for slides (below) and dilution with the optimum signal strength was chosen for each probe.

Paraffin was removed from slides with histoclear II (National Diagnostics, USA) and tissue was hydrated with an ethanol series comprising 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, SDW. Tissues were hydrolysed for 20 minutes in 0.2M hydrochloric acid. Slides were washed for five minutes each in SDW, 2x SSC (0.3M NaCl, 0.03M sodium citrate), and SDW; incubated for 18 minutes in 1µg/mL proteinase K in 100mM Tris (pH 8), and 50mM EDTA (pH 8) at 37°C; and washed for 2 minutes in PBS (0.137M NaCl, 2.5mM KCl, 0.01M Na<sub>2</sub>HPO<sub>4</sub>, 2mM KH<sub>2</sub>PO<sub>4</sub>). Proteinase K was blocked with a 2 minute incubation in 2mg/mL glycine in PBS. Slides were washed twice for 2 minutes in PBS, post-fixed for 10 minutes in 4% formaldehyde in PBS and washed twice for 5 minutes in PBS. Tissue was dehydrated with an ethanol series comprising SDW, 30% ethanol, 50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol.

Probes were denatured at 80°C for 2-10 minutes. 'Sandwiches' of two slides joined with 350µL probe in contact with both tissue faces were incubated overnight in a humidified box at hybridisation temperature (53-55°C). Slides were separated

and washed twice for 1.5 hours in 2X SSC and 50% formamide at hybridisation temperature. Slides were then washed for 5 minutes in TBS (0.1M Tris pH 7.5, 0.4M NaCl) and incubated for 1 hour with blocking agent (0.5% Roche blocking agent in TBS). Slides were washed for 30 minutes in TTB (1% BSA, 0.3% Triton X-100 in TBS) and incubated for 1.5 hours with antibody (AP conjugated anti-DIG, Fab fragment, Roche) at a concentration of 1:3000 in TTB. Slides were washed three times for 20 minutes each in TTB, then washed for 5 minutes in detection buffer (100mM Tris pH9.5, 100mM NaCl, 50mM MgCl<sub>2</sub>) and incubated in 50mL detection buffer containing 75µL NBT (Roche) and 75µL BCIP (Roche) for 12-36 hours until signal was visible, then the reaction was stopped in SDW.

The *FDa* sense probe was used to confirm absence of background signal in wild-type plants (data not shown). The photos presented in Section 5.3.1 show results for hybridisation of RNA antisense probes for *FDa*, *VEG1* and *PIM*.

### 5.2.3 Grafting experiment

Grafting techniques were employed to determine if *FDa* could be transmitted across a graft union. Plants used for stocks were grown for 3 weeks after sowing under LD conditions, and had several fully expanded leaves at the time of grafting. Stock plants were decapitated above the uppermost fully expanded leaf, and a longitudinal slit was cut down the centre of the stem for the scion. Seedlings used as scions were grown for 1 week after sowing then decapitated at the epicotyl, approximately 1cm above the cotyledonary node. A graft wedge was prepared from the cut end of the scion, inserted into the stock, and secured with a small piece of silicon tubing. Pots were enclosed in plastic bottles for 1-2 weeks to maintain high humidity during establishment of the graft union. Lateral shoots arising from the scion and stock were excised regularly. Grafts were grown under 18h LD conditions in the UTAS phytotron. This experiment was conducted with the technical assistance of J. Vander Schoor.

#### 5.2.4 Gateway entry clones

Entry clones were created using the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> vector (Invitrogen Corporation, Carlsbad, CA, USA) with inserts comprising full-length coding sequence for *FDa*, *DET*, *LF*, *PsGF14-1*, *PsGF14-2*, *PsGF14-3*, *PsGF14-4*, *PsGF14-5*, *PsGF14-6* and *PsGF14-7* from wild-type pea (NGB5839) cDNA. Entry clones for controls were created in the same way using full-length coding sequence for *AtFD*, *AtFT* and *AtTFL1* from wild-type *Arabidopsis* (Columbia) cDNA, and SPGB and SP from wild-type tomato (cv. Moneymaker) cDNA. Primer details are given in Appendix 1. Isolation and cloning of entry constructs for *FTa1*, *FTa2*, *FTb1*, *FTb2* and *FTc* from wild-type pea (NGB5839) cDNA was performed by V. Hecht. The same entry clones were used for creating constructs for yeast two-hybrid, yeast three-hybrid and BiFC analyses, with inserts shuttled to destination vectors by LR reaction (see Section 2.7.2 for details of Gateway cloning method).

#### 5.2.5 Yeast two-hybrid analysis

Yeast two-hybrid analysis was conducted using the ProQuest<sup>™</sup> Two-Hybrid System (Invitrogen Corporation, Carlsbad, CA, USA). For creation of yeast two-hybrid constructs, inserts from entry constructs (Section 5.2.4) were transferred by LR recombination reaction into pDEST32 (bait; Invitrogen) and pDEST22 (prey; Invitrogen) destination vectors, to create both a bait and prey construct for each gene. Bait and prey constructs were transformed into MaV203 yeast cells (Vidal, 1997). A negative transformation control (without bait and prey constructs), was included with each batch of transformations, in accordance with the manufacturer's instructions (ProQuest<sup>™</sup> Two-Hybrid System, Invitrogen Corporation, Carlsbad, CA, USA). Each interaction was tested in both directions, for example: PsFDa as bait with putative interactor as prey, putative interactor as bait with PsFDa as prey. Four yeast clones were obtained for each direction. For each protein of interest, two yeast clones containing the bait construct with an empty prey vector, and four yeast clones containing the prey construct with an empty bait vector, were obtained as controls to test for auto-activation of bait or prey constructs. Two clones for each kit interaction control were obtained using constructs provided with the Invitrogen ProQuest<sup>™</sup> Two-Hybrid System (strong positive interaction control pEXP32/Krev1 with pEXP22/RalGDS-WT; weak positive interaction control pEXP32/Krev1

pEXP22/RalGDS-m1; negative interaction control pEXP32/Krev1 pEXP22/RalGDS-m2) and two clones containing empty bait and prey vectors were obtained as negative activation controls.

Yeast growth media was made using pre-prepared powder mixes from Sunrise Science Products (San Diego, CA, USA). Rich, routine growth medium (YPAD) comprised 20 g/L peptone, 10g/L yeast extract, 20g/L dextrose and 40 mg/L adenine. This was manually supplemented with 60 mg/L adenine hemisulfate. Dropout media was prepared from dropout base (1.71 g/L yeast nitrogen base, 5 g/L ammonium sulfate and 20 g/L dextrose) combined with dropout mixes with a Synthetic Complete/Hopkins mixture base (21 mg/L adenine, 85.6 mg/L of each of L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, glutamine, L-glutamic acid, glycine, L-histidine, myo-inositol, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, uracil and L-valine, 173.4 mg/L L-leucine and 8.6 mg/L para-aminobenzoic acid) with certain amino acids omitted as appropriate for each purpose. Dropout media was used for transformation (SC-Leu-Trp), testing for *URA3* reporter gene induction (SC-Leu-Trp-Ura), and testing for *HIS3* reporter gene induction (SC-Leu-Trp-His supplemented with 1-50mM 3AT).

To test for interactions, fresh colonies of interaction clones and appropriate controls were grown on SC-Leu-Trp at 25°C or 30°C for 18 hours then replica plated on to SC-Leu-Trp-Ura, SC-Leu-Trp-His+3AT and YPAD plates. SC-Leu-Trp-His+3AT plates were replica cleaned immediately. After 24h incubation at 25°C or 30°C, filter-lift X-gal assays were performed to test for  $\beta$ -Galactosidase induction. SC-Leu-Trp-His+3AT and SC-Leu-Trp-Ura plates were replica cleaned and incubated for a further 2 days at 25°C or 30°C. For X-gal assays, colour was checked after 1 hour (colouration indicated strong interaction) and after 24 hours (colouration indicated weak interaction, no colouration indicated lack of interaction). Interactions were considered to be ‘weak positive’ interactions where a majority of clones for both interaction directions tested positive in tests for *HIS3* and X-gal, and ‘strong positive’ interactions if clones also tested positive for *URA3*, with no background auto-activation by bait or prey constructs. Interactions were considered to be ‘negative’ if a majority of clones tested negative for all three tests. If clones tested positive for *URA3* induction, but not *HIS3* or X-gal tests, results were considered to

be ‘false positive’. For testing previously published interactions of *Arabidopsis* and tomato FD, FT, and TFL1 homologs, only *HIS3* and *URA3* reporter gene induction was tested, with interactions considered to be strong if positive for both *HIS3* and *URA* tests, weak if positive for *HIS3* alone, and negative if negative for both tests.

### 5.2.6 Isolation of 14-3-3 genes from pea

Nucleotide and protein sequences for the 16 expressed members of the soybean 14-3-3 gene family (Li and Dhaubhadel, 2011) and protein sequences for 14-3-3 proteins identified as interactors of OsFD1 and OsHd3a in rice (GF14b, BAF14917; GF14c, BAF23770; Taoka et al., 2011) and of SP in tomato (SITFT6, AAL04424; SITFT7, AAL04425; Pnueli et al., 2001) were used as queries in BLAST searches against *Medicago* and *Lotus* genome and expressed sequence resources (Mt3.5; *Lotus* build 2.5; DFCI TGI; GenBank dbEST). BLASTn searches were conducted using coding sequence from identified members of the *Medicago* 14-3-3 protein family as queries, to identify pea sequence for corresponding pea genes from available resources (GenBank TSA sequence and nucleotide databases). Genes identified in *Medicago*, *Lotus* and pea were named with numerical suffixes, similar to those used in tomato, to avoid confusing overlap with the non-compatible nomenclature previously used for the soybean 14-3-3 protein family.

### 5.2.7 Yeast three-hybrid analysis

To test interactions between FDa with each of FTa1, FTb2 and DET separately in the presence of each pea 14-3-3 protein isolated, yeast three-hybrid analysis was performed. For expression of a third protein for yeast three-hybrid analysis, plasmid pARC351 was kindly provided by R. Immink (Wageningen Plant Research International, the Netherlands). pARC351 was derived from pRED-NLSa plasmid (P. Ouwerkerk, University of Leiden, the Netherlands) with modifications to introduce Gateway compatibility by R. Immink. Constructs for the seven pea genes encoding 14-3-3 proteins isolated, were created by transferring inserts from entry constructs for each gene (Section 5.2.4) into the pARC351 destination vector by LR recombination reaction (Section 2.7.2). A yeast strain with separate mating types, PJ69-4A and PJ69-4 $\alpha$  (James et al., 1996), was kindly provided by R. Immink (Wageningen Plant Research International, the Netherlands). Based on the advice of

R. Immink and P. Wigge, a new yeast strain was used to avoid the reduced efficiency of co-transforming three constructs simultaneously and to test pea interactions in an alternative yeast strain. pDEST32 bait constructs and pDEST22 prey constructs, made earlier for *Fda*, *FTa1*, *FTb2* and *DET* for yeast two-hybrid analysis (Section 5.2.5), were co-transformed into yeast strain PJ69-4 $\alpha$ . Constructs of each isolated pea 14-3-3 gene in the pARC351 vector, were transformed into yeast strain PJ69-4A. Transformed PJ69-4 $\alpha$  and PJ69-4A clones were mated together to obtain diploid yeast cells containing the three target constructs, using a previously described protocol (Folter and Immink, 2011). Appropriate negative controls, with empty vectors included with bait, prey and bridging protein constructs, were also created to test for auto-activation. In addition, constructs for the strong, weak and negative yeast two-hybrid interaction controls provided with the Invitrogen ProQuest™ Two-Hybrid System, were transformed separately into PJ69-4 $\alpha$  and PJ69-4A clones that were mated together to obtain diploid yeast clones as interaction controls.

Rich growth medium (YPAD) was prepared as previously described (Section 5.2.5). Yeast dropout media was prepared from dropout base (1.71 g/L yeast nitrogen base, 5 g/L ammonium sulfate and 20 g/L dextrose) from Sunrise Science Products (San Diego, CA, USA) combined with dropout mixes (30 mg/L L-isoleucine, 150 mg/L L-valine, 20 mg/L L-adenine hemisulfate salt, 20 mg/L L-arginine HCl, 20 mg/L L-histidine HCl monohydrate, 100 mg/L L-leucine, 30 mg/L L-lysine HCl, 20 mg/L L-methionine, 50 mg/L L-phenylalanine, 200 mg/L L-threonine, 20 mg/L L-tryptophan, 30 mg/L L-tyrosine, 20 mg/L L-uracil) with specific amino acids omitted as required. Dropout media was used for selection of transformants (SC-Leu-Trp for PJ69-4 $\alpha$  transformants; SC-Ura for PJ69-4A transformants; SC-Leu-Trp-Ura for selection of diploid yeast clones containing all three constructs after mating) and testing interactions (SC-Leu-Trp-His-Ura supplemented with 1, 5 or 10mM 3AT).

To test for interactions, fresh colonies for interaction clones and appropriate controls were grown on SC-Leu-Trp-His-Ura+3AT plates incubated at 20°C, 25°C or 30°C to test for *HIS3* induction as described in Section 5.2.5, with longer incubation times allowed for growth at lower temperatures. Yeast clones were also replica plated on to SC-Leu-Trp-His+3AT plates where growth of yeast two-hybrid strong, weak and negative interaction controls could also be tested.



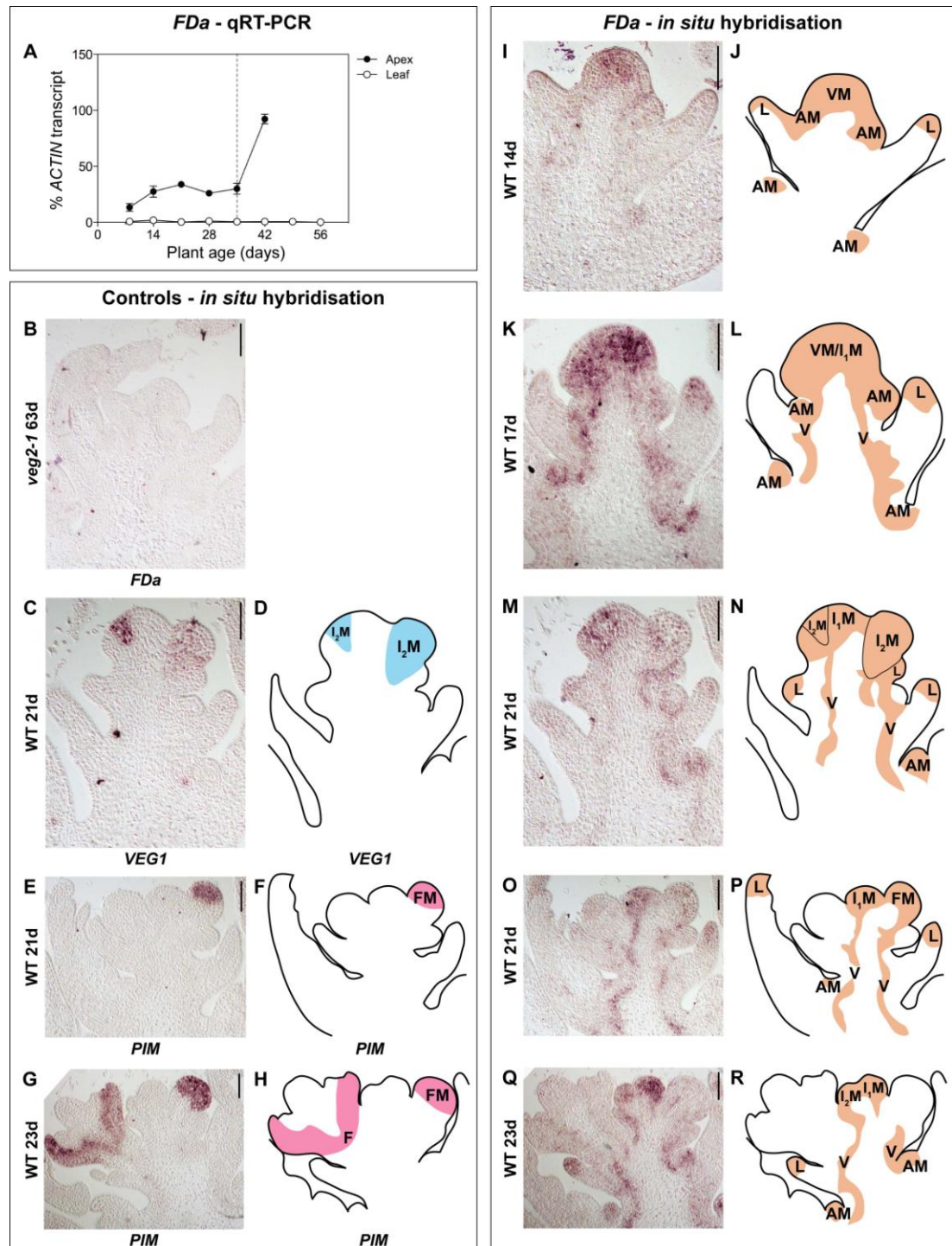
### 5.2.8 Bimolecular fluorescence complementation (BiFC) analysis

BiFC analysis was performed through collaboration with A. Berbel, C. Ferrándiz and F. Madueño at the Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia, Spain. pYFC43 and pYFN43 destination vectors (Belda-Palazón et al., 2012) used for creation of BiFC constructs were kindly shared by A. Ferrando, Universidad Politécnica de Valencia, Spain. Inserts from entry constructs comprising full-length coding sequence for *FDa*, *FTa1*, *FTa2*, *FTb1*, *FTb2*, *FTc*, *LF* and *DET* amplified from cDNA (see Section 5.2.4 for details), were transferred by LR recombination reaction (see Section 2.7.2) into pYFC43 and pYFN43 destination vectors. All constructs for pea genes were created at UTAS and sent to A. Berbel who completed all BiFC experiments in Valencia. Constructs for the positive control interaction between *Arabidopsis* proteins AKIN10 and AKIN $\beta$ 2 were provided by A. Ferrando, as described previously (Belda-Palazón et al., 2012). Constructs were introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) and used to infiltrate young fully expanded leaves of 4-week-old tobacco plants as previously described (Scacchi et al., 2009). Leaves were examined after 3 to 4 days with a Leica TCS-SL confocal microscope and a laser scanning confocal imaging system.

## 5.3 Results

### 5.3.1 Wild-type expression patterns of *FDa*

The expression of *FDa* was first investigated by qRT-PCR in apex and leaf tissues of wild-type plants throughout plant development from seedling to flowering adult plant, under a LD photoperiod. Figure 5.1A shows that *FDa* was expressed in the apex throughout development, with expression increasing with plant age, whereas expression in expanded leaves was negligible. *FDa* expression patterns in the wild-type apex were next investigated in more detail by *in situ* hybridisation during the vegetative phase (Figure 5.1I-J), the V/I<sub>1</sub> transition (Figure 5.1K-L) and early flowering stages (Figure 5.1M-R). Apical samples from the deletion mutant *veg2-1*, were included as a negative control for the *FDa in situ* probe (Figure 5.1B). Expression patterns for *VEG1* (Figure 5.1C-D) and *PIM* (Figure 5.1E-F) were determined on serial sections of the same apices used for *FDa*, as these genes serve as indicators of I<sub>2</sub> and floral meristems, respectively (see Chapter 3). *VEG1* was expressed in I<sub>2</sub> meristems, and *PIM* was expressed in floral meristems and floral primordia (in the petal region of the petal/stamen common primordia and in the sepals), as expected from previous studies (Taylor et al., 2002; Berbel et al., 2012). Within the apex, *FDa* was found to be expressed in the vegetative SAM, axillary meristems, the I<sub>1</sub> meristem, I<sub>2</sub> meristems, vasculature and tips of leaf primordia (Figure 5.1I-R). *FDa* also appeared to be expressed in floral meristems during early development (Figure 5.1O-P). However, *FDa* was not expressed in floral meristems or floral primordia during later floral development (Figure 5.1Q-R).



**Figure 5.1.** Wild-type *FDa* expression patterns throughout development in pea under LD conditions.

(A) Expression of *FDa* in dissected shoot apices and the uppermost fully expanded leaf of wild-type (NGB5839) plants throughout development. Relative transcript levels were determined by qRT-PCR, normalised to the transcript level of *ACTIN*, and represent mean  $\pm$  SE for  $n = 2$  biological replicates, each consisting of pooled material from two plants. Developing floral buds were first macroscopically visible in the wild-type apex 35 days after sowing (grey line).

(B-H) *In situ* hybridisation results for control experiments. (B) *FDa* in the shoot apex of the *veg2-1* deletion mutant, as a negative control for the *FDa* probe, 63 days after sowing. (C-D) *VEG1* expression domain 21 days after sowing, as a marker for *I*<sub>2</sub> meristems. (E-H) *PIM* expression domain (E, F) 21 days after sowing and (G, H) 23 days after sowing, as a marker for floral meristems. (continued next page)

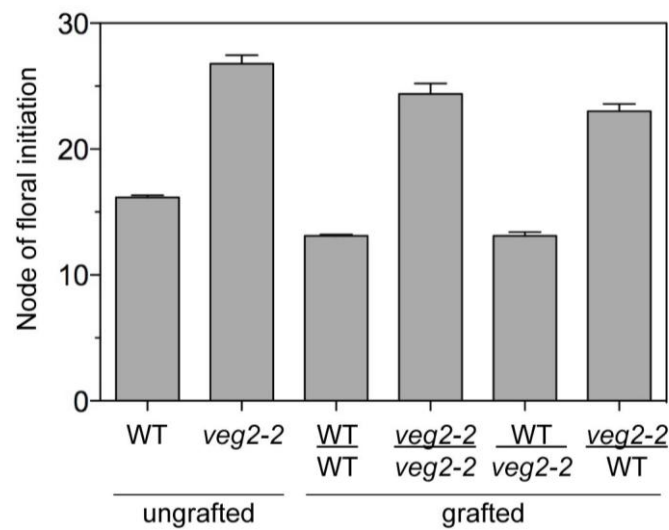
**Figure 5.1. (continued) (I-R)** *In situ* hybridisation results for expression of *FDa* in wild-type (NGB5839) shoot apices throughout plant development. **(I-J)** *FDa* expression in the vegetative apex, 14 days after sowing. **(K-L)** *FDa* expression in the apex, 17 days after sowing, at the approximate time of the transition from vegetative to  $I_1$  meristem identity. **(M-N)** *FDa* expression in the inflorescence apex, 21 days after sowing, in a serial section from the same apex shown in (C). **(O-P)** *FDa* expression in the inflorescence apex, 21 days after sowing, in a serial section from the same apex shown in (E). **(Q-R)** *FDa* expression in the inflorescence apex during development of floral primordia, 23 days after sowing, in a serial section from the same apex shown in (G).

Abbreviations shown on diagrams are as follows: axillary meristem (AM), floral meristem (FM), developing flower (F), leaf primordia (L), primary inflorescence meristem ( $I_1$ M), secondary inflorescence meristem ( $I_2$ M), vasculature (V), vegetative shoot apical meristem (VM). Expression domains are coloured for *FDa* (orange), *VEG1* (blue) and *PIM* (pink).

### 5.3.2 Grafting experiment

There is no precedent for *FD* mobility in other species, in fact, ectopic expression of *FD* in leaf vasculature in transgenic *Arabidopsis* plants is incapable of rescuing *fd* mutant phenotype, indicating that *FD* in the leaves does not constitute or regulate a mobile signal (Abe et al., 2005). However, detection of expression of *FDa* in the vasculature and axillary meristems within the wild-type pea apex (Figure 5.1), left open the somewhat remote possibility that *FDa* may be able to move through the vasculature. Grafting techniques were employed to investigate if *FDa* may act as or regulate a long-distance signal that could be transmitted across a graft union. For each graft, the epicotyl of a seven-day-old seedling was grafted onto a three-week-old stock possessing several expanded leaves. *veg2-2* mutant scions were grafted on to wild-type stocks to test if this could rescue the late-flowering phenotype of the *veg2-2* mutant. In addition, grafts were made to test if *veg2-2* mutant stocks would affect the flowering time of wild-type scions. Controls comprising ungrafted plants and grafts of stocks and scions of the same genotype were also included.

There was no significant difference in flowering time between *veg2-2* control grafts and grafts with *veg2-2* scions on wild-type graft stocks (Figure 5.2;  $p = 0.729$ ). Similarly, flowering time was not significantly different between wild-type control grafts and grafts with wild-type scions grafted to *veg2-2* mutant stocks (Figure 5.2;  $p = 1.000$ ). These results indicate that flowering time was dependent on the genotype of the graft scion and was not significantly influenced by stock genotype. The results of this grafting experiment indicate that *FDa*/*VEG2* and/or associated gene products are not capable of travelling over a graft-union to influence flowering time.



**Figure 5.2.** Grafting experiment to determine if *FDa/VEG2* might act as or regulate a long-distance signal across a graft union.

Graft combinations of wild-type (NGB5839) and *veg2-2* were created by grafting seven-day-old scions on to three-week-old graft stocks. Controls comprising grafted plants with stock and scion of identical genotype, and ungrafted plants were included for comparison. For each graft combination, the genotypes of scion (top) and stock (bottom) are shown, separated by a horizontal line. Plants were grown under 18h LD conditions. Values represent mean  $\pm$  standard error for  $n = 5-11$  plants.

### 5.3.3 Protein interactions of *FDa*

As FD proteins have been shown to interact with FT and TFL1 homologs in a wide range of angiosperm species, it could be expected that these interactions also occur in legumes. In this study, interactions between *FDa* protein and each of the five pea FT proteins (FTa1, FTa2, FTb1, FTb2, FTc), and the two pea TFL1 homologs that share the roles of *Arabidopsis* TFL1 (LF and DET), were investigated by yeast two- and three-hybrid analyses, and BiFC.

#### 5.3.3.1 Yeast two-hybrid analysis

Firstly, specific interactions between *FDa* and each of FTa1, FTa2, FTb1, FTb2, FTc, DET and LF were tested, and the possibility of *FDa* self-interaction was also examined. Four clones for each interaction were tested in each direction of interaction, i.e. *FDa* as bait with putative interactor as prey and putative interactor as bait with *FDa* as prey. In addition, at least two clones of recommended controls were tested, including strong, weak and negative interaction controls, and controls to test for auto-activation. Induction of reporter genes *HIS3*, *URA3* and *lacZ* was determined at 25°C and 30°C. Although evidence of interaction was seen in some

individual clones, no interactions were detected between the pea proteins of interest that were consistent across a majority of clones, between tests and in both directions (Table 5.1). Tests for induction of reporter genes were successful for the strong and weak interaction controls included on each plate (Table 5.1).

To test whether these negative results could reflect a problem with the yeast two-hybrid system being used for testing this type of interaction, constructs were created to replicate previously published interactions of FD, FT and TFL1 homologs from tomato and *Arabidopsis* (Pnueli et al., 2001; Abe et al., 2005; Wigge et al., 2005). AtFD and SlSPGB both showed auto-activation when tested as bait with an empty prey vector (data not shown). Consequently, interactions could only be tested in one direction, with AtFD or SlSPGB as prey. No interaction was detected between AtTFL1-AtFD, AtTFL1-SlSPGB or SlSP-SPGB (Table 5.1). However, only one clone was obtained for SlSP-SPGB, and other researchers have also had difficulty detecting interactions between FD and TFL1 in yeast two-hybrid systems (Abe et al., 2005; Jang et al., 2009). Weak interactions were detected for AtFT-AtFD and AtFT-SlSPGB using this yeast two-hybrid system (Table 5.1).

These results indicate that FDa could not interact with any of the pea FT homologs in this yeast system, which could detect interactions between FD and FT homologs from other species. Results for interactions between FDa and TFL1 homologs LF and DET were inconclusive as no FT-TFL1 interactions could be detected using this system.

**Table 5.1.** Summary of results from yeast two-hybrid analysis of protein interactions between FDa and pea FT/TFL1 homologs.

Interactions between pea (Ps) proteins were tested in both directions (FDa as bait/prey), and strong (Krev1 - RalGDS WT), weak (Krev1 - RalGDS m1) and negative (Krev1 - RalGDS m2) interaction controls and controls of homologous genes from *Arabidopsis* (At) and tomato (Sl) were also included. Each cell represents a specific interaction in one direction. Within each cell, 1-4 yeast clones are represented by symbols arranged horizontally in lines, with one line per test, and clone order preserved in each line. Clones were tested for growth on SC-Leu-Trp-His+3AT (first line; + indicates strong/weak interaction; - indicates no interaction), blue colouration in X-gal assays (second line) after 1 hour (strong interaction, S) or 24 hours (weak interaction, W; no colouration/interaction, -) and growth on SC-Leu-Trp-Ura (third line; + indicates strong interaction; - indicates weak/no interaction). Each cell is coloured according to the conclusion for the majority of clones: strong interaction (dark green), weak interaction (pale green) or no interaction (red). Interactions that were not tested are shown as grey.

		Empty vector		Krev1 as...	PsFDa as...		AtFD as...	SISPGB as...
		Bait	Prey	Bait	Bait	Prey	Prey	Prey
Interaction tested with...	Empty vector	-- -- --			-- -- --	---- ---- +++	---- ---- ----	---- ---- ----
	RalGDS WT (S)			++ SS ++				
	RalGDS m1 (W)			++ WW --				
	RalGDS m2 (-)			-- -- --				
	PsFDa				--++ ---- ----			
	PsFTa1	---- ---- ----	-- -- --		---- ---- +-	---- ---- --++		
	PsFTa2	---- ---- --++	-- -- --		---- ---- ++++	---- ---- ----		
	PsFTb1	---- ---- ----	-- -- -+		---- ---- --++	--++ ---- ----		
	PsFTb2	---- ---- ----	-- -- --		++-- --W- +--	---- ---- ++--		
	PsFTc	---- ---- ----	-- -- --		---- ---- ++--	---- ---- ----		
	PsDET	---- ---- ----	-- -- --		---- ---- ----	---- ---- ----		
	PsLF	---- ---- ----	-- -- --		---- ---- -+-	+++ +++ ----		
	AtFT	---- ----					++- ---	++ --
	AtTFL1	---- ----					---- ----	-- --
	SISP	---- ----						- -

### 5.3.3.2 *Isolation of 14-3-3 genes from pea*

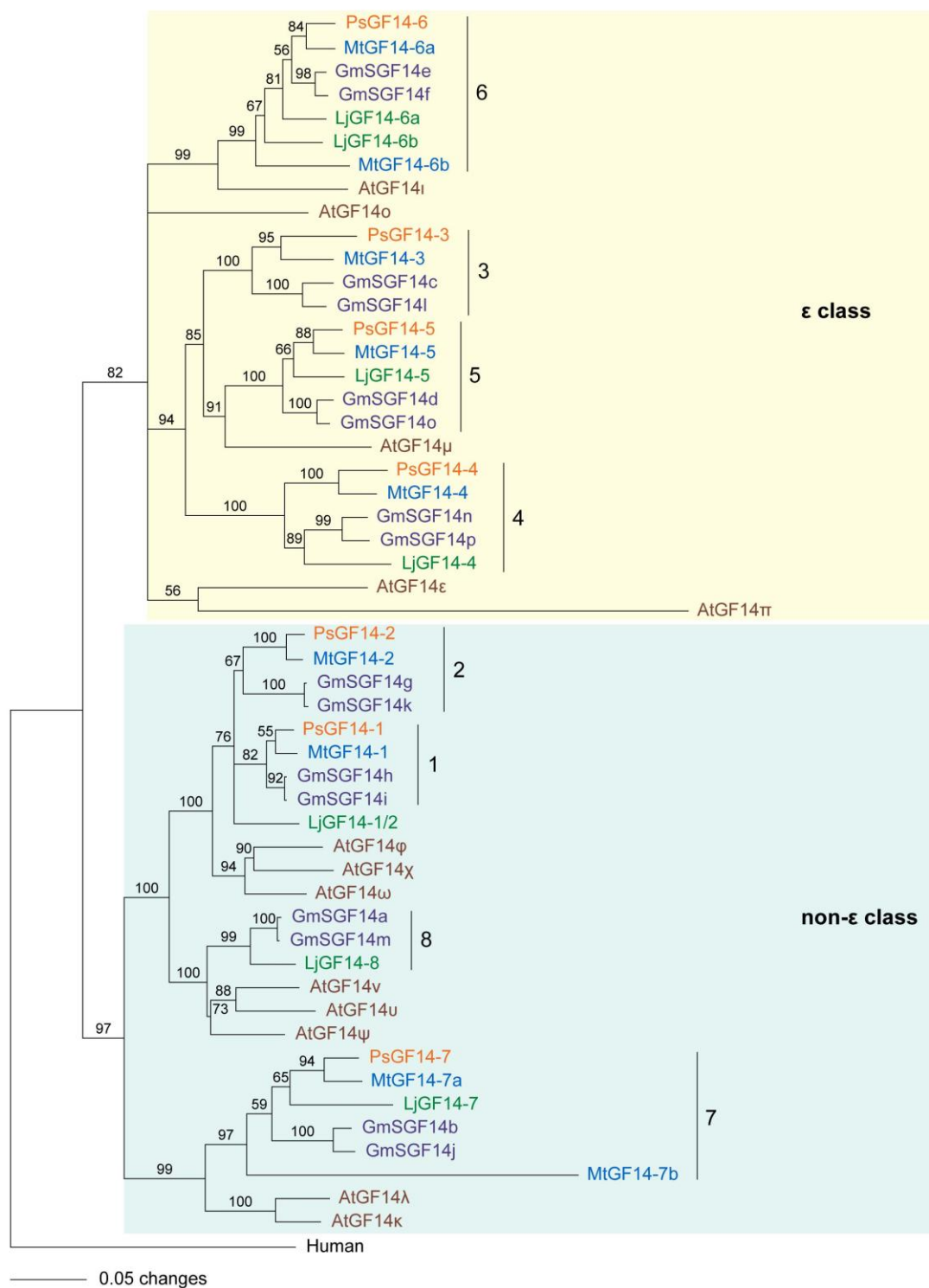
Given the possibility of false negative results from yeast two-hybrid analyses (e.g. Legrain and Selig, 2000), it seems more likely that yeast two-hybrid analysis in this study yielded false negative results than that the interaction between FD and FT homologs, which is central to flowering in diverse angiosperms, is not conserved in pea. When it was reported that 14-3-3 proteins facilitate the interaction between rice FT and FD homologs (Taoka et al., 2011), the strategy for testing interactions between pea proteins was re-evaluated. Two endogenous yeast 14-3-3 proteins (BMH1 and BMH2) are present in yeast two-hybrid systems, and given successful use of yeast systems for detecting interactions between FD and FT/TFL1 proteins from other species (e.g. Wigge et al., 2005; Danilevskaya et al., 2008a; Taoka et al., 2011), it seems that BMH1 and BMH2 are usually able to substitute for plant 14-3-3 proteins as bridging molecules. However, it is conceivable that the endogenous 14-3-3 proteins present in the yeast system used in this study, were somehow inadequate to facilitate an interaction that could be occurring within pea plants. If this was the limiting factor, inclusion of pea 14-3-3 proteins within the yeast system could potentially improve interactions between the pea proteins of interest. With this view, the legume 14-3-3 family was characterised with the aim of isolating the pea 14-3-3 genes for later use in yeast three-hybrid analysis (Section 5.3.3.3).

The soybean 14-3-3 gene family and two 14-3-3 genes from pea have been characterised previously (Stanković et al., 1995; May and Soll, 2000; Li and Dhaubhadel, 2011). In this study, 14-3-3 genes were identified in *Medicago* and *Lotus* by performing BLAST searches against available genome and transcript resources. Nine 14-3-3 genes were identified in *Medicago* and seven were identified in *Lotus*. BLASTn searches were conducted against pea sequence resources using the coding sequence for each *Medicago* 14-3-3 gene, and sequences for seven pea 14-3-3 genes were identified. These legume sequences were included in a phylogenetic analysis that also contained sequences from the expressed soybean and *Arabidopsis* 14-3-3 genes.



Figure 5.3 shows that the legume 14-3-3 proteins fell within the two major classes of plant 14-3-3 proteins: epsilon and non-epsilon (Ferl et al., 1994; DeLille et al., 2001). Across both classes there were a total of eight legume subclades of 14-3-3 proteins (Figure 5.3). There were two soybean paralogs within every legume subclade, which is not surprising, as soybean has a tetraploid origin (Shoemaker et al., 1996; Schmutz et al., 2010). However, the published soybean nomenclature uses alphabetical suffixes that do not reflect phylogenetic relationships and thus cannot be directly applied to the genes from other legumes. To avoid confusion, the pea, *Medicago* and *Lotus* sequences were named (or renamed) using numerical suffixes that reflect the relationships between subclade members (Figure 5.3).

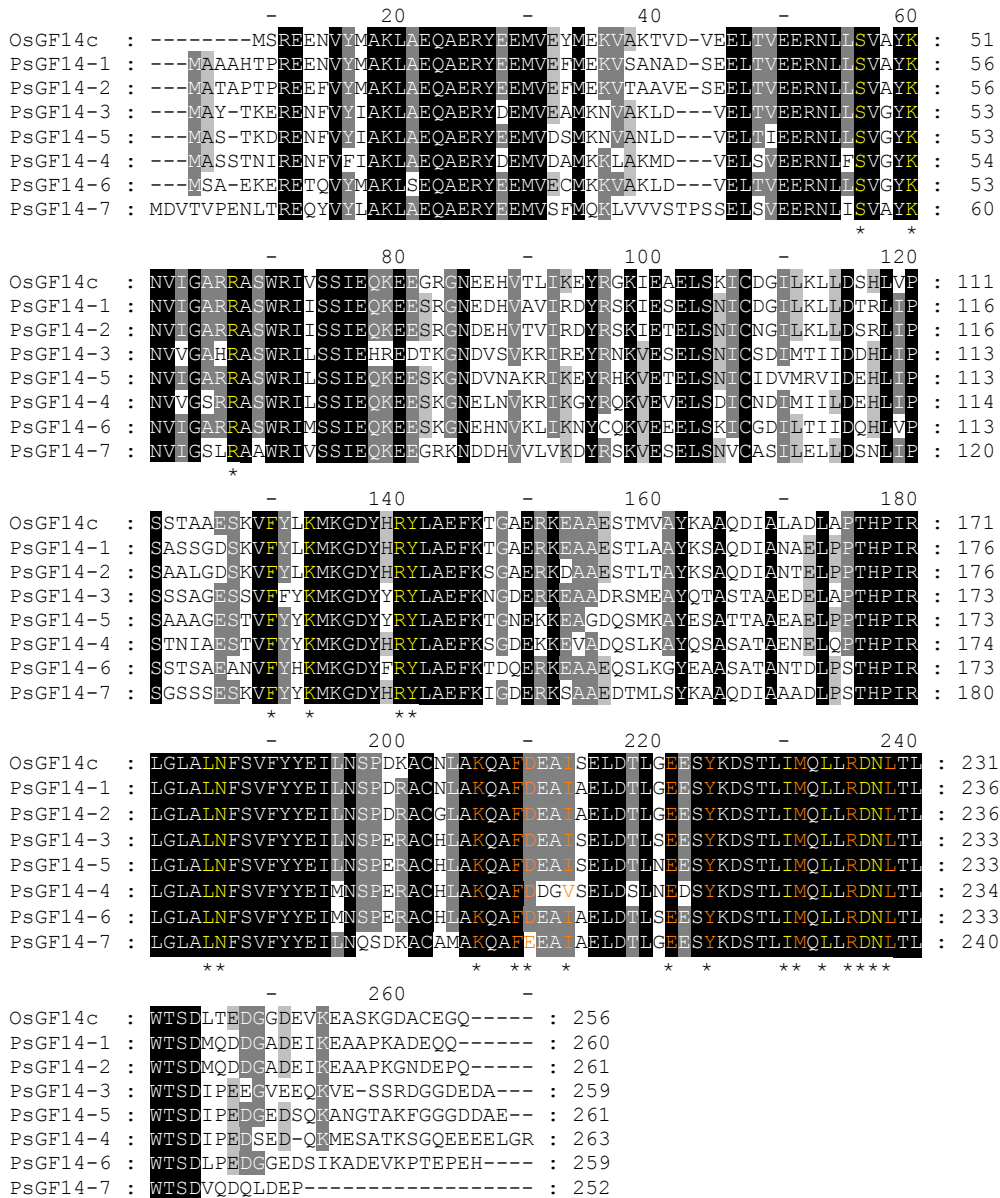
The pea 14-3-3 genes identified in this study include five novel genes (*PsGF14-3* to *PsGF14-7*). *PsGF14-1* (originally designated PSGF14a by Stanković et al., 1995) and *PsGF14-2* (corresponds to AJ238682 from May and Soll, 2000) were identified previously. The pea genes represented seven of the eight legume subclades (Figure 5.3). The eighth legume subclade was present in soybean (*GmSG14a*, *GmSG14m*) and *Lotus* (*LjGF14-8*) but no corresponding genes could be identified in *Medicago* or pea (Figure 5.3). This suggests that this subclade may have been lost prior to divergence of the Trifolieae and Viceae tribes. Overall, the seven 14-3-3 genes identified in pea were thought to be representative of the diversity within the legume 14-3-3 gene family.



**Figure 5.3.** Phylogenetic neighbour-joining tree of *Arabidopsis* and legume 14-3-3 proteins

Predicted protein sequence for 14-3-3 genes identified in this study in *Lotus* (Lj), *Medicago* (Mt) and pea (Ps) are included with transcribed members of the previously characterised soybean (Gm; Li and Dhaubhadel, 2011) and *Arabidopsis* 14-3-3 families (At; Rosenquist et al., 2001). Numbers indicate legume subclades. The phylogram was created from full-length protein sequences, except for LjGF14-7, for which only partial sequence was available. The phylogram is rooted to the Human 14-3-3 protein 0. Bootstrap values obtained from 1000 trees are indicated as a percentage above or next to each branch. Sequence and alignment details are shown in Appendix 4.

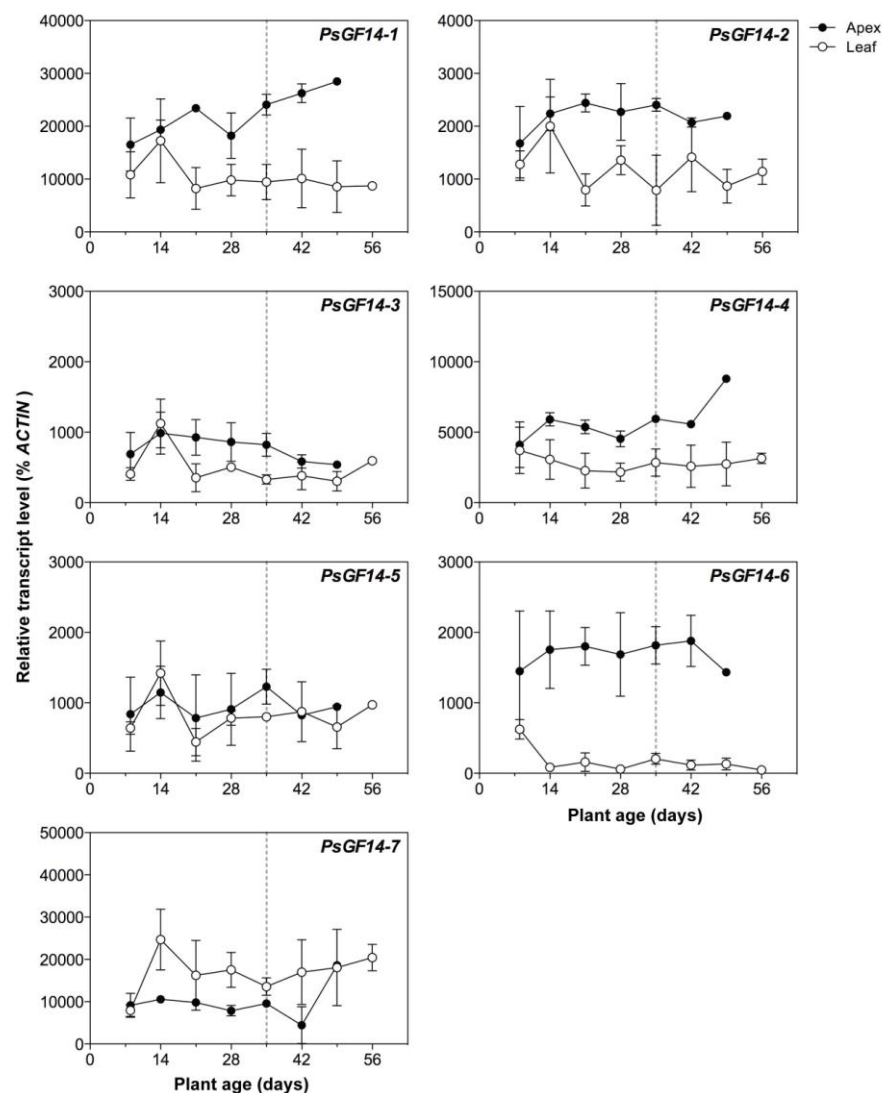
The sequence and expression patterns of the identified pea 14-3-3 proteins were investigated in more detail to determine if any or all of these proteins were suitable candidates for bridging interactions between *FDa* and pea FT/TFL1 homologs. Appropriate candidates should retain all structural features known to be critical for interactions with FD or FT proteins and be expressed in the same tissues as *FDa*. In rice, OsGF14c is known to bridge the interaction between OsFD1 and Hd3a in the florigen activation complex, and the binding sites within OsGF14c have been determined by modelling and site-directed mutagenesis (Taoka et al., 2011). To determine if these sites are conserved in the pea 14-3-3 genes, full-length predicted protein sequences were aligned against OsGF14c. Figure 5.4 shows that the residues at these putative binding sites were highly conserved in all pea 14-3-3 proteins. PsGF14-7 was the only pea 14-3-3 protein with a single residue difference at a putative FT-binding site, when compared with OsGF14c, but this difference was minor involving two amino acids of similar size and properties: glutamate and aspartate (Figure 5.4). Thus, in terms of structure, all pea 14-3-3 proteins were considered to be potential candidate bridging proteins for the pea FD-FT/TFL1 interactions.



**Figure 5.4.** Conservation of residues between pea 14-3-3 proteins and OsGF14c.

Alignment of full-length predicted protein sequence for the pea 14-3-3 gene family (PsGF14-1-7) and rice protein OsGF14c, which bridges the interaction between OsFD1 and Hd3a in rice (Taoka et al., 2011). Residues corresponding to GF14c binding sites with Hd3a and OsFD1 are coloured orange and yellow, respectively, and marked with asterisks. Shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%.

qRT-PCR was conducted to analyse expression of the seven pea 14-3-3 genes in wild-type shoot apices and leaves throughout plant development under a LD photoperiod. Figure 5.5 shows that all seven genes were expressed in the apex throughout development, where *FDa* is also expressed (Figure 5.1). All of the 14-3-3 genes were also expressed in leaves at some stage during development (Figure 5.5). *PsGF14-6* exhibited some tissue specificity with considerably higher expression in the apex than in the leaf for most stages of development (Figure 5.5). As neither structural comparisons nor expression pattern gave any reason to exclude any of the genes, all were retained for yeast three-hybrid analysis.



**Figure 5.5.** Expression patterns of pea 14-3-3 genes.

Expression during development in dissected shoot apices and the uppermost fully expanded leaf of wild-type (NGB5839) plants throughout plant development for the seven pea 14-3-3 genes isolated in this study. Relative transcript levels were determined by qRT-PCR analysis, normalised to the transcript level of the *ACTIN* gene, and represent mean  $\pm$  SE for  $n = 2$  biological replicates, each consisting of pooled material from two plants.

### 5.3.3.3 *Yeast three-hybrid analysis*

Yeast three-hybrid analysis was conducted to test interactions between FDa with each of FTa1, FTb2 and DET separately in the presence of each pea 14-3-3 protein identified. A subset of pea CETS proteins were selected, as these were considered to be likely candidates for interaction with FDa. *FTb2* encodes a putative florigen signal, *FTa1/GIGAS* has a role in specification of I<sub>2</sub> and floral meristems through upregulation of MADS-box genes, and *DET/TFL1a* maintains I<sub>1</sub> indeterminacy by repressing floral genes within the SAM (Chapter 3; Singer et al., 1990; Hecht et al., 2011; Berbel et al., 2012). Interactions were tested in only one direction, with FDa as prey, with the aim of identifying any strong/weak interactions, and later confirming these with subsequent testing in the other direction (with FDa as bait). A new yeast strain was used for yeast three-hybrid analysis, which had separate mating types: PJ69-4α and PJ69-4A (James et al., 1996). Interactions were tested at 20°C, 25°C and 30°C in diploid yeast clones.

Table 5.2 shows that no growth was detected on selective medium lacking histidine for FTa1-FDa, FTb2-FDa and DET-FDa, in the presence of any of the seven pea 14-3-3 proteins isolated. Growth was seen for positive controls (data not shown), which indicates that no interactions were occurring between FDa and FTa1, FTb2 or DET, bridged by 14-3-3 proteins in this yeast three-hybrid system.

**Table 5.2** Results of yeast three-hybrid analysis testing specific protein interactions between FDa with FTa1, FTb2 and DET in the presence of pea 14-3-3 proteins.

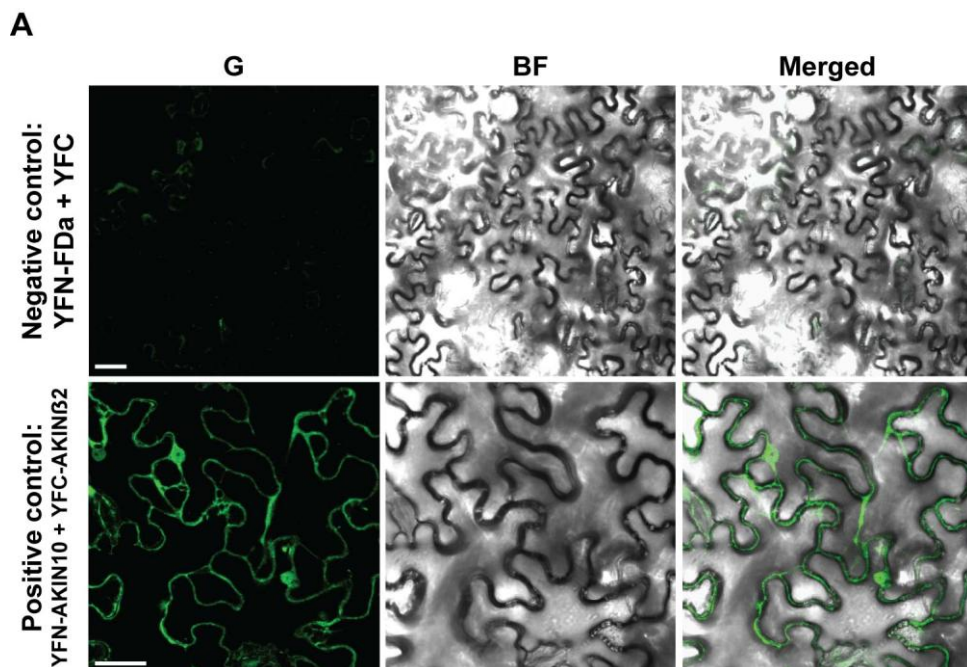
Diploid yeast clones were tested for growth on SC-Leu-Trp-His-Ura+3AT. Growth (+) indicates the occurrence of strong or weak interactions between bait and prey fusion proteins. No growth (-) indicates the absence of interaction. Replica plates of clones on SC-Leu-Trp-His+3AT plates, grown at the same time, showed the same results, but growth occurred for strong (KREV1 - RalGDS WT) and weak (KREV1 - RalGDS m1) two-hybrid interaction controls.

		Bait	Empty	FTa1	FTb2	DET
		Prey	Empty	FDa	FDa	FDa
Bridging protein	PsGF14-1	-	-	-	-	-
	PsGF14-2	-	-	-	-	-
	PsGF14-3	-	-	-	-	-
	PsGF14-4	-	-	-	-	-
	PsGF14-5	-	-	-	-	-
	PsGF14-6	-	-	-	-	-
	PsGF14-7	-	-	-	-	-

#### 5.3.3.4 *Bimolecular fluorescence complementation*

As an alternative approach to testing protein interaction, BiFC analysis was used to examine the possible interactions of FDa with each pea FT protein, and LF and DET *in planta*. This involved co-expressing proteins of interest fused to the N- or C-terminal halves of yellow fluorescent protein (YFP) in tobacco leaf epidermal cells. Figure 5.6A shows the background levels of YFP fluorescence observed when FDa fused to the N-terminal half of YFP (YFN-FDa) was co-expressed with the C-terminal half of YFP alone (YFC), as a negative control. When constructs for two *Arabidopsis* proteins known to interact, AKIN10 and AKIN $\beta$ 2 (Belda-Palaz3n et al., 2012), were co-expressed as a positive control, YFP fluorescence was observed in the nucleus and cytoplasm of transfected cells (Figure 5.6A).

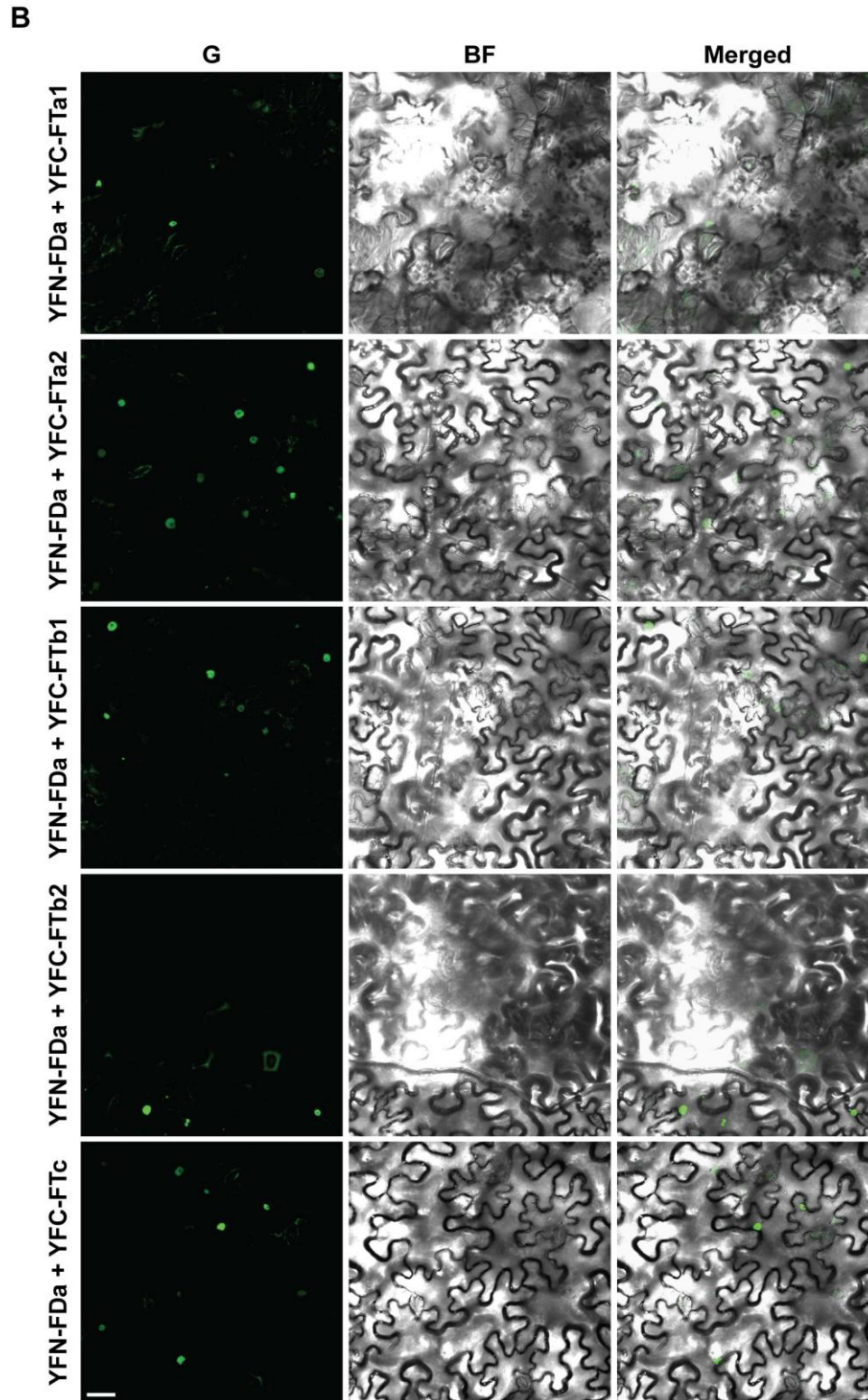
YFP fluorescence was observed in nuclei when YFN-FDa was co-expressed separately with each of the five pea FT proteins fused to YFC (Figure 5.6B). Of the two pea TFL1 homologs tested, fluorescence was observed when YFN-FDa was co-expressed with YFC-DET but not when co-expressed with YFC-LF (Figure 5.6C). These results indicate that FDa can interact with all five FT proteins and DET, but not LF *in planta*.



**Figure 5.6.** Results of BiFC analysis testing specific protein interactions between FDa and pea FT and TFL1 homologs *in planta*.

(A) Negative control comprising FDa fused to the N-terminal half of YFP (YFN-FDa) co-expressed with the C-terminal half of YFP (YFC), and positive control with co-expression of constructs for a known interaction (Belda-Palaz3n et al., 2012). (continued next page)





**Figure 5.6. (continued) (B-C)** YFN-FDa co-expressed separately with (B) each pea FT homolog and (C, on next page) the pea TFL1 homologs LF and DET fused to YFC.

For each interaction, photos from left to right comprise the green channel image showing fluorescence of YFP (G), the bright field image (BF), and the merged G and BF image. Scale bars indicate 40µm. (continued next page)



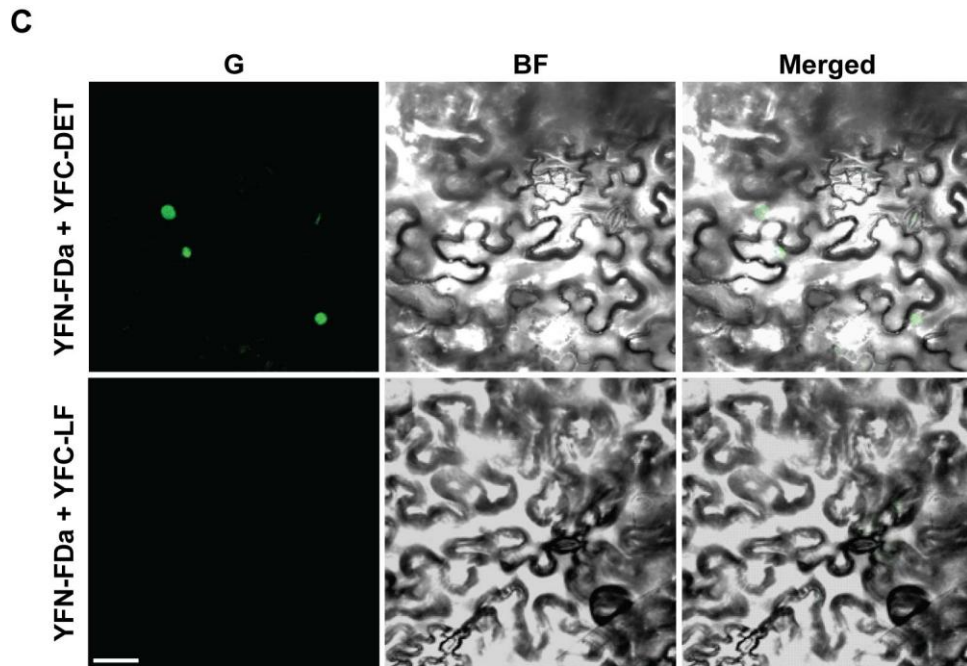


Figure 5.6. (continued)

#### 5.3.4 Identification of genes regulated by *FDa/VEG2*

In order to identify the pea genes that are direct or indirect targets of *FDa/VEG2* transcriptional activity, qRT-PCR was used to investigate expression of floral integrator and floral meristem identity genes in the two *veg2* mutants under LD conditions. The missense mutation in the DNA-binding region of the bZIP domain of *FDa* in the *veg2-2* mutant, is likely to reduce binding specificity to target DNA (Chapter 4). Thus direct and indirect transcriptional targets of *FDa* could be expected to exhibit reduced and/or delayed upregulation in the *veg2-2* mutant relative to wild-type. Genes that are misregulated in the *veg2-2* partial function mutant, should also show misregulation in the more severe *veg2-1* deletion mutant, to an equal or greater extent. For a specific gene, absence of expression would indicate complete dependence on *FDa* for induction, whereas delayed or reduced upregulation in the *veg2-1* deletion mutant would indicate contribution from an *FDa*-independent pathway.

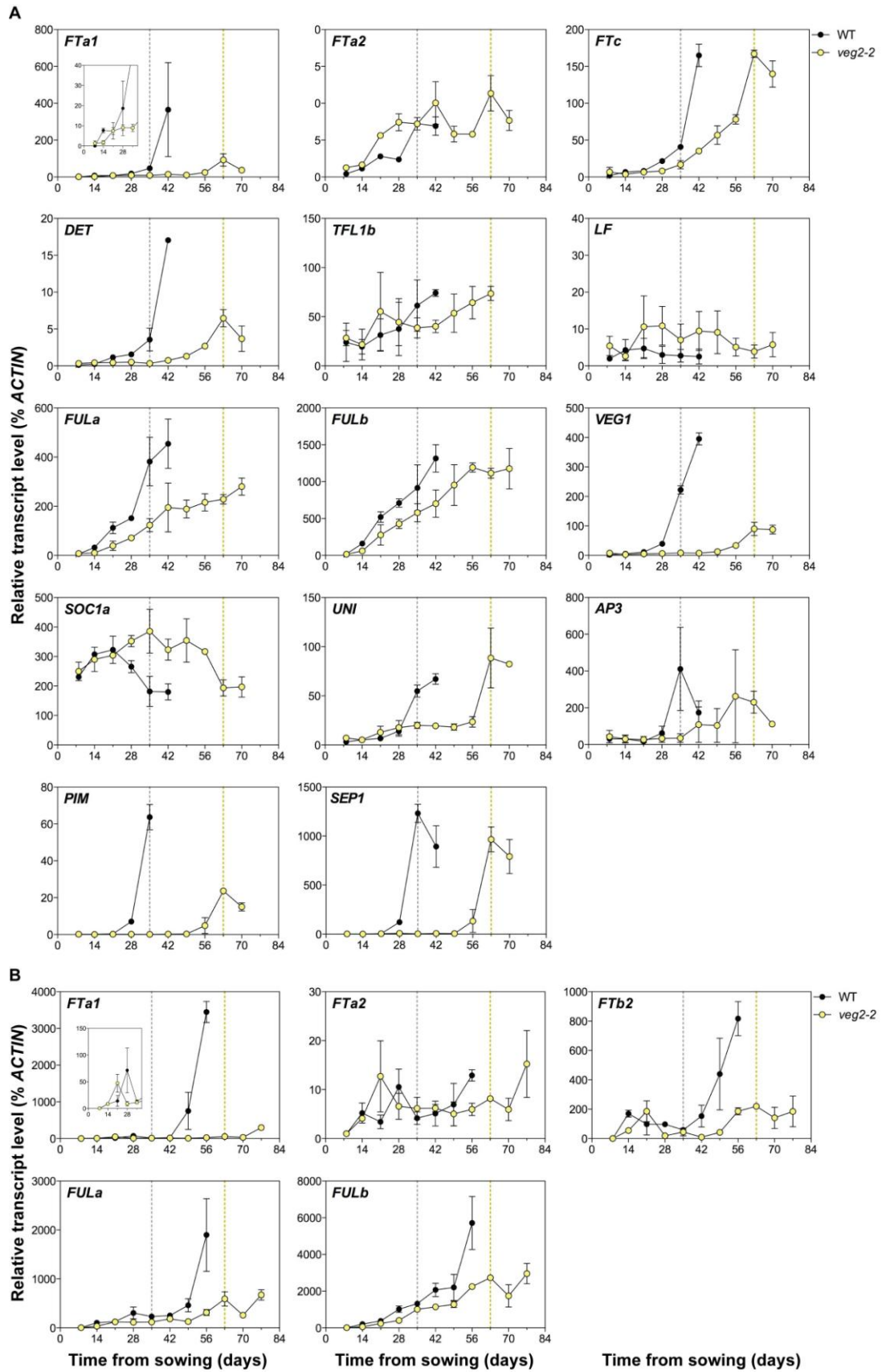
In the *veg2-2* mutant, gene expression was examined in a developmental series containing tissue samples harvested each week throughout development from seedling to flowering adult plant, under LD conditions. In this experiment, floral buds were first macroscopically visible 35 days after sowing in wild-type plant

apices and 63 days after sowing in the apices of *veg2-2* mutant plants. As expected, a number of MADS-box genes showed delayed induction and/or reduced apical expression in the *veg2-2* mutant. *PIM* and *SEPI*, which mark early floral development (Hecht et al., 2011), were induced a week before developing floral buds were first visible in wild-type and *veg2-2* plants (Figure 5.7A). Induction of *VEG1*, a marker for I<sub>2</sub> identity, was induced at a similar time, and also showed a four to five week delay in *veg2-2* plants (Figure 5.7A). Induction of *AP3* was delayed in the *veg2-2* mutant, but the exact timing of induction was unclear due to large variation between replicates (Figure 5.7A). Two pea *FUL* homologs, *FULa* and *FULb* (Berbel et al., 2012), showed a steady increase in expression with plant age, but the level of expression for both genes was reduced in *veg2-2* (Figure 5.7A). Curiously, induction of *SOC1a* was comparable between genotypes, but downregulation was delayed in the *veg2-2* mutant, corresponding with early floral development (Figure 5.7A).

Apical expression of some *CETS* genes was also affected in the *veg2-2* mutant. The induction of *FTc* prior to flowering was delayed in the *veg2-2* mutant (Figure 5.7A). Early apical induction of *FTa1* and *FTa2*, 14 to 21 days after sowing, was comparable between genotypes, but the peak of *FTa1* expression which occurred during late floral development in wild-type (42 days after sowing) was delayed to coincide with flowering in *veg2-2* (56 days after sowing; Figure 5.7A). Induction of *DET/TFL1a*, a marker for I<sub>1</sub> identity, was delayed by approximately four weeks in *veg2-2*, relative to wild-type (Figure 5.7A). Expression of *LF/TFL1c* appeared slightly higher throughout development in *veg2-2* relative to wild-type (Figure 5.7A). Expression of the third pea *TFL1* homolog, *TFL1b*, was similar between the two genotypes (Figure 5.7A).

Initial induction of *UNI*, the pea ortholog of *LFY*, 21-28 days after sowing, was comparable between genotypes (Figure 5.7A). However, the upregulation of *UNI* associated with the appearance of flowers was delayed in the *veg2-2* mutant in accordance with the delay in flowering time (Figure 5.7A).

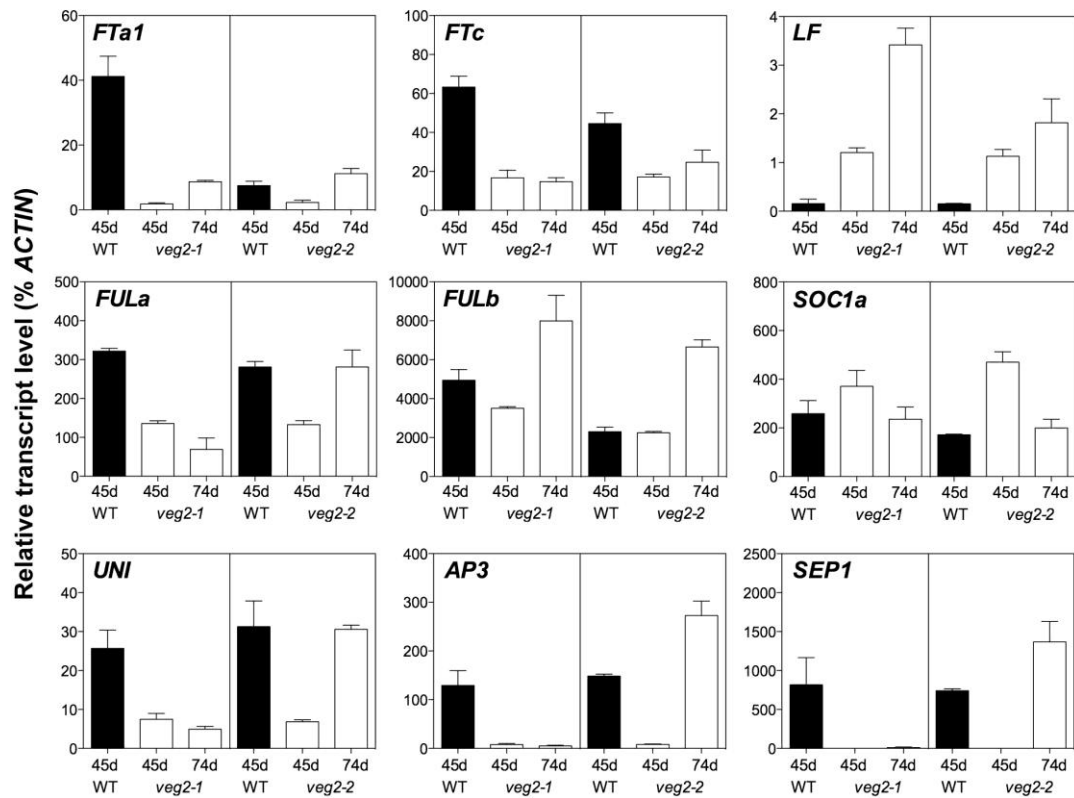
In the leaf, the timing of early induction of *FT* genes was comparable between wild-type and *veg2-2*, but the upregulation of these genes, which occurred after the appearance of floral buds in wild-type, was delayed in *veg2-2* (Figure 5.7B). A similar pattern was observed for induction of *FULa*, and the level of *FULb* expression was consistently lower throughout development in *veg2-2* (Figure 5.7B).



**Figure 5.7.** Expression of flowering genes in the *veg2-2* mutant. (continued next page)

**Figure 5.7. (continued)** Gene expression in wild-type (black circles) and the *veg2-2* mutant (yellow circles) in (A) the dissected shoot apex and (B) the uppermost fully expanded leaf, during plant development under a 24h LD photoperiod. Values have been normalised to the transcript level of *ACTIN* and represent mean  $\pm$  standard error for  $n = 2$  biological replicates, each consisting of pooled material from two plants. Developing floral buds were first macroscopically visible in the wild-type apex 35 days after sowing (grey line) and first visible in the *veg2-2* apex 63 days after sowing (yellow line). For *FTa1*, initial time-points are magnified inset to show early induction which is masked by high expression levels during later floral development.

Genes that exhibited misregulation in the apex of *veg2-2* were further investigated in the *veg2-1* mutant in a separate experiment, which also included the *veg2-2* mutant for comparison. Gene expression was examined in apex samples by qRT-PCR at two time-points, 45 and 74 days after sowing, after the appearance of flowers in wild-type and late-flowering *veg2-2* mutant plants, respectively. Expression of *FTa1*, *FTc*, *FULA* and *UNI*, were consistently lower in *veg2-1* than in wild-type, and expression of *AP3* and *SEP1* was negligible at both time-points in *veg2-1* (Figure 5.8). *FULb* showed a reduced level of expression in *veg2-1* at day 45, but expression had increased by day 74, similar to *veg2-2* (Figure 5.8). The level of *LF* expression was higher in both *veg2* mutants than in wild-type and showed an increase in expression level between 45 and 74 days after sowing (Figure 5.8). *SOC1a* was expressed at a slightly higher level in *veg2-1* than in wild-type at day 45, but decreased by day 74, similar to the pattern seen in the *veg2-2* mutant (Figure 5.8). Results from this experiment showing delayed induction of *DET*, and absence of *VEG1* and *PIM* expression in *veg2-1* were shown in Chapter 3 (Figures 3.2 and 3.14). These results confirm altered expression of these genes in both *veg2* mutants and indicate that *Fda* regulates their expression, either directly or indirectly.



**Figure 5.8.** Apical expression of flowering genes in the *veg2* mutants.

Gene expression in dissected shoot apices of *veg2-1* and *veg2-2* mutants and corresponding wild-type plants, at time-points 45 and 74 days after sowing, under LD conditions. Values have been normalised to the transcript level of *ACTIN* and represent mean  $\pm$  standard error for 2-3 biological replicates. Wild-type lines are wild-type siblings of *veg2-1* (left) and NGB5839 (right).

## 5.4 Discussion

### 5.4.1 Wild-type expression patterns of *FDa*

Expression analysis by qRT-PCR revealed that *FDa* is expressed in the pea apex throughout development in wild-type plants under LD conditions (Figure 5.1A). An increase in *FDa* expression was visible after the macroscopic appearance of floral buds (Figure 5.1A). This could be due to the increased proportion of  $I_2$  and young floral meristem tissue present within the dissected apex samples, as subsequent reproductive nodes were generated. In *Arabidopsis*, there is a similar increase in *FD* expression associated with floral induction (Searle et al., 2006).

The spatial pattern of *FDa* expression within the pea apex was also investigated using *in situ* hybridisation, and was generally consistent with the roles of *FDa* identified earlier in this study through characterisation of the *veg2* mutants (Chapter 3). *FDa* was expressed in the vegetative SAM, the  $I_1$  meristem,  $I_2$  meristems, and floral meristems during early development (Figure 5.1I-R), consistent with roles in regulation of the  $V/I_1$  transition and in  $I_2$  and floral identity (Chapter 3). *FDa* was lost from floral meristems during later development in pea (5.1G-H and Q-R), which supports a model in which *PIM* downregulates *FDa*, in a manner similar to the downregulation of *FD* by *API* in *Arabidopsis* (Wigge et al., 2005; Kaufmann et al., 2010). In a previous study, *FDa* was also shown to be expressed in the plant apex throughout development under SD conditions (Hecht et al., 2011), which is consistent with a role for *FDa/VEG2* under both LD and SD photoperiods (Chapter 3). Overall, the expression pattern of *FDa* appears similar to that of *Arabidopsis FD*, which is expressed predominantly in the shoot apex under LD and SD conditions, specifically within the SAM and in the leaf anlagen and floral primordia (Abe et al., 2005; Wigge et al., 2005).

The detection of expression of *FDa* within the vasculature and axillary buds (Figure 5.1), prompted a grafting experiment to determine if *FDa* and/or its gene products were able to cross a graft union from basal to aerial portions of the plant to affect flowering time. Grafting of a wild-type graft stock to a *veg2-2* scion did not rescue mutant phenotype, and reciprocal grafting of a *veg2-2* mutant stock to a wild-type scion did not delay flowering time (Figure 5.2). These results indicate that *FDa/VEG2* does not act as or regulate a long-distance signal that can be transmitted across a graft union. However, these results do not rule out movement on a smaller

scale within the apex. Based on the results of this experiment, the significance, if any, of expression of *FDa* in the vasculature of the apex is not clear.

qRT-PCR results showed a negligible level of *FDa* expression in expanded leaves throughout development (Figure 5.1A). In contrast to *FDa*, homologous genes in rice, maize and tomato, show expression in expanded leaves (Lifschitz et al., 2006; Muszynski et al., 2006; Tsuji et al., 2013b). Accordingly, *OsFD2* is believed to have a role in leaf development in rice (Tsuji et al., 2013b). Similarly, in maize, *DLF1* is expressed in leaves and DLF1 protein interacts with the FT homolog *ZCN8* (Muszynski et al., 2006; Danilevskaya et al., 2008a). Transgenic plants with reduced *ZCN8* expression have altered leaf morphology, suggesting that *ZCN8* has a role in leaf development in wild-type plants (Danilevskaya et al., 2011), and based on expression pattern and interaction results, it is possible that *DLF1* acts in conjunction with *ZCN8* to fulfil this role (Muszynski et al., 2006; Danilevskaya et al., 2008a). Likewise, tomato *FT* and *TFL1* homologs, *SFT* and *SP*, have been found to have important roles in compound leaf development in tomato, and again based on expression pattern and interactions, it seems likely that SPGB may be acting in complexes with these proteins to facilitate these roles in leaf development (Pnueli et al., 2001; Lifschitz et al., 2006; Shalit et al., 2009). As expression of *FDa* in leaf tissue in pea was negligible, and leaf morphology did not appear to be affected in the *veg2-1* mutant, it is unlikely that *FDa* has any role in leaf development in pea.

#### 5.4.2 Protein interactions

Results from BiFC analysis indicate that *FDa* can interact with all five pea FT homologs, and the TFL1 homolog DET *in planta* (Figure 5.6). However, these interactions could not be shown in the yeast two- and three-hybrid systems used for this study (Tables 5.1-5.2). In yeast two-hybrid analysis, interactions were detected for strong and weak interaction controls, and the previously published interactions AtFT-AtFD and AtFT-SISPGB, but no interactions were detected between *FDa* and pea FT or TFL1 homologs, or for previously published controls involving *Arabidopsis* and tomato FD and TFL1 homologs (Table 5.1). It is not particularly surprising that there was difficulty in detecting interactions between FD and TFL1 homologs using this technique, as various published studies have reported difficulty detecting similar interactions in yeast-based systems (Abe et al., 2005; Jang et al.,

2009). However, based on positive results for FDa-FT interactions in BiFC (Figure 5.6B) and results from other studies testing interactions between homologous proteins from other species (e.g. Wigge et al., 2005; Taoka et al., 2011), it is surprising that no interactions were detected between pea FD and FT proteins in yeast in this study. One possibility considered, was that the endogenous yeast 14-3-3 proteins present in the yeast two-hybrid system used for testing interactions were insufficient for bridging interactions between pea FD and FT homologs. However, testing interactions in a new yeast strain in the presence of pea 14-3-3 proteins in yeast three-hybrid analysis, also failed to reveal interactions detected by BiFC analysis (Table 5.2; Figure 5.6). This indicated that some other factor prevented detection of interactions between the pea proteins in yeast.

There are several common reasons for obtaining false negative results from yeast two- and three-hybrid analyses. Firstly, protein misfolding can occur, obscuring interaction domains. Secondly post-translational modifications that occur normally in plant cells, may not occur in the yeast host cell. Thirdly, co-factors which normally facilitate interaction or provide stability to protein complexes may be absent in yeast. It is unclear why interactions could not be detected between pea proteins of interest in yeast in this study, when this system has been used effectively to detect interactions between homologous proteins from other species. The issues encountered in this study highlight the benefits of using multiple techniques when testing protein interactions.

#### 5.4.2.1 Interactions between *FDa* and *FT* homologs

Previously, the pea *FT* genes were found to differ in their ability to promote flowering in the *Arabidopsis ft* mutant, suggesting that the small differences present in protein sequence between the pea FT proteins, can affect their inherent activity (Hecht et al., 2011). In addition, the pea *FT* genes exhibit differences in expression pattern, in terms of timing, tissue specificity, and regulation by photoperiod (Hecht et al., 2011). Furthermore, the severe phenotype of the *gigas-2/fta1* deletion mutant, strongly indicates that there is little redundancy between *FTa1/GIGAS* and other *FT* genes for induction of key genes including *VEG1* and *PIM* for flowering under LD conditions (Chapter 3). These observations strongly suggest that there has been divergence of function between the five *FT* family members in pea (Hecht et al.,



2011). However, BiFC results from this study indicate that all pea FT proteins have retained the ability to interact with FDa *in planta* (Figure 5.6B).

In other species where expansion of the *FT* family has resulted in divergence of function between *FT* family members, there is evidence of specificity in protein interactions with bZIP transcription factors. In maize, there are 15 *FT* family members (Danilevskaya et al., 2008a), but out of a subset of the seven considered most likely to be functional homologs of *Arabidopsis FT*, only two encode proteins that show convincing interactions with DLF1 in yeast (Danilevskaya et al., 2008a; Meng et al., 2011). Maize has at least two other FD homologs (Figure 4.4; Tsuji et al., 2013b), so it seems likely that the remaining FT homologs act with specific FD proteins to fulfil divergent roles. In wheat, TaFT interacts with TaFDL2 to bind to the promoter of the *API/FUL* homolog *VRN1*, but TaFT2 interacts specifically with TaFDL13 in a complex that cannot bind to *VRN1* (Li and Dubcovsky, 2008). It is yet to be determined if the FT proteins in pea can interact with other bZIP transcription factors, and if so whether they show specificity in these other interactions.

Based on findings in rice, it is likely that the pea FDa-FT interactions are facilitated by 14-3-3 proteins (Taoka et al., 2011; Tsuji et al., 2013b). The exact mechanism for differences in the inherent activity between FT proteins is yet to be determined, but one possibility is that they may interact differently with 14-3-3 proteins. Modelling of the rice complex involving Hd3a, GF14c and OsFD1, indicates that Hd3a binds directly to 14-3-3 proteins, which in turn facilitate binding to the DNA-binding FD proteins (Taoka et al., 2011). The rice 14-3-3 proteins differ in their ability to bind with Hd3a, and mutation to key residues at the 14-3-3 binding interface in Hd3a abolished binding with GF14c and GF14b (Taoka et al., 2011). Not all of the corresponding residues are well conserved between pea FT homologs (Appendix 4), and it is possible that these sequence differences could confer differences in specificity of binding to pea 14-3-3 proteins. Although BiFC results from this study show that these sequence differences do not abolish interaction with FDa (Figure 5.6B), preferential binding to different 14-3-3 proteins, could conceivably alter the shape and functionality of the resulting complex. This could be investigated through testing of interactions (e.g. by co-immunoprecipitation) and modelling of the protein complex structure in a future study.

Alternatively, divergence in the sequence of segment B between pea FT proteins may affect their inherent activity (Hecht et al., 2011). In *Arabidopsis*, Y85 and segment B are regions of the FT protein that are critical for the floral-promoting function of FT (Hanzawa et al., 2005; Ahn et al., 2006). However, modelling in rice indicates that these regions are not involved in interaction with 14-3-3 proteins, and are instead on the surface of the complex, where there is a putative ligand-binding pocket (Taoka et al., 2011). It has been proposed that this ligand-binding pocket has an important role in binding with one or more co-factors, with members of the TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) family suggested as possible candidates (Taoka et al., 2011; Ho and Weigel, 2014). Thus it is possible that the differences in segment B between pea FT proteins may alter binding with co-factors, which have not yet been identified, with important implications for function. The importance of segment B for the inherent activity of each FT protein could be examined by swapping this domain between proteins and testing relative activity in terms of flowering time when overexpressed in the *Arabidopsis ft* mutant.

Modelling in rice indicates that FT homologs and 14-3-3 proteins involved in the florigen activation complex do not come into direct contact with the promoter regions of target genes (Taoka et al., 2011). Instead, DNA-binding is restricted to the duplicate FD proteins present within this complex, which suggests that target specificity is determined by the structure of the DNA-binding, basic region of the FD protein. The detection of interaction between all five pea FT homologs and FDa (Figure 5.6B), suggests that all pea FT homologs should share a high level of redundancy in their ability to upregulate the same target genes via binding with FDa. However, the fact that the *gigas* mutant completely fails to upregulate *VEG1* and *PIM* under LD conditions (Figure 3.14), shows that the remaining four *FT* genes do not act redundantly with *FTa1* in this role. In contrast, in SD conditions, *FTa1* is not essential for upregulation of *VEG1* or *PIM* (Figure 3.15), suggesting that a different *FT* gene may fulfil this role.

Based on current knowledge, it is not clear (a) why the remaining FT proteins cannot compensate for the absence of *FTa1* in the *gigas* mutant, through interaction with FDa under LD conditions, and (b) why photoperiod may alter the roles of *FT* genes. One possibility is that the differential expression patterns of *FT* genes may influence the availability of each FT protein for interaction with FDa in the apex

(Hecht et al., 2011). However, this explanation is not adequate, as *FTc* is eventually expressed at reasonably high levels within the apex of the *gigas* mutant in LD conditions, yet *VEG1* expression is not induced (Figure 3.14; Hecht et al., 2011; Berbel et al., 2012). In addition, there is little increase in the expression of other *FT* genes under SD conditions that could account for redundancy of *FTa1* in promoting *VEG1* and *PIM* expression under this photoperiod (Figure 3.15; Hecht et al., 2011). An alternative explanation is that each FT homolog may bind different co-factors that are photoperiod-dependent and affect target specificity. Alternatively, transcription of the 14-3-3 gene family could show photoperiod dependency, which could affect which complexes form, especially if FT proteins show preferential binding to different 14-3-3 proteins. Lastly, it remains a possibility that the pea FT proteins may be able to interact with other bZIP transcription factors (e.g. FDb if present in pea, or AREB3 homologs), to regulate specific sets of target genes, but *veg2-1* mutant phenotype indicates that FDa is the only transcription factor capable of regulating the key flowering genes *VEG1* and *PIM* (Figures 3.14 and 3.15).

In contrast to the *gigas* mutant, *veg2* mutant phenotypes are comparable between LD and SD conditions (Chapter 3). This indicates that the roles of *FDa* are not dependent on photoperiod. It is possible that FDa acts in complexes with different FT proteins for each stages of pea inflorescence development. Based on BiFC results, all five FT proteins are candidates for formation of complexes with FDa. Once *fta2*, *ftb1*, *ftb2* and *ftc* mutants are available, single and multiple mutants could be used to determine which *FT* genes are important for each stage of pea inflorescence development, under different conditions.

#### 5.4.2.2 Interactions between *FDa* and *TFL1* homologs

The two pea TFL1 homologs that share the roles of *Arabidopsis* TFL1, showed specificity in interaction with FDa (Figure 5.6C). The results of BiFC analysis indicate that DET can bind to FDa *in planta* (Figure 5.6C), supporting a model in which DET maintains SAM indeterminacy in wild-type plants by acting in a complex with FDa to repress expression of floral genes within the SAM (see Chapter 8). This interaction is consistent with the roles of homologous proteins in *Arabidopsis* (Wigge et al., 2005; Hanano and Goto, 2011; Ryu et al., 2013). In contrast, no interaction was detected between LF and FDa *in planta* (Figure 5.6C),

indicating that *LF* must act to delay flowering independently of *FDa*. The exact mechanism of *LF* action is not currently clear. It is possible that a second *FD* homolog, *FDb*, exists in pea that could not be isolated during this study (Chapter 4), so it is conceivable that *LF* could be acting in an anti-florigenic complex with this putative *FDb*. An alternative theory is that *LF* may act in a complex with a related bZIP transcription factor, similar to interactions between *FT* and *AREB3/DPBF4* homologs in wheat (Li and Dubcovsky, 2008; Tsuji et al., 2013b).

Previously it has been suggested that *LF* may function by repressing *VEG1* in newly initiated axillary meristems (Beveridge et al., 2003). In support of this theory, *LF* has recently been found to be expressed predominantly in vegetative axillary buds (A. Berbel, C. Ferrándiz and F. Madueño, unpublished data). In addition, *veg1* is thought to be epistatic to *lf*, indicating that the function of *LF* could be dependent on *VEG1* (Reid and Murfet, 1984). Intriguingly, *lf* is itself epistatic to *gigas* under both LD and SD conditions and *I*<sub>2</sub> specification occurs normally in the *lf gigas* double mutant (Hecht et al., 2011; personal observation). This suggests that *LF* is responsible for blocking *VEG1* expression and associated specification of *I*<sub>2</sub> meristems in the *gigas* single mutant under LD conditions, and indicates that repression of *LF* is an important aspect of the role of *FTa1/GIGAS* in the upregulation of *VEG1* for *I*<sub>2</sub> specification in LD conditions. One explanation, that *FTa1* directly represses *LF* transcription, seems unlikely, as *LF* expression increased rather than decreased during the transition to flowering (Hecht et al., 2011). In addition, this increase was delayed in the *gigas* mutant (Hecht et al., 2011), pointing to a positive influence of *FTa1/GIGAS* on *LF* transcription, whether direct or indirect. However, it is possible that *FTa1* may negatively regulate *LF* action through some other mechanism, such as competition for binding of common partners required for complex formation, or through competition for the same promoter regions of target genes.

The epistasis of *veg2-2* over *lf* indicates that the function of *LF* is dependent on *FDa/VEG2* (Susmilch, 2008), but unlike the relationship between *FDa* and *DET*, this cannot be explained by binding of *FDa* and *LF* in the same protein complex (Figure 5.6C). If *FDa* and *LF* are acting in the same linear pathway, epistasis of *veg2-2* over *lf* could result from upregulation of *LF* by *FDa/VEG2*, or repression of *FDa/VEG2* by *LF*. However, an increased level of *LF* expression was seen in *veg2-1*

and *veg2-2* mutant apex samples used for the qRT-PCR experiments in this study (Figures 5.7A and 5.8), suggesting that *FDa* normally inhibits *LF* transcription in wild-type plants. Previously it was suggested that *LF* may act by repressing expression of *VEG2/FDa* in axillary meristems (Beveridge et al., 2003), but *in situ* results from this study show that *FDa* is expressed in vegetative axillary meristems, indicating that this is not the mechanism of *LF* action (Figure 5.1). An alternative possibility is that *LF* acts in a complex that binds to promoter regions of *FDa* target genes, and prevents *FDa* binding, in order to inhibit upregulation of these target genes. The precise mechanism of *LF* action remains an interesting area for future investigation.

#### 5.4.2.3 *The legume 14-3-3 gene family*

Investigation of the legume 14-3-3 family was undertaken with the aim of using pea 14-3-3 proteins to facilitate interactions between FD and FT/TFL1 homologs in yeast three-hybrid analysis. Although no interactions were detected in yeast-three hybrid analysis (Table 5.2), characterisation of the legume 14-3-3 family conducted in this study provides a useful foundation for future research. Pea representatives were isolated for seven of the eight legume subclades identified in phylogenetic analysis (Figure 5.3). No genes identified in *Medicago* or pea fell within the eighth legume subclade, which suggests that this subclade was lost prior to divergence of the Trifolieae and Viceae tribes. Based on the close synteny between pea and *Medicago* (Choi et al., 2004; Kaló et al., 2004), the number of 14-3-3 genes could be expected to be similar between these two species. Two extra 14-3-3 genes were identified in *Medicago* (*MtGF14-6b* and *MtGF14-7b*), for which no corresponding gene was identified in pea. It is possible that these genes are present in pea, but not represented in available sequence resources. Future availability of genome sequence resources for pea will allow this to be determined. A high level of conservation of all amino acids at putative FT-binding sites within each 14-3-3 protein identified in pea (Figure 5.4), and expression of each gene in the apex under LD conditions, suggests that all seven proteins are candidate members of pea florigenic or anti-florigenic complexes (Figure 5.5). This could be investigated by testing interactions between the seven 14-3-3 proteins, *FDa*, and pea FT/TFL1 homologs in a future study (e.g. by co-immunoprecipitation). In addition, it would be

interesting to determine if these 14-3-3 genes exhibit different expression patterns under SD conditions, as photoperiod-dependent expression of specific 14-3-3 genes could potentially account for variation in the function of the pea *FT* homologs under different photoperiods (see Section 5.4.2.1).

### 5.4.3 Identification of genes regulated by *FDa/VEG2*

Based on prior knowledge from other species, it could be expected that a pea homolog of *FD* would regulate the expression of numerous MADS-box genes (e.g. Abe et al., 2005; Danilevskaya et al., 2008b; Taoka et al., 2011). In this study, the results of qRT-PCR indicate that *FDa* acts upstream of transcription of a number of different groups of flowering genes in the apex, including pea homologs of *FT*, *TFL1* and *LFY* in addition to MADS-box genes.

As expected, a number of MADS-box genes showed reduced and/or delayed expression in *veg2-2* and reduced or no expression in *veg2-1*, indicating that *FDa/VEG2* acts transcriptionally upstream of these genes. *PIM* and *SEP1* acted as early markers of floral development and showed a similar 4-5 week delay in *veg2-2* (Figure 5.7A), but were not expressed in the *veg2-1* mutant, consistent with the absence of floral meristems in this mutant (Figures 3.14 and 5.8). This indicates that *FDa/VEG2* is critical for induction of these genes. The pea *FUL* homologs, *FULa* and *FULb*, exhibited a reduced level of apical expression in both *veg2* mutants relative to wild-type at the same time-point (Figures 5.7A and 5.8). This indicates that *FDa/VEG2* has a role in upregulating *FULa* and *FULb*, but is not critical for the induction of these genes. Although the roles of *FULa* and *FULb* have not yet been characterised in pea, the early upregulation of these genes suggest that they may have an early role in pea inflorescence development (Figure 5.7A). *FUL* and *SEP* genes have previously been identified as downstream targets of *FD* in *Arabidopsis* (Teper-Bamnolker and Samach, 2005). Likewise, *API* homologs have been identified as targets of *FD* genes in *Arabidopsis* and rice, although there is still some confusion over whether this transcriptional regulation is direct or indirect (Abe et al., 2005; Wigge et al., 2005; Benlloch et al., 2011; Taoka et al., 2011). In *Arabidopsis*, Wigge et al. (2005) mapped the *FD* response element in the *API* promoter to a 130bp region that includes a C-box motif. Benlloch et al. (2011) could find no evidence of binding between *FD* and this C-box motif, leading to their conclusion that *FD* either does not

bind directly to the *API* promoter, or binds to an atypical binding site that could not be identified during their study. However, Taoka et al. (2011) demonstrated binding of OsFD1 to this C-box element from the *API* promoter by electrophoretic mobility shift assay (EMSA). Whether *PIM*, *SEPI*, *FULa* and *FULb* are direct or indirect targets of *FDa* in pea could be investigated in a future study.

*FULa* and *FULb* also exhibited a reduced level of expression in the leaf in *veg2-2* relative to wild-type (Figure 5.7B). In *Arabidopsis*, accumulation of *FUL* was significantly reduced in the leaves of the *fd* mutant relative to wild-type plants, suggesting that *FD* is normally involved in upregulation of *FUL* in leaves (Teper-Bamnolker and Samach, 2005). As *FD* is expressed in the apex and developing leaf primordia, and shows only minimal expression in mature leaves (Abe et al., 2005; Wigge et al., 2005), it is likely that the role of *FD* in upregulation of these genes could be occurring when these leaves are developing in the apex. In this experiment, the same explanation could be used, as induction of *FULa* and *FULb* in the leaves is delayed relative to the apex (Figure 5.7). It would be interesting to examine whether *FULa* and *FULb* affect any aspects of leaf development in a future experiment.

Curiously, induction of *SOC1a* was not affected in the *veg2-2* mutant (Figure 5.7), and *SOC1a* was expressed at a slightly higher level in the *veg2-1* mutant than in wild-type at the first time-point tested (Figure 5.8). These results indicate that *FDa/VEG2* does not have an important role in the induction of *SOC1a*. In *Arabidopsis*, *SOC1* induction is delayed in the *fd* mutant, and *SOC1* is thought to be one of the earliest targets of the *FT/FD* pathway (Searle et al., 2006; Wang et al., 2009). As *veg2-2* is a partial function mutant, it would be worth examining *SOC1a* induction at an earlier time-point in the *veg2-1* deletion mutant, to confirm that *SOC1a* induction is not affected in the absence of *FDa/VEG2* function. Downregulation of *SOC1a* was delayed in accordance with the delay in flowering in *veg2-2* (Figure 5.7A), and appeared similarly delayed in *veg2-1* (Figure 5.8), indicating that *SOC1a* may be downregulated by genes that are transcriptionally downstream of *FDa/VEG2*. *SOC1a* expression is also higher in the *gigas* and *veg1* mutants, suggesting that downregulation of *SOC1a* may be downstream of *GIGAS/FTa1* and *VEG1* also, (Appendix 5). In *Arabidopsis*, *SOC1* expression is downregulated in floral meristems by *API* (Liu et al., 2007). It is possible that *PIM* downregulates *SOC1a*, in a similar manner in pea, as the decrease in *SOC1a*

expression in wild-type and the *veg2-2* mutant coincides with the induction of *PIM* (Figure 5.7A). However, one or more other genes must also be involved, as the downregulation of *SOC1a* also occurs in the *veg2-1* mutant, which does not exhibit *PIM* expression (Figures 3.14 and 5.8). The mechanism of *SOC1a* induction and role of *SOC1a* during flowering in pea is yet to be the subject of detailed investigation, but it is intriguing that induction of *SOC1a* appears to be independent of *FDa/VEG2* in pea.

In terms of inflorescence architecture, *VEG1* is perhaps the most interesting MADS-box gene known in pea. The novel role of *VEG1* in specification of  $I_2$  meristems, could underlie the compound inflorescence architecture seen in pea and related legume species (Berbel et al., 2012). In Chapter 3, induction of *VEG1* was found to be delayed in the *veg2-2* mutant and did not occur in the *veg2-1* or *gigas* mutants under LD conditions (Figure 3.14). The 4-5 week delay in *VEG1* expression in the *veg2-2* mutant can be seen clearly in the developmental series examined in this chapter (Figure 5.7A). These observations support a model in which *FDa* and *FTa1* act to upregulate *VEG1* under LD conditions (Chapter 3; Berbel et al., 2012). Little is known about the transcriptional regulation of *AGL79* homologs in other species, where expression is predominantly in root tissue (Parenicova et al., 2003; Kim et al., 2005).

Upregulation of a *LFY* homolog by an *FD* homolog has not previously been reported in any other species, but results from this study indicate that this occurs in pea. Initial induction of *UNI*, the pea *LFY* ortholog, was comparable between *veg2-2* and wild-type (Figure 5.7A). However, the upregulation of *UNI* associated with flowering was delayed in the *veg2-2* mutant in accordance with the delay in flowering (Figure 5.7A), and *UNI* expression was reduced in the *veg2-1* mutant, relative to wild-type plants (Figure 5.8). Similar results were obtained for the *gigas* mutant, wherein initial *UNI* induction occurs, but *UNI* is not subsequently upregulated (Appendix 5; Hecht et al., 2011). These results indicate that *FDa* and *FTa1* both control the upregulation of *UNI* expression associated with flowering. Direct or indirect regulation of *UNI* by *FDa* and *FTa1* is remarkably different from the relationship between the corresponding genes in *Arabidopsis*. In *Arabidopsis*, *LFY* acts independently of and redundantly with *FT/FD* to promote floral development (Abe et al., 2005; Wigge et al., 2005). Indeed, *LFY* expression is not



altered by overexpression of *FD* or *FT* in transgenic *Arabidopsis* plants (Moon et al., 2005; Wigge et al., 2005). This difference between pea and *Arabidopsis* may explain the severity of phenotype for the non-flowering *veg2-1* and *gigas* mutants, relative to the late-flowering *ft* and *fd* null mutants in *Arabidopsis* (Chapter 3; Abe et al., 2005; Wigge et al., 2005). In the absence of an *FDa/FTa1*-independent pathway for induction of *VEG1* under LD conditions, flowering cannot occur when this pathway is blocked in pea. *UNI* expression is also reduced in the *veg1* mutant (Appendix 5), thus it appears that the upregulation of *UNI* associated with flowering occurs downstream of *VEG1*. The mechanism for *UNI* induction remains an interesting area for future research.

The initial induction of *FT* genes in the leaf of the *veg2-2* mutant occurred at a similar time to wild-type plants (Figure 5.7B), suggesting that the time of arrival of the FT-based florigen signal is unlikely to be affected in the *veg2-2* mutant, as expected based on knowledge of *FD* and *FT* from *Arabidopsis* (Abe et al., 2005; Wigge et al., 2005). This conclusion is further supported by results from the grafting experiment in this chapter, in which grafting a *veg2-2* scion to wild-type graft stock did not rescue the late-flowering phenotype of the *veg2-2* mutant (Figure 5.2). The initial induction of *FTa1* and *FTa2* in the apex also occurred at a similar time as wild-type in the *veg2-2* mutant, but induction of *FTc* was delayed by approximately one week (Figure 5.7A). A reduced level of *FTc* expression was also observed in the *veg2-1* mutant (Figure 5.8), and previously *FTc* induction was shown to be delayed in the *gigas* mutant (Hecht et al., 2011). These findings support a model in which *FDa/VEG2* acts with one or more FT proteins (including *FTa1/GIGAS*) to upregulate expression of *FTc* within the plant apex, during the V/I<sub>1</sub> transition. In this manner, *FTc* is proposed to integrate signals from other FT family members (Hecht et al., 2011). Curiously, there was a dramatic increase in the expression level of *FTa1* in the leaf and apex and *FTb2* in the leaf in wild-type plants, after the appearance of flowers in the apex, which was delayed in the *veg2-2* mutant (Figure 5.7). It is possible that this may be part of a positive feedback loop to ensure the commitment to flowering or this could be linked to processes beyond flowering such as fruit and seed development. Alternatively, the increases in *FTa1* and *FTb2* expression observed in expanded leaves in this study may reflect the apical environment at the time of leaf development, which seems likely given that *FDa* is expressed in

developing leaf primordia but not in expanded leaves (Figure 5.1). A comparable increase in *FT* expression has been observed in cauline leaves in *Arabidopsis*, after the floral transition, and it is thought that these elevated *FT* levels prevent reversion from reproductive to vegetative development (Liu et al., 2014). Overall, results from this study indicate that *FDa/VEG2* acts upstream of *FTc* and may be part of auto-regulation of expression of *FTa1*, *FTa2* and *FTb2* in pea.

Induction of *DET* was delayed by approximately four weeks in the *veg2-2* mutant, which corresponded to the four week delay in the appearance of visible floral buds within the apex of the *veg2-2* mutant relative to wild-type (Figure 5.7A). As *DET* is a marker for  $I_1$  identity (Chapter 3; Berbel et al., 2012), this suggests that the  $V/I_1$  transition takes approximately four weeks longer to occur when *FDa* function is disrupted in the *veg2-2* mutant, relative to wild-type plants. The induction of *FTc*, prior to induction of *DET*, indicates that *FTc* may have a role during the  $V/I_1$  transition (Figure 5.7A). Previously, the importance of altered *DET* expression in the *veg2-1* and *veg2-2* mutants was discussed only in terms of meristem identity (Chapter 3). However, this finding also indicates that *FDa* normally acts in conjunction with an FT-based florigen signal to upregulate the expression of *DET* at the time of the  $V/I_1$  transition in wild-type plants, which is both a novel and significant finding. Transcriptional regulation of a *TFL1* homolog, by an FD homolog has not been reported in any other species. However, a recent computational model designed to simulate flowering in *Arabidopsis*, incorporated upregulation of *TFL1* by FT/FD as a modification to overcome issues with the model, which originally predicted termination of the *Arabidopsis* SAM in a flower (Jaeger et al., 2013). When *TFL1* was included as a target of FT/FD, SAM indeterminacy could be maintained during flowering (Jaeger et al., 2013). This is consistent with the observations that the level of *TFL1* expression in the *Arabidopsis* SAM is strongly upregulated at the onset of flowering and *TFL1* is expressed in proportion to *FT* (Jaeger et al., 2013). As *DET* is eventually upregulated in the *veg2-1* mutant (Figure 3.2), *DET* must also be regulated by an *FDa/VEG2*-independent pathway in pea. In *Arabidopsis*, *TFL1* is a direct target of *LFY* (Winter et al., 2011), and similarly in *Antirrhinum* upregulation of the *TFL1* ortholog *CEN* is dependent on *FLORICAULA* (*FLO*), the *LFY* ortholog (Bradley et al., 1996). *UNI* is expressed at a low level in the apex prior to flowering (Figure 5.7A), so it is

conceivable that *UNI* may be able to upregulate *DET* in the absence of functional *FDa/VEG2*. As flowering time does not appear to be affected in the *uni* mutant (personal observations), it is likely that any role for *UNI* in the V/I<sub>1</sub> transition would be redundant with *FDa/VEG2*, so this possibility should be investigated further in *veg2 uni* double mutant plants.

Expression of a second pea *TFL1* homolog, *LF*, was also altered in the *veg2* mutants, but *LF* showed an increased level of expression in both *veg2* mutants relative to wild-type (Figures 5.7A and 5.8). This suggests that *FDa/VEG2* normally represses *LF* expression in wild-type plants. It is particularly interesting that *FDa* appears to regulate two *TFL1* homologs in opposite ways: promoting induction of *DET* and repressing expression of *VEG1*. The exact mechanism for this regulation would be an interest focus for a future study.

These qRT-PCR results provide a useful insight into which genes are regulated (either directly or indirectly) by *FDa* in pea. It is possible that *FDa* protein acts in separate complexes with different FT proteins to upregulate target genes during pea inflorescence development. Direct targets of different pea florigen complexes could be identified by chromatin immunoprecipitation (ChIP). Alternatively, direct regulation of putative target genes could be confirmed by EMSA. This remains an area for future research interest.

#### 5.4.4 Conclusions

This chapter has focussed on the molecular mechanisms of *FDa* action during pea inflorescence development. The expression of *FDa* within the vegetative SAM, I<sub>1</sub> meristem, I<sub>2</sub> meristems and young floral meristems (Figure 5.1) is consistent with the roles of *FDa/VEG2* in the V/I<sub>1</sub> transition and I<sub>2</sub> and floral meristem specification, as characterised in Chapter 3. The results of BiFC analysis suggest that *FDa* may act in florigenic complexes with all pea FT proteins to promote I<sub>1</sub>, I<sub>2</sub> and floral identity, as well as in an antiflorigenic complex with *DET* to maintain SAM indeterminacy (Figure 5.6), which is similar to the systems characterised in other species (e.g. Wigge et al., 2005; Hanano and Goto, 2011; Tsuji et al., 2013a). Also similar to *FD* homologs in other species (e.g. Teper-Bamnolker and Samach, 2005; Danilevskaya et al., 2008b), *FDa* upregulates expression of a range of MADS-box genes in pea (Figures 5.7 and 5.8). However, *FDa* also regulates expression of *UNI*, *DET* and *LF*

(Figures 3.2, 5.7 and 5.8; directly or indirectly), and regulation of *LFY* or *TFL1* homologs has not previously been shown in any other species. Overall, the results from this chapter show that the mechanism of *FD* action is largely conserved between *Arabidopsis* and pea. Differences in the regulatory roles of *FD* genes between these species, including regulation of *VEG1* and *UNI*, and possibly also *LF* and *DET*, may contribute to the differences in inflorescence development that exist between *Arabidopsis* and pea.

## CHAPTER 6: Investigation of the *LATE5* locus

### 6.1 Introduction

The *LATE BLOOMER 5* (*LATE5*) locus has not previously been characterised in any detail. A single *late5* mutant allele was identified as one of several late-flowering mutants isolated in a relatively recent EMS mutagenesis screen of NGB5839 (Hecht et al., 2007; Weller, 2007). Preliminary observations indicated that *late5* was distinct in that this mutant also appeared to have mild inflorescence identity defects (Weller, 2007). Similar to *veg2-2*, the *late5* mutant exhibits delayed flowering, partial loss of I<sub>2</sub> identity and floral defects under both LD and SD photoperiods (Weller, 2007; Sussmilch, 2008).

#### 6.1.1 Genetic interactions

The genes involved in pea inflorescence development differ in their genetic interactions with *DET* and *LF*. *lf* is epistatic to *gigas* under both *LD* and *SD* conditions (Hecht et al., 2011; personal observation), but the relationship between *FTa1/GIGAS* and *DET* is photoperiod-dependent, possibly due to differences in the timing of the V/I<sub>1</sub> transition in the *gigas* mutant between photoperiods (Chapter 3). Under *LD* conditions, *det* is epistatic to *gigas*, but under *SD* conditions, the two loci have additive effects and double mutants flower late, similar to the *gigas* single mutant, but with a terminal I<sub>2</sub>, as seen in the *det* single mutant (Taylor, 1998). *veg2* is epistatic to both *lf* and *det* (Reid et al., 1996; Taylor, 1998; Sussmilch, 2008). Similarly, *veg1* is thought to be epistatic to *lf* as the *veg1 lf* double mutant could not be distinguished from the *veg1* single mutant (Reid and Murfet, 1984). However, the relationship between *VEG1* and *DET* is more interesting, as the main stem (I<sub>1</sub>) of *veg1 det* double mutants appears to terminate in a flower (Singer et al., 1994; Singer et al., 1999; Berbel et al., 2012). Similar to *veg2* and *veg1*, *late5* is also epistatic to *lf* and the *late5 lf* double mutant resembles the *late5* single mutant phenotype, except for a minor decrease in flowering time (Sussmilch, 2008). Initial observation of the *late5 det* double mutant, indicated that *LATE5* and *DET* interact in a similar manner to *VEG1* and *DET* (Sussmilch, 2008). In the *late5 det* double mutant, the main stem apex also appears to terminate in a flower and a similar phenotype was noted for the

*late5 lf det* triple mutant (Sussmilch, 2008). However, limited availability of *late5 det* and *late5 lf det* mutants prevented thorough investigation of these mutants.

### 6.1.2 Mapping of the *LATE5* locus

Results of preliminary linkage analysis, place *LATE5* close to where *VEG2/FDa* is located towards the base of linkage group I (Sussmilch, 2008; see Chapter 4). However, *LATE5* is not allelic with *VEG2/FDa* (Weller, 2007). Close synteny between pea and *Medicago* has previously enabled successful identification of candidate genes for pea mutant loci using available *Medicago* genome resources with a comparative mapping strategy (e.g. Hecht et al., 2007; Liew et al., 2009; Hecht et al., 2011). *LATE5* is located towards the base of pea linkage group I, which corresponds to a region towards the base of *Medicago* chromosome 5 (Aubert et al., 2006; Sussmilch, 2008; Bordat et al., 2011). *PsSVPa*, a pea *SVP*-like gene located in this region, was initially investigated and ruled out as a candidate gene for the *LATE5* locus (Sussmilch, 2008; see also Chapter 7).

### 6.1.3 Chapter aims

The overall aim for this chapter was to further investigate the *LATE5* locus, with particular focus on its roles, genetic interactions, and molecular identity. Firstly, some aspects of the *late5* single mutant phenotype were characterised in detail for the first time, including ontogenetic variation in internode length, branching, and  $I_2$  and floral morphology. Secondly, genetic interactions of *LATE5* with *DET* and *LF* were investigated through examination of *late5 det* and *late5 lf det* mutants and phenotypic comparisons with other mutant combinations. Thirdly, the location of *LATE5* within the flowering pathway was investigated through analysis of gene expression in the *late5* mutant. Lastly, attempts were made to identify the molecular nature of the *LATE5* locus, through a mapping and candidate gene-based approach.

## 6.2 Materials and methods

This section contains specific details of materials and methods for studies included in this chapter. General materials and methods also relevant to this chapter are described in Chapter 2.

### 6.2.1 Plant material, growth conditions and measurements

Details of the plant lines and growth conditions used for the experiments presented in this chapter are outlined in Table 6.1. Plants were either grown in the UTAS phytotron with total photoperiod comprising a base photoperiod of 8 hours of natural daylight extended with fluorescent light for longer photoperiods, or in controlled environment growth cabinets at 20°C under fluorescent light for the full photoperiod. Ontogenetic variation in internode length and branching were measured as described previously, in the same experiment presented in Chapter 3 (see Section 3.2.4). The length of the  $I_2$  axis was measured from the axil/ $I_2$  junction to the tip of the stub, on all determinate  $I_2$  structures present at nodes on the main stem at time of plant harvest (LD: 97 days after sowing; SD: 152 days after sowing). Seed for all double and triple mutants used during this study was obtained from previous experiments (Sussmilch, 2008), or was provided by J. Weller and J. Vander Schoor. The phenotypes of *veg1 det* and *veg1 lf det* mutants were observed in segregants grown for expression experiments performed as a contribution to collaborative works with A. Berbel, C. Ferrándiz and F. Madueño at the Universidad Politécnica de Valencia, Spain (Berbel et al., 2012). Photos of variation in  $I_2$  morphology in *det* and *lf det* mutants depict a combination of control plants and *det* and *lf det* segregants from NGB5839 x *veg1 det* and *veg1 det* x *lf* families which had no *veg1* segregants. Similar phenotypes were observed between *det* and *lf det* control plants and segregants. The *late5* x TER F<sub>2</sub> population used for fine-mapping of the *LATE5* locus (Section 6.3.4) was grown by J. Weller, J. Vander Schoor and S. Davidson prior to commencement of this study.

**Table 6.1.** Details of plant material and growth conditions used for experiments presented in Chapter 7. Number of plants (*n*) is shown. For the expression study (Section 6.3.3), *n* represents the number of plants at each time-point.

Purpose	Growth conditions	Chapter section(s)	Genotypes	<i>n</i>
Characterisation of ontogenetic variation in internode length, <i>l</i> <sub>2</sub> length, and branching	8h SD (Phytotron)	6.3.1.1	WT (NGB5839)	6
		6.3.1.2	<i>late5</i>	6
	24h LD (Phytotron)	6.3.1.1	WT (NGB5839)	6
		6.3.1.2 6.3.1.3	<i>late5</i>	6
Characterisation of <i>l</i> <sub>2</sub> determinacy	18h LD (Phytotron)	6.3.1.3	<i>late5</i>	6
Characterisation of floral morphology	24h LD (Phytotron)	6.3.1.4	<i>late5</i>	7
Characterisation of <i>l</i> <sub>2</sub> morphology in <i>det</i> and <i>lf det</i> mutants	18h LD (Phytotron)	6.3.2	<i>det</i>	23
			<i>lf det</i>	35
Characterisation of the interaction between <i>VEG1</i> and <i>DET</i>	18h LD (Phytotron)	6.3.2	WT (segregants)	45
			<i>det</i>	12
			<i>lf det</i>	34
			<i>veg1 det</i>	10
			<i>veg1 lf det</i>	26
Characterisation of the interaction between <i>LATE5</i> and <i>DET</i>	18h LD (Phytotron)	6.3.2	WT (NGB5839)	5
			<i>det</i>	37
			<i>lf det</i>	32
			<i>late5</i>	6
			<i>late5 det</i>	29
			<i>late5 lf</i>	5
			<i>late5 lf det</i>	25
Expression of flowering genes	24h LD (Cabinet)	6.3.3	WT (NGB5839)	4
			<i>late5</i>	4
Fine-mapping of the <i>late5</i> locus	18h LD (Phytotron)	6.3.4	<i>late5</i> x TER F <sub>2</sub>	79
			<i>late5</i> x 1794 F <sub>2</sub>	93
			<i>late5</i> x JI399 F <sub>2</sub>	184

### 6.2.2 qRT-PCR analysis

The qRT-PCR experiment presented in Section 6.3.3 is part of the same experiment involving a developmental series for *veg2-2* that was presented in Chapter 5. The details for this experiment are outlined in Section 5.2.1. qRT-PCR primer details are given in Appendix 1.



### 6.2.3 Genotyping for *VEG1*

*veg1 det* and *veg1 lf det* mutants were identified in populations segregating at the *VEG1* locus, by genotyping plants with PCR-based markers, as described in Section 3.2.2. *VEG1* PCRs were repeated 2-3 times in conjunction with control PCRs with primer pair PsTFL1a-1F and PsTFL1a-REV03 to confirm that the absence of *VEG1* PCR product was due to absence of the gene rather than PCR failure or poor DNA quality. Plants homozygous for *veg1* were further confirmed by distinct phenotype in a *det* and *lf det* background, whereby flowering time was delayed and normal I<sub>2</sub> structures were rarely observed (see Section 6.3.2).

### 6.2.4 Fine mapping of the *LATE5* locus

Prior to commencement of this study, DNA for the *late5* x TER F<sub>2</sub> population, comprising 79 plants, was extracted and markers for *AS2*, *SVPa*, *RGP*, R11-730, *FENR1*, *LATE5*, *AF*, *I* and Q20-950 were scored (Sussmilch, 2008). In addition, up to twelve progeny from each F<sub>2</sub> plant were grown to distinguish heterozygous from homozygous dominant individuals for loci with morphological markers: *LATE5* and *AF* (Sussmilch, 2008).

During this study, additional markers were designed for the *late5* x TER and *late5* x JI399 mapping populations. Pea sequence availability was extremely limited during early stages of this study. When pea sequence was not available, primers for isolation of putative marker loci were designed directly from *Medicago* sequence, in exon regions found to be conserved between *Medicago*, soybean and *Lotus*. Later, when pea TSA sequences became available (Franssen et al., 2011; Kaur et al., 2012), primers were designed directly from pea sequences identified in BLASTn searches using *Medicago* coding sequence against the GenBank TSA Sequence Database (<http://blast.ncbi.nlm.nih.gov>). The basic details of the markers included in Section 6.3.4 are given in Table 6.2. Full details of molecular markers are given in Appendix 1 (Table A1.4).

In the *late5* x JI399 F<sub>2</sub> mapping population, segregants were genotyped for *sn-1* in PCRs containing four primers: PsLUX-FLF, PsLUX-sn1.2, PsLUX-7R and PsLUX-8R (see Appendix 1 for details). PCRs yielded products of 600bp and 400bp for plants homozygous for *SN*, 600bp and 200bp for plants homozygous for *sn-1*, and 600bp, 400bp and 200bp for plants that were heterozygous at this locus.

**Table 6.2.** Marker loci on pea linkage group I used in Chapter 6. Primer details for molecular markers are given in Appendix 1 (Table A1.4).

Name	Corresponding <i>Medicago</i> locus (Mt3.5)	Population	Marker type	Source
<i>AF</i>	Medtr5g014400	<i>late5</i> x TER	Morphological	Weeden et al. (1993)
<i>AP2</i>	Medtr5g016810	<i>late5</i> x JI399	HRM	This study
<i>AP3</i>	Medtr5g021270	<i>late5</i> x JI399	CAPS	This study
<i>AS2</i>	Medtr5g071360	<i>late5</i> x TER	CAPS	Aubert et al. (2006)
		<i>late5</i> x JI399	HRM	This study
<i>CPK16</i>	Medtr5g022030	<i>late5</i> x JI399	HRM	This study
<i>DUF151</i>	Medtr5g023110	<i>late5</i> x TER	CAPS	This study
		<i>late5</i> x JI399	HRM	This study
<i>DUF343</i>	Medtr5g022760	<i>late5</i> x TER	HRM	This study
<i>EMB2754</i>	Medtr5g020140	<i>late5</i> x JI399	HRM	This study
<i>F-box</i>	Medtr5g025560	<i>late5</i> x TER	CAPS	This study
		<i>late5</i> x JI399	HRM	This study
<i>FENR1</i>	Medtr5g022300	<i>late5</i> x TER	CAPS	J. Hofer, unpublished
<i>FG-GAP</i>	Medtr5g021150	<i>late5</i> x JI399	HRM	This study
<i>GATA-TF</i>	Medtr5g021340	<i>late5</i> x JI399	dCAPS	This study
<i>I</i>	Medtr5g011120	<i>late5</i> x TER <i>late5</i> x JI399	Morphological	Weeden et al. (1993)
<i>LARP1C</i>	Medtr5g022790	<i>late5</i> x JI399	CAPS	This study
Q20-950	-	<i>late5</i> x TER	RAPD	Laucou et al. (1998)
R11-730	-	<i>late5</i> x TER	RAPD	Laucou et al. (1998)
<i>RGP</i>	Medtr5g046030	<i>late5</i> x TER	Size	Aubert et al. (2006)
		<i>late5</i> x JI399	HRM	This study
<i>RING finger</i>	Medtr5g022770	<i>late5</i> x JI399	HRM	This study
<i>SVPa</i>	Medtr5g032150/ Medtr5g032520	<i>late5</i> x TER	dCAPS	Sussmilch (2008)
		<i>late5</i> x JI399	HRM	This study
<i>SVPc</i>	Medtr5g066180	<i>late5</i> x TER	CAPS	This study
		<i>late5</i> x JI399	HRM	
<i>UNK1</i>	Medtr5g016100	<i>late5</i> x TER	dCAPS	This study
		<i>late5</i> x JI399	dCAPS	This study
<i>WUS</i>	Medtr5g021930	<i>late5</i> x JI399	HRM	This study

To identify candidate genes for *LATE5*, the syntenic regions were identified in soybean and common bean genomes by identifying homologs of the flanking markers *FENR1* and *GATA-TF*, by reciprocal blast searches between *Medicago*, soybean and common bean (Table 6.3). BLASTx searches using the intervening regions on soybean chromosome 16 (Gm16:27560086-28259800) and common bean chromosome 2 (Chr02:21838334-22846653) in 25kb blocks as queries, were performed against *Arabidopsis* proteins at TAIR.

**Table 6.3.** Homologs of the marker loci flanking *LATE5* on pea linkage group I. The top hits from reciprocal blast searches are given.

Name	<i>Medicago</i> locus (Mt3.5)	<i>P. vulgaris</i> locus (v1.0)	<i>G. max</i> locus (v1.1)
<i>FENR1</i>	Medtr5g022300	Phvul.002G108000	Glyma16g23710
<i>GATA-TF</i>	Medtr5g021340	Phvul.002G112000	Glyma16g24381

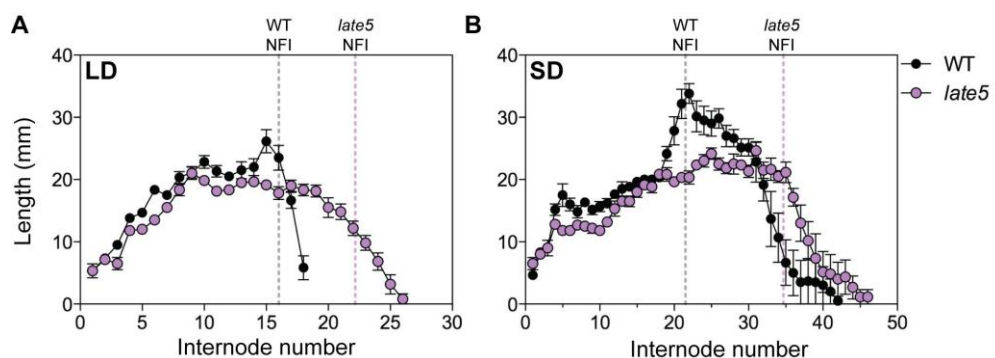
## 6.3 Results

### 6.3.1 Further investigation of *late5* mutant morphology

#### 6.3.1.1 Ontogenetic variation in internode length

The delay in flowering time described for the *late5* mutant, suggests that the V/I<sub>1</sub> transition may be delayed in this mutant (Weller, 2007; Sussmilch, 2008). In Chapter 3, ontogenetic variation in internode length was altered in mutants exhibiting altered timing of expression of *DET*, a marker for I<sub>1</sub> identity, suggesting that internode length may reflect differences in timing of the V/I<sub>1</sub> transition (Figures 3.3 and 3.4). In this study, internode length was measured in *late5* mutants under LD and SD photoperiods.

In the *late5* mutant, the node of floral initiation was delayed relative to wild-type plants in both LD (mean NFI  $\pm$  SE: wild-type =  $16.0 \pm 0.0$ , *late5* =  $22.2 \pm 0.2$ ) and SD conditions (mean NFI  $\pm$  SE: wild-type =  $21.5 \pm 0.4$ , *late5* =  $34.7 \pm 1.2$ ). Figure 6.1 shows that in wild-type plants, internode length decreased at the onset of flowering, as previously described (Chapter 3; Ross et al., 1992), and this decrease was delayed in the *late5* mutant. As seen for *veg2-2*, the decrease in internode length in *late5* preceded the NFI under LD conditions (Figure 6.1A). These results suggest the V/I<sub>1</sub> transition may be delayed in the *late5* mutant, indicating that *LATE5* may have an important role during the V/I<sub>1</sub> transition under both LD and SD photoperiods.



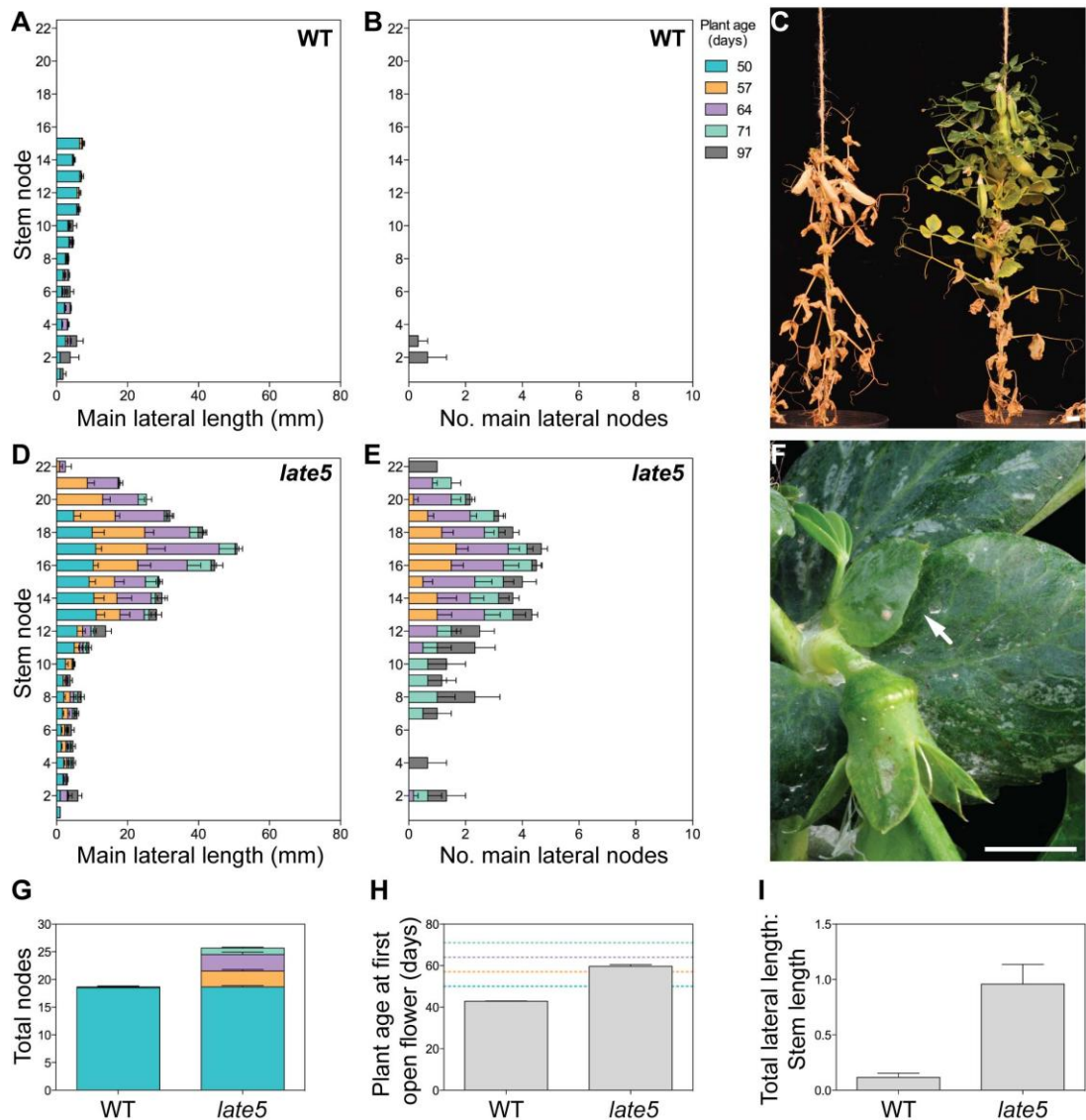
**Figure 6.1.** Ontogenetic variation in internode length as an indicator for relative timing of the V/I<sub>1</sub> transition in the late-flowering *late5* mutant.

(A-B) Ontogenetic variation in internode length in wild-type (NGB5839) and *late5* mutant plants under (A) LD (24h) and (B) SD (8h) conditions. Internodes are numbered with internode 1 between the first and second scale leaf. Data points represent mean  $\pm$  standard error for  $n = 6$  plants per genotype. Broken vertical lines indicate mean node of floral initiation for each genotype.

### 6.3.1.2 *Lateral outgrowth*

Increased axillary branching has previously been noted as a feature of the *late5* mutant, as well as several other late- and non-flowering pea mutants, and in deflowered wild-type pea plants (see Chapter 3; Sussmilch, 2008), but branching has not previously been characterised in detail in the *late5* mutant. In this study, lateral outgrowth was measured in the *late5* mutant under both LD and SD conditions.

Overall, *late5* mutants exhibited significantly more branching than wild-type plants under LD conditions (Figure 6.2I;  $p = 0.004$ ). Wild-type plants exhibited some early outgrowth of axillary buds (Figure 6.2A), but there was a tendency for these buds to become dormant and die without developing into branches with expanded leaves (Figure 6.2B). In contrast, axillary outgrowth was more sustained in the *late5* mutant, and axillary branches with expanded leaves were more likely to develop (Figure 6.2D-E). In *late5*, lateral outgrowth began at aerial nodes, sometimes at multiple nodes simultaneously, and radiated to higher and lower nodes with time (Figure 6.2D-E), as previously described for other mutants (Chapter 3). In some *late5* plants, branches were borne as additional axillary structures at flowering nodes, which also bore  $I_2$  structures (Figure 6.2F).



**Figure 6.2.** Lateral outgrowth in the *late5* mutant under LD conditions.

(A, B, D, E) Graphical representations of lateral development based on measurements taken at weekly time-points (50-71 days after sowing) and at plant harvest (97 days after sowing) in (A-B) wild-type (NGB5839) and (D-E) *late5* mutant plants. (A, D) Increase in length of the main lateral at each node. (B, E) Increase in number of nodes on the main lateral at each node. Stem node (main stem) is shown on the y-axis and branch length on the x-axis to represent an upright plant.

(C) Photo of wild-type (left) and *late5* mutant plants (right), shown 93 days after sowing.

(F) Photo of a branch developing as a second axillary structure (arrow), next to an  $I_2$  on the main stem of a *late5* mutant plant, shown 73 days after sowing.

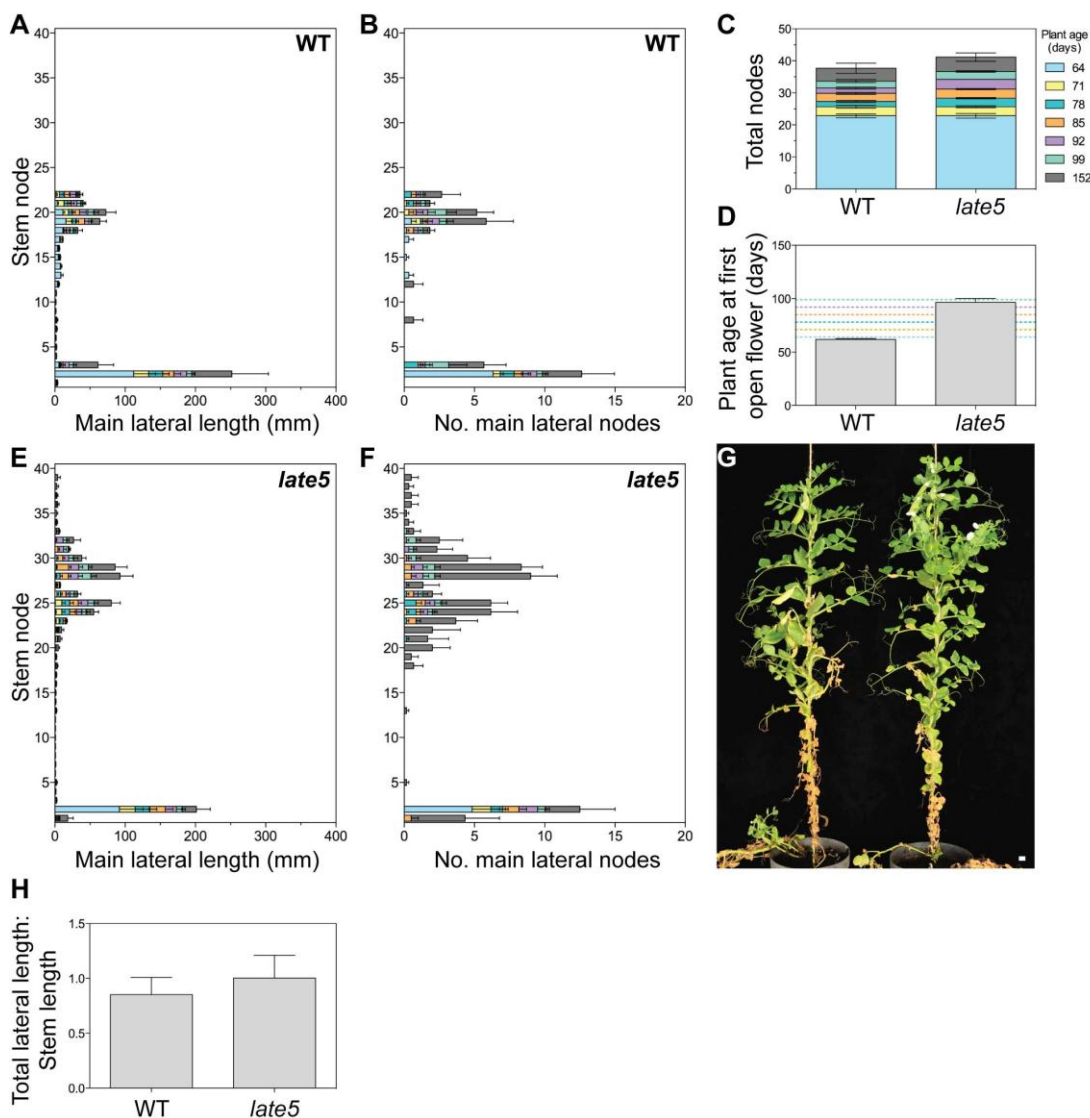
(G) Total nodes with expanded leaves at each time-point used for branching measurements, as an indicator for relative timing of plant senescence. Colours correspond to time-points in (A, B, D, E, H).

(H) Flowering time measured as plant age at first open flower. Horizontal lines indicate weekly time-points in (A, B, D, E, G).

(I) Ratio of total length of all laterals greater than 5mm in length, divided by stem length, as measured at time of plant harvest (97 days after sowing).

In all graphs, mean values  $\pm$  standard error for  $n = 6$  plants are shown.  $I_2$  structures are not included in lateral measurements.

Figure 6.3 shows that under SD conditions, the overall branching phenotype of the *late5* mutant was similar to wild-type. Both wild-type and *late5* mutant plants exhibited branch development at aerial and basal nodes (Figure 6.3A, B, E, F). There was no significant difference in the ratio of total lateral length to stem length between wild-type and *late5* plants under SD conditions (Figure 6.3H;  $p = 0.574$ ).



**Figure 6.3.** Lateral outgrowth in the *late5* mutant under SD conditions.

(A, B, E, F) Graphical representations of lateral development based on measurements taken at weekly time-points (64-99 days after sowing) and at plant harvest (152 days after sowing) in (A-B) wild-type (NGB5839) and (E-F) *late5* mutant plants. (A, E) Increase in length of the main lateral at each node. (B, F) Increase in number of nodes on the main lateral at each node. Stem node (main stem) is shown on the y-axis and branch length on the x-axis to represent an upright plant.

(C) Total nodes with expanded leaves at each time-point used for branching measurements, as an indicator for relative timing of plant senescence. Colours correspond to time-points in (A, B, D, E, F).

(D) Flowering time measured as plant age at first open flower. Horizontal lines indicate weekly time-points in (A, B, C, E, F). (continued next page)

**Figure 6.3. (continued) (G)** Photo of wild-type (left) and *late5* mutant plants (right), shown 113 days after sowing.

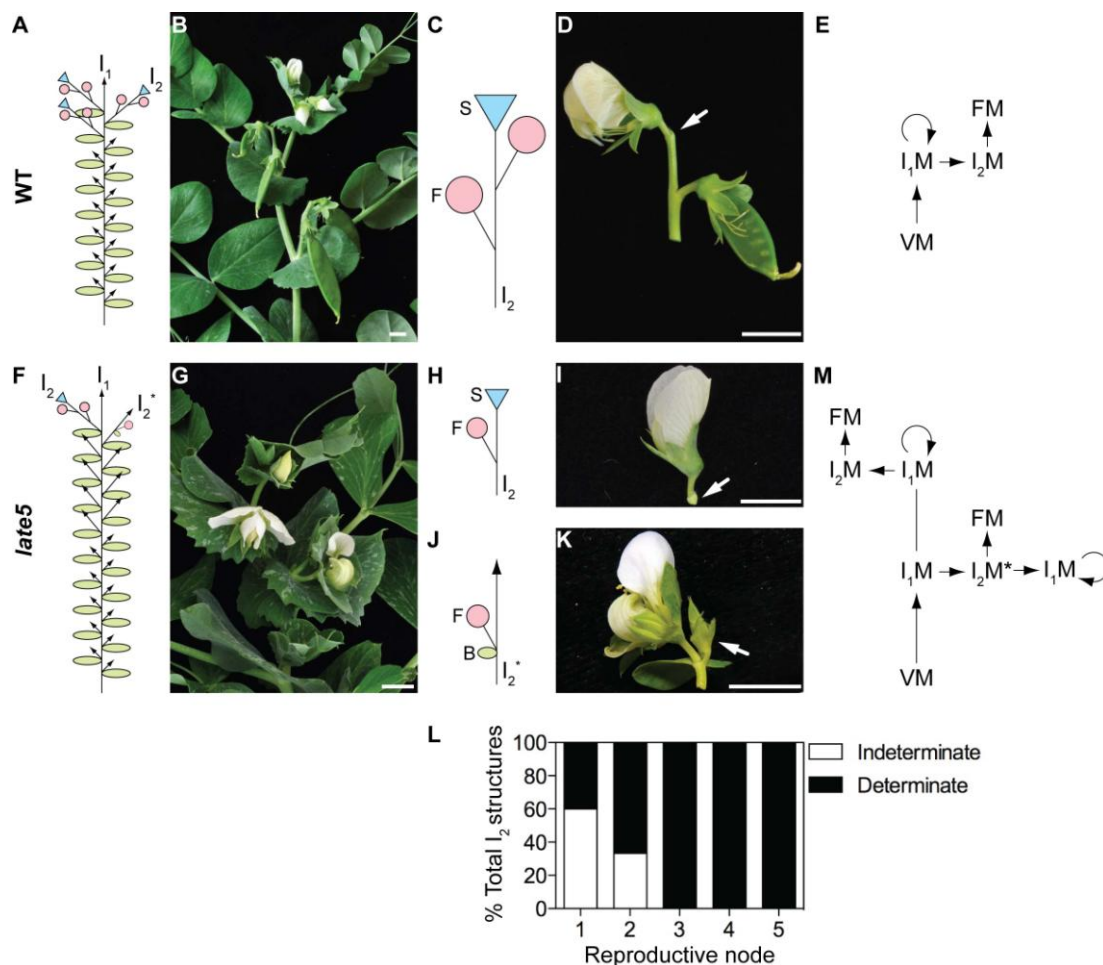
**(H)** Ratio of total length of all laterals greater than 5mm in length, divided by stem length, as measured at time of plant harvest (152 days after sowing).

In all graphs, mean values  $\pm$  standard error for  $n = 6$  plants are shown.

### 6.3.1.3 *I<sub>2</sub>* morphology

The previously reported *I<sub>2</sub>* identity defects in the *late5* mutant were next examined in more detail. In wild-type plants, each *I<sub>2</sub>* exhibits complete suppression of leaves, bears one or two axillary flowers, and terminates in a hairy stub (Figure 6.4C-D). In the *late5* mutant, the majority of *I<sub>2</sub>* structures were normal (Figure 6.4H-I), but the *I<sub>2</sub>* structure(s) at the first one or two reproductive nodes showed abnormal, indeterminate features superficially resembling those in the *veg2-2* mutant (Figure 6.4J-L). Like wild-type, abnormal *I<sub>2</sub>* structures bore axillary flowers at the first one or two nodes, but these flowers usually had abnormal morphology and were subtended by stipule-like bracts comprising leaf tissue but lacking the rachis and leaflets of a normal compound leaf (Figure 6.4J-K). Rather than terminating in a stub, these abnormal *I<sub>2</sub>* structures retained an indeterminate apex that continued to develop, producing full compound leaves. Interestingly, this inflorescence defect was not seen in all *late5* mutant plants, and some plants exhibited normal, determinate *I<sub>2</sub>* structures at all reproductive nodes (Figure 6.4L). Curiously, determinate *I<sub>2</sub>* structures in the *late5* mutant appeared to be consistently shorter than wild-type *I<sub>2</sub>* structures (Figure 6.4D and I).





**Figure 6.4.** *I*<sub>2</sub> morphology in the *late5* mutant.

(A-E) The wild-type pea inflorescence. (A) Diagram of wild-type plant architecture with the primary inflorescence (*I*<sub>1</sub>) and secondary inflorescence (*I*<sub>2</sub>) indicated. (B) Photo of reproductive nodes on the wild-type stem. (C) Diagram and (D) photo of the wild-type *I*<sub>2</sub> which bears axillary flowers (F) and terminates in a stub (S; arrow in photo). (E) Schematic of meristem transitions involved in wild-type inflorescence development.

(F-M) The *late5* mutant inflorescence. (F) Diagram of plant architecture in the *late5* mutant. (G) Photo of reproductive nodes on the *late5* stem. (H) Diagram and (I) photo of a determinate *I*<sub>2</sub> structure, seen at later flowering nodes of *late5*, which has normal *I*<sub>2</sub> morphology and terminates in a stub (arrow). (J) Diagram and (K) photo of an indeterminate *I*<sub>2</sub> structure, which has an abnormal flower and subtending bract (B) at the first node, and has retained an indeterminate apex (arrow in photo). (L) Graph showing the percentage of *I*<sub>2</sub> structures that are indeterminate or determinate at each reproductive node on the main stem for *n* = 6 *late5* mutant plants under LD conditions. The node of floral initiation corresponds to reproductive node 1. (M) Schematic of meristem identity transitions during inflorescence development in the *late5* mutant, inferred from morphology.

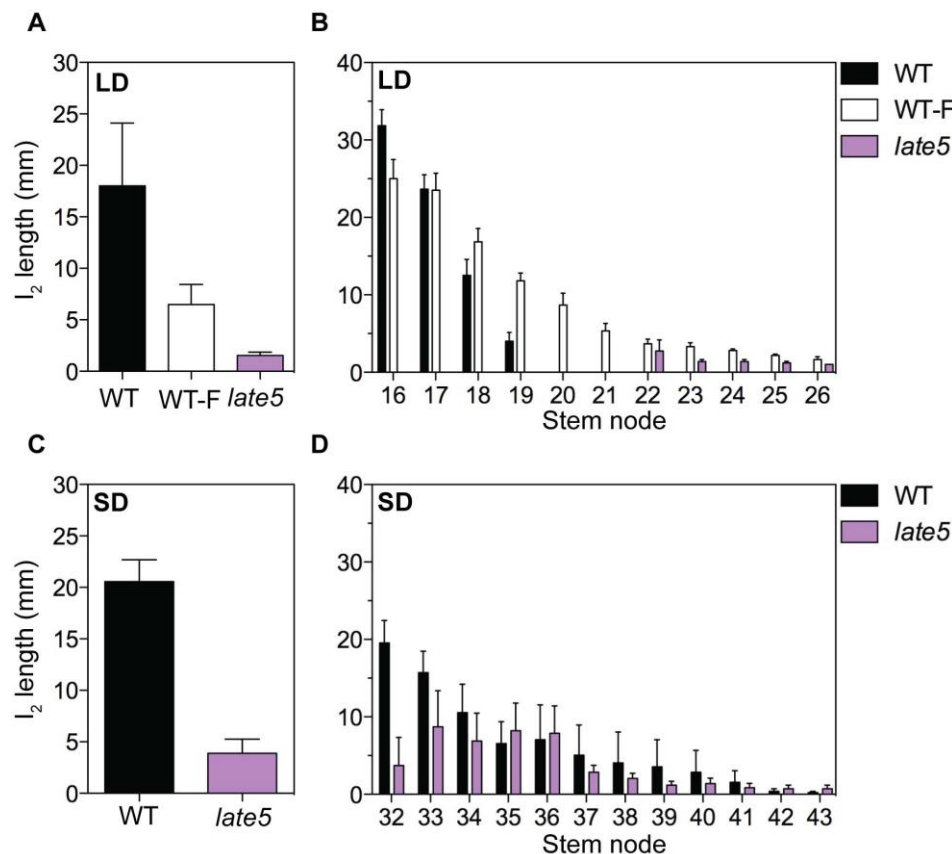
In diagrams, arrows indicate indeterminate growth, circles are flowers, triangles are terminal stubs, and ovals are leaves or bracts. Asterisks indicate abnormal nature of structures.

In photos, scale bars indicate 1 cm.

In schematics, meristems are: vegetative meristem (VM), primary inflorescence meristem (*I*<sub>1</sub>M), secondary inflorescence meristem (*I*<sub>2</sub>M), floral meristem (FM). Straight arrows indicate meristem transitions and products. Circular arrows indicate meristem indeterminacy. Straight lines lacking arrow heads indicate continued growth.



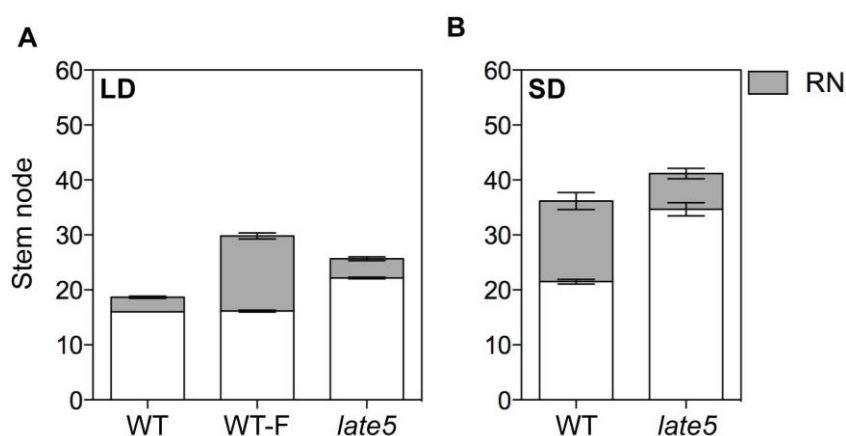
Next,  $I_2$  length was characterised in more detail in the *late5* mutant under both LD and SD conditions by measuring the length of determinate  $I_2$  structures from the  $I_2$ /axil junction to the tip of the  $I_2$  stub. Under both photoperiods, the mean length of all  $I_2$  structures on the main stem, was significantly higher in wild-type than *late5* mutant plants (Figure 6.5A and C;  $p < 0.000$ ). However, in wild-type plants, the length of  $I_2$  structures decreased at higher reproductive nodes (Figure 6.5B and D), and flowering is delayed in the *late5* mutant, thus  $I_2$  structures begin at a later node in the *late5* mutant, than in wild-type (Figure 6.6). Wild-type plants cease apical growth at a node lower than the first flowering node of *late5* plants, so the range of  $I_2$ -bearing nodes does not overlap between these genotypes (Figure 6.6A). To investigate the possibility that the  $I_2$  length in the *late5* mutant could be similar to that of wild-type at corresponding nodes, flowers on wild-type plants were removed after anthesis, which delayed senescence and resulted in continued production of  $I_2$ -bearing nodes (Figure 6.6A). As predicted,  $I_2$  length was similar between  $I_2$  structures borne at the same node in deflowered wild-type plants and intact *late5* mutant plants, under LD conditions (Figure 6.5B). In SD conditions, the delay in senescence of wild-type plants allowed a direct comparison with *late5*, and  $I_2$  length was again similar for  $I_2$  structures borne at corresponding nodes in the two genotypes (Figures 6.5D and 6.6B).



**Figure 6.5.** I<sub>2</sub> length in the *late5* mutant.

Characterisation of I<sub>2</sub> length in intact wild-type (WT; NGB5839), deflowered wild-type (WT-F; NGB5839; LD only) and *late5* mutant plants grown under (A-B) 24h LD or (C-D) 8h SD conditions. (A, C) Mean length of all determinate I<sub>2</sub> structures on the main stem of wild-type and *late5* plants. (B, D) Mean length of determinate I<sub>2</sub> structures at each stem node. Only stem nodes with structures present in more than one plant group are shown.

Mean values  $\pm$  standard error for  $n = 6$  plants are shown.



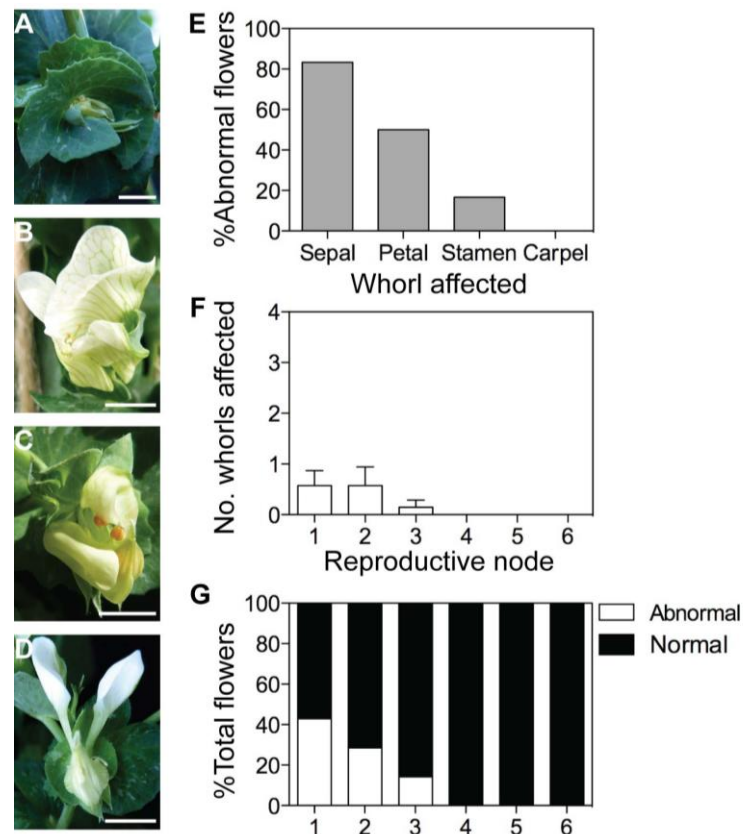
**Figure 6.6.** Location of reproductive nodes in the *late5* mutant, relative to wild-type.

The reproductive nodes (grey) between the mean node of floral initiation and mean total nodes are indicated for wild-type (WT; NGB5839), deflowered wild-type (WT-F; NGB5839; LD only) and *late5* mutant plants grown under (A) 24h LD or (B) 8h SD conditions.

Mean values  $\pm$  standard error for  $n = 6$  plants are shown.

### 6.3.1.4 *Floral morphology*

Floral defects were previously noted as another feature of the *late5* mutant (Weller, 2007; Sussmilch, 2008), and these floral defects were characterised in more detail in this study. Flowers in the *late5* mutant grown under LD conditions ranged from completely normal to severely abnormal (Figures 6.4G, 6.7A-D). In abnormal flowers, floral defects most commonly affected the outer sepal and petal whorls (Figure 6.7E). Defects in the stamen whorl were also seen occasionally (Figure 6.7E). Common defects included fusion to leaf or petal tissue, displacement of floral organs and a reduced number of floral organs (Figure 6.7A-D). Both the occurrence and severity of floral defects was greater at early reproductive nodes, and decreased until all flowers were normal at higher reproductive nodes (Figure 6.7F-G).



**Figure 6.7.** Floral morphology in the *late5* mutant under LD conditions.

(A-D) Flowers from *late5* mutant plants exhibiting a range of floral defects. (E) Percentage of abnormal flowers defective in each of the four floral whorls. (F) Mean number of whorls affected by floral defects at each reproductive node. Values represent mean  $\pm$  standard error. (G) Percentage of total flowers exhibiting floral defects at each reproductive node.

This figure is based on characterisation of all flowers borne at reproductive nodes on the main stem of 7 *late5* plants (31 flowers total). For (E)  $n = 6$  abnormal flowers, for (F-G)  $n = 1-7$  flowers per reproductive node.

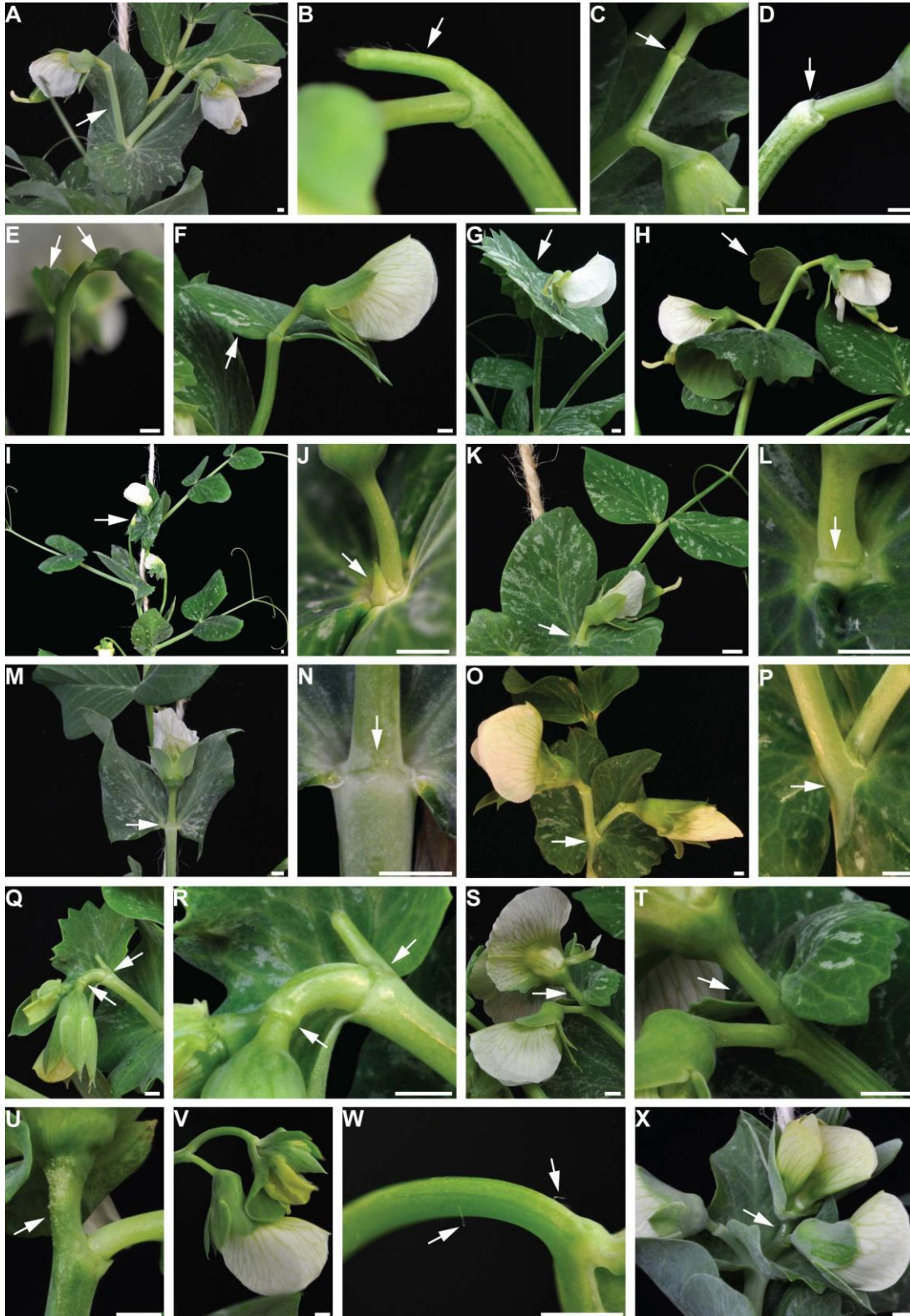
### 6.3.2 Investigation of genetic interactions with *DET*

Preliminary observations of the *late5 det* double mutant and *late5 lf det* triple mutant indicated an interesting relationship between *LATE5* and *DET*. However, the number of mutants previously obtained among F<sub>2</sub> segregants was extremely limited, preventing thorough investigation. In both *late5 det* and *late5 lf det* mutants, the main stem apex appeared to terminate in a flower (Sussmilch, 2008). A similar phenotype has previously been described for the *veg1 det* double mutant (Singer et al., 1999; Berbel et al., 2012). In this study, the *late5 det* double mutant and *late5 lf det* triple mutant were observed more closely in comparison to *det*, *lf det*, *veg1 det* and *veg1 lf det* mutants.

#### 6.3.2.1 Morphology of *det* and *lf det* mutants

*DET* and *LF* exhibit an additive genetic relationship, whereby the only phenotypic difference between *det* and *lf det* mutant phenotypes is flowering time (Murfet, 1989b). The *det* mutant flowers at the same time as wild-type plants, while the *lf det* mutant flowers earlier, at the same time as *lf* mutant plants (Foucher et al., 2003). In both the *det* single mutant and the *lf det* double mutant, one or more axillary I<sub>2</sub> structures are produced before the main stem (I<sub>1</sub>) terminates in an ectopically placed I<sub>2</sub> (Figure 6.8A; Murfet, 1989a; Singer et al., 1990). Flowering branches, which develop after the termination of the main stem, have the same fate as the main stem. In this study, considerable variation was observed in I<sub>2</sub> structures in *det* and *lf det* mutant plants, with respect to both the appearance of the end of I<sub>2</sub> structures and to the suppression of leaf tissue. I<sub>2</sub> structures normally end in a determinate stub which protrudes after the last I<sub>2</sub> node, and is covered in small epidermal hairs (Figure 6.8B). In *det* and *lf det* mutant plants, the length of the terminal stub was seen to vary from an obvious, protruding stub to a subtle, reduced ring of I<sub>2</sub> tissue at the I<sub>2</sub>/pedicel junction (Figure 6.8B-D). Some I<sub>2</sub> structures exhibited complete suppression of laminae (Figure 6.8A-D), as is seen in wild-type plants (Figure 6.4C-D). Other I<sub>2</sub> structures bore bracts that ranged in size from small bracts to large circles of leaf tissue that enclosed the stem (Figure 6.8E-H). These bracts were particularly common at nodes on the terminal I<sub>2</sub>, but were also noted on I<sub>2</sub> structures at other reproductive nodes. Bract-like organs usually had serrated margins (Figure 6.8E-H), similar to the base of the stipules which attach to the stem

in a normal compound leaf (Figure 6.8A). In some cases a full compound leaf, including stipules, rachis and leaflets, was present subtending the flower borne on the terminal  $I_2$ , which gave the appearance that the main stem terminated in a flower (Figure 6.8I, K and M). However, in each case observed in this study, a stub or a ring of tissue was visible at the point of leaf attachment to the stem (Figure 6.8J, L and N). There is no ring or junction present on a normal stem node where a leaf is borne in wild-type, or at the point where the  $I_1$  changes to an ectopic  $I_2$  in *det* or *lf det* mutant plants (Figure 6.8O-P). The presence of a stub or a ring of tissue indicated that the main stem terminated in an  $I_2$  with an axillary flower, rather than a terminal flower. Curiously, in place of a flower, the terminal  $I_2$  structures in some mutant plants produced an axillary  $I_3$  structure that had the same appearance as an  $I_2$ , and bore axillary flowers (Figure 6.8Q-R). In contrast, some axillary  $I_2$  structures appeared to terminate in a flower instead of a stub, with no ring separating pedicel tissue from the  $I_2$  (Figure 6.8S-X). The pedicels of these flowers were sometimes thick, like an  $I_2$  rather than a pedicel, and often bore a bract (Figure 6.8S-T) or a small bump (Figure 6.8U). In other flowers that appeared to terminate  $I_2$  structures, the pedicels lacked these features but had hairs that are reminiscent of the hairs present on a normal pea  $I_2$  stub (Figure 6.8B, V-X). These observations indicate that *det* and *lf det* plants can appear to terminate in flowers, with only subtle signs that they actually terminate in ectopic  $I_2$  structures, and that axillary  $I_2$  structures themselves, can end in flowers, rather than stubs in these genotypes.



**Figure 6.8.** Variation in  $I_2$  morphology observed in *det* and *lf det* mutant plants.

(A) Axillary and terminal (arrow)  $I_2$  structures.

(B) Protruding stub (arrow) of an  $I_2$ .

(C-D) Ring of  $I_2$  tissue (arrow) surrounding the last flower produced on an  $I_2$ .

(E-H) Bracts (arrows) of various size present at  $I_2$  nodes. **(continued next page)**

**Figure 6.8. (continued) (I-N)** Terminal  $I_2$  structures, each with a full compound leaf at the  $I_2$  node, giving the appearance of a terminal flower, except for the presence of **(I-J)** a distinctive stub (arrow) or **(K-N)** a ring of  $I_2$  tissue (arrows) at the junction between the  $I_2$  and the floral pedicel. In **(M-N)** stipule tissue was cut away to show the ring of  $I_2$  tissue.

**(O-P)** The point of attachment of the last leaf on the  $I_1$ , where the  $I_1$  becomes an ectopic  $I_2$  in *det* and *lf det* mutant plants. This photo shows that there is no ring around the stem at normal stem nodes (arrows).

**(Q-R)** An  $I_2$  with an axillary  $I_3$ , borne in place of a flower on a terminal  $I_2$ .

**(S-X)**  $I_2$  structures from *det* and *lf det* mutant plants, each with a flower that appears to terminate the  $I_2$ . **(S-T)** Terminal flower with a bract present on the floral pedicel (arrow). **(U)** Terminal flower with a small bump (arrow) present on the pedicel. **(V-X)** Terminal flowers with no bumps or bracts but hairs (arrows) present on the pedicel. The pedicel in **(V)** is shown magnified in **(W)**.

Photos depict either *det* or *lf det* mutant plants. Similar characteristics were seen in both mutants. Scale bars indicate 2mm.

#### 6.3.2.2 *Phenotypic characterisation of veg1 det and veg1 lf det mutants*

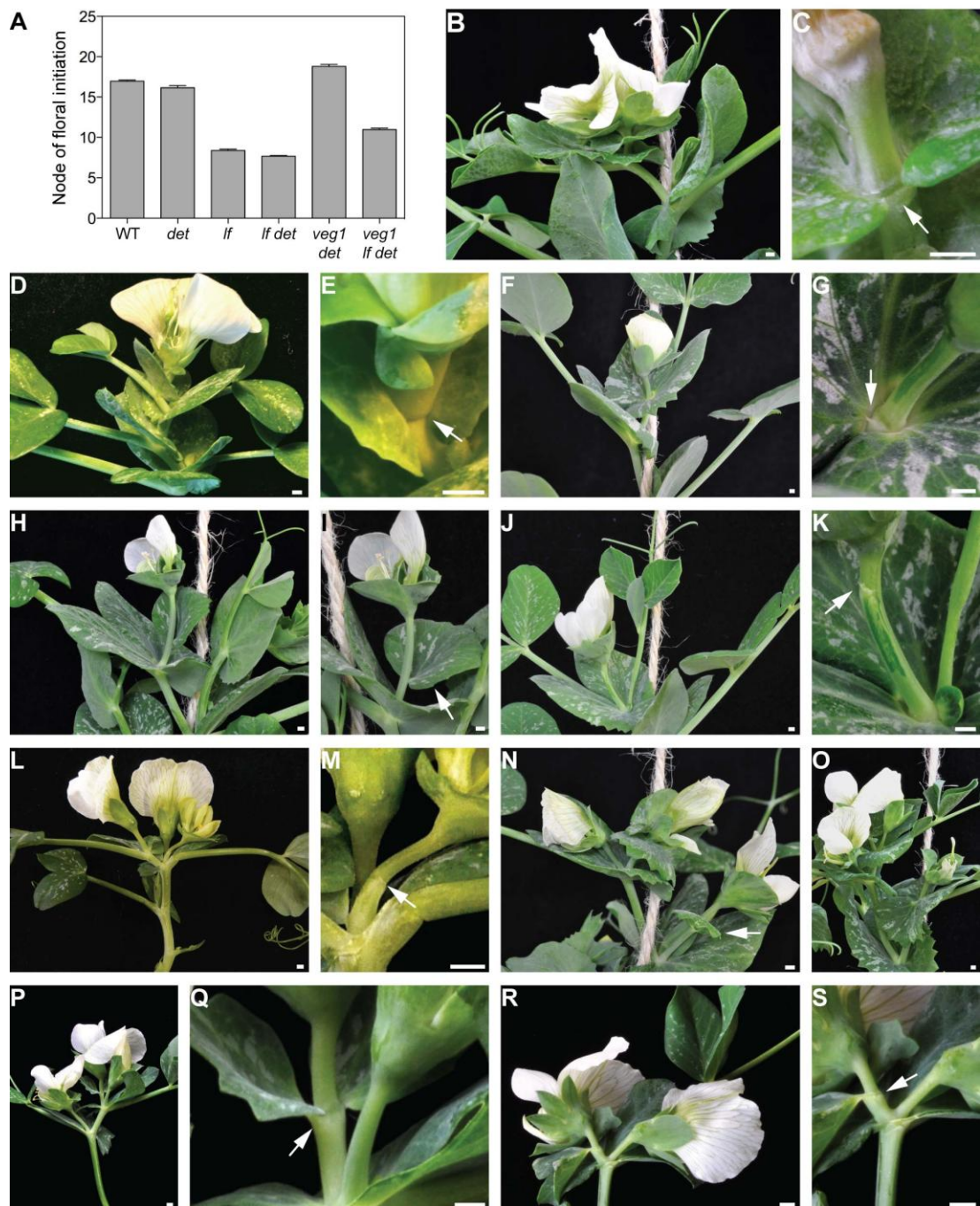
The *veg1 det* double mutant has been described previously as the only pea mutant in which the main stem terminates in a flower (Singer et al., 1999). The phenotype of *veg1 det* was revisited in this study, and *veg1 lf det* phenotype was also characterised in detail for the first time. In *veg1 det*, the main stem and flowering branches, which share the same fate as the main stem, all showed determinate growth (Figure 6.9B, D). A single flower, or two flowers fused at the pedicel, appeared to terminate the main stem and branches (Figure 6.9B, D). However, in some plants observed in this study, an  $I_2$  stub or ring of tissue was visible at the junction between the floral pedicel and the stem (Figure 6.9C, E), similar to observations for some terminal  $I_2$  structures in *det* and *lf det* mutant plants (Figure 6.8J, L, N).

Similarly, in *veg1 lf det* triple mutant plants, the main stem and flowering branches showed determinate growth with one or two flowers borne at the end of the structure (Figure 6.9F, H). However, once again a small hairy stub or ring of tissue was often present at the junction between the floral pedicel and the stem (Figure 6.9G, Q, S), indicating that the stem terminated in the rudimentary stub of an  $I_2$ . In other cases, a bract or bump was associated with the floral pedicel, or the pedicel itself was unusually long and covered in small hairs (Figure 6.9H-K). This was similar to those observations in some *det* and *lf det* mutants, as described above (Figure 6.8S-W). Occasionally, a bract present on the floral pedicel spiralled around the pedicel and became the sepal whorl for the flower (Figure 6.9N), suggesting an incomplete specification of the boundary between  $I_2$  structure and flower. In *veg1 lf det* triple mutant plants, there was often more than one reproductive node on each

shoot axis. In such cases, the node of floral initiation bore an  $I_2$ -like structure with axillary flowers subtended by bracts or full leaves, and a final flower, often with a visible ring of tissue separating the pedicel from the ' $I_2$ ' axis (Figure 6.9N-Q). On some branches, relatively normal  $I_2$  structures were present, ending in a ring of tissue with no leaf or bract subtending the flower (Figure 6.9L-M).

These observations indicate that *veg1 det* and *veg1 lf det* mutants may exhibit conversion of the  $I_1$  to an abnormal, terminal  $I_2$ , rather than a terminal flower. This suggests that *veg1* alters the severity but not the fundamental nature of the *det* and *lf det* phenotypes. This provides a reference point for examination of *LATE5* interactions with *DET* and *LF*.





**Figure 6.9.** Characterisation of *veg1 det* and *veg1 lf det* mutant phenotypes.

(A) Node of floral initiation in single, double and triple mutants and wild-type plants grown under LD (18h) conditions. Values represent mean  $\pm$  standard error for 6-45 plants per genotype.

(B-E) Photos of the *veg1 det* mutant phenotype. (B) Two flowers fused at the pedicel, appearing to terminate the main stem. (C) A ring of tissue (arrow) at the junction between a floral pedicel and the main stem. (D-E) A single flower appearing to terminate a flowering branch, but with a ring of tissue (arrow) at the junction between floral pedicel and branch stem. (continued next page)

**Figure 6.9. (continued) (F-S)** Photos of the *veg1 lf det* mutant phenotype. **(F-G)** A flower appearing to terminate the main stem, but with a small, hairy stub (arrow) visible at the point of pedicel attachment. **(H-I)** A flower on the main stem, with a bract (arrow) on the ‘pedicel’. **(J-K)** A flower on the main stem, with a stub-like bump on the ‘pedicel’. **(L-M)** A flowering branch with an axillary  $I_2$  (arrow pointing to  $I_2$  ring) at the first reproductive node. Stipules have been cut away from the branch stem for clarity. **(N)** The main stem with a flower that has a bract (arrow) on the pedicel at the first reproductive node, an axillary flower at the second reproductive node and a flower that appears to be terminal. A bract on the pedicel of the last flower spirals around the pedicel to become the sepal whorl of the flower. **(O-Q)** An  $I_2$ -like structure at the NFI with axillary flowers subtended by full compound leaves at the first two nodes of the structure and a third flower subtended by a bract with a visible ring of tissue separating the pedicel from the branch stem (arrow). **(R-S)** A flowering branch with several nodes bearing axillary buds (not shown), followed by an axillary flower subtended by a full compound leaf and a flower subtended by a bract. A ring of tissue is visible at the junction between the pedicel of the last flower and the branch stem (arrow). Stipule and bract tissue have been cut away from the branch stem for clarity.

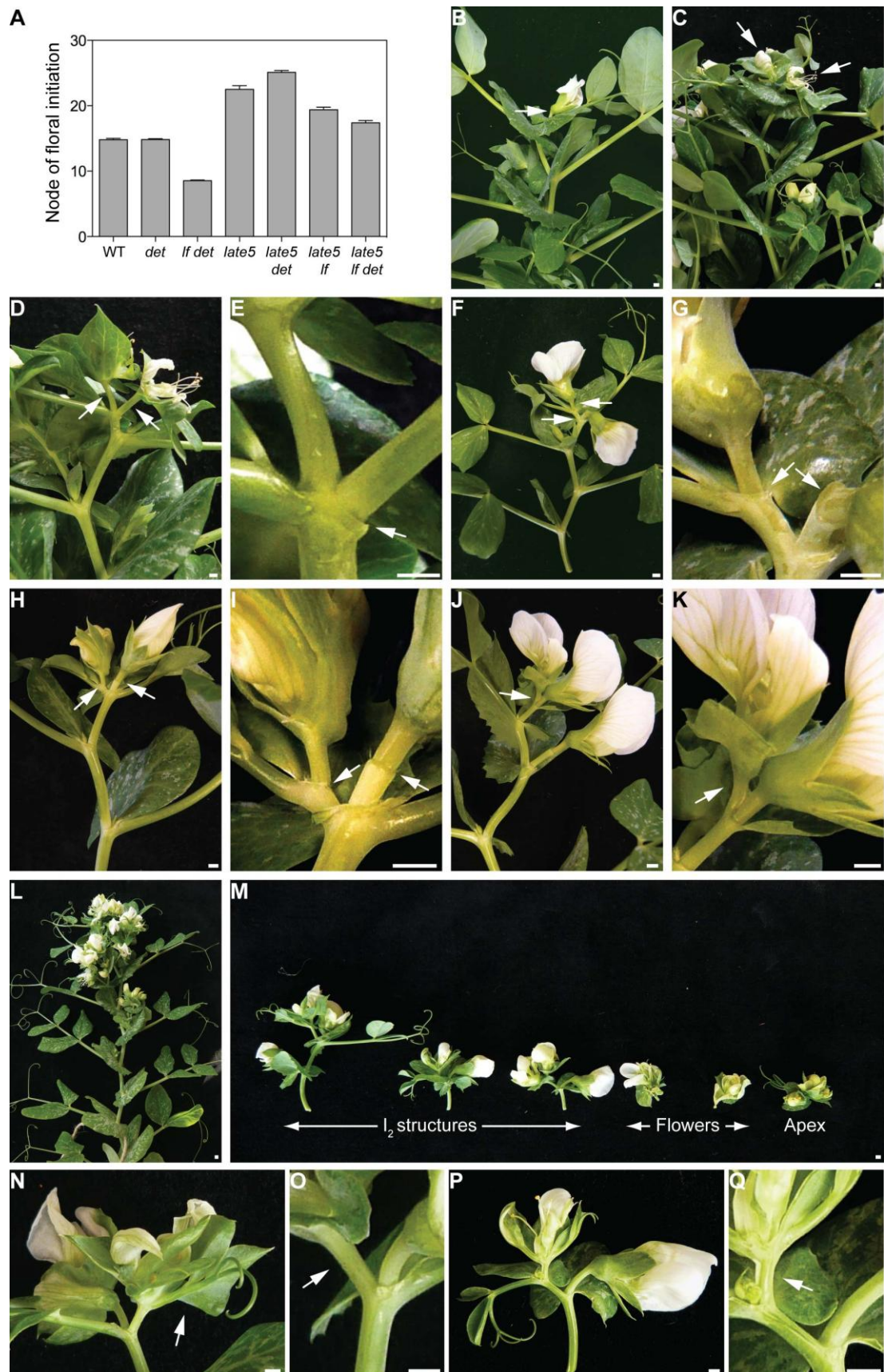
Scale bars indicate 2mm.

### 6.3.2.3 *Phenotypic characterisation of late5 det and late5 lf det mutants*

Similar to the *late5* single mutant, *late5 det* and *late5 lf det* mutants flowered later than wild-type plants (Figure 6.10A). As for *veg1 det*, the main stem and flowering branches of *late5 det* mutant plants appeared to terminate in a single flower, or two flowers coming from the same node (Figure 6.10B-C). However, closer inspection revealed that these flowers were usually separated from the main stem by a ring of tissue (Figure 6.10D-I). Again, this was similar to some terminal  $I_2$  structures seen in *det* and *lf det* mutants, indicating that the main stem of these *late5 det* plants terminated in an  $I_2$  rather than a flower. In addition, some reproductive nodes on flowering branches of *late5 det* plants bore relatively normal  $I_2$  structures that terminated in a ring of tissue and lacked bracts/leaves (Figure 6.10F-J). Again, similar to observations in *det* and *lf det*, some  $I_2$  structures on flowering branches of *late5 det* plants appeared to terminate in a flower instead of a stub (Figure 6.10J-K).

*late5 lf det* mutant plants generally had numerous reproductive nodes on the main stem, gradually increasing in determinacy from  $I_2$  structures to axillary flowers (Figure 6.10L-M). The  $I_2$  structures had axillary flowers subtended by bracts or full leaves and often terminated in a flower with no visible ring of tissue, bump or bract on the pedicel (Figure 6.10N-Q). The pedicel of this terminal flower was often thick, and the sepal whorl usually had leaf-like characteristics (Figure 6.10M). Overall, these observations suggest that the *late5 det* and *late5 lf det* mutants exhibit a more severe version of the terminal  $I_2$  phenotype exhibited by *det* and *lf det* mutant plants.





**Figure 6.10.** Characterisation of *late5 det* and *late5 lf det* mutant phenotypes. (continued next page)

**Figure 6.10. (continued)** (A) The node of floral initiation in single, double and triple mutants and wild-type plants grown under 18h LD conditions. Values represent mean  $\pm$  standard error for 5-37 plants per genotype.

(B-K) Photos of the *late5 det* mutant phenotype. (B) A single flower (arrow) appearing to terminate the main stem. (C-D) Two flowers (arrows) appearing to terminate the main stem, but (E) a ring of tissue was present (arrow) separating the pedicels from the main stem tissue, indicating that neither flower was truly terminal. (F-I) Two flowering branches, both with several vegetative nodes, followed by an axillary  $I_2$  (arrow) and an axillary flower with the floral pedicel separated from the branch stem by a ring of tissue (arrow). (J-K) A flowering branch with an axillary  $I_2$  followed by a terminal  $I_2$ . The terminal  $I_2$  appears to terminate in a flower instead of an  $I_2$  ring or stub. The pedicel of this flower is fused to the pedicel of an axillary flower.

(L-Q) Photos of the *late5 lf det* mutant phenotype. (L) Reproductive nodes on the main stem. (M) Axillary structures with increasing determinacy, removed from the six reproductive nodes on the main stem of one plant. Structures comprised three axillary ' $I_2$ ' structures each with axillary flowers borne before a flower appeared to terminate the structure, two axillary flowers, and two flowers borne at the apex. (N-O) An  $I_2$  structure with an axillary flower subtended by a full compound leaf (cut away for clarity), followed by a flower that appeared to terminate the  $I_2$  structure (no ring of tissue separating pedicel from stem; arrow). This flower had a sepal whorl with compound leaf characteristics including two stipule like structures and tendrils (arrow). (P-Q) An  $I_2$  structure with an axillary flower subtended by a bract (cut away for clarity), followed by a flower bud subtended by a full compound leaf (cut away), followed by a flower (front cut away) that appeared to terminate the  $I_2$  structure (no ring of tissue separating pedicel from stem; arrow).

Scale bars in photos indicate 2mm.

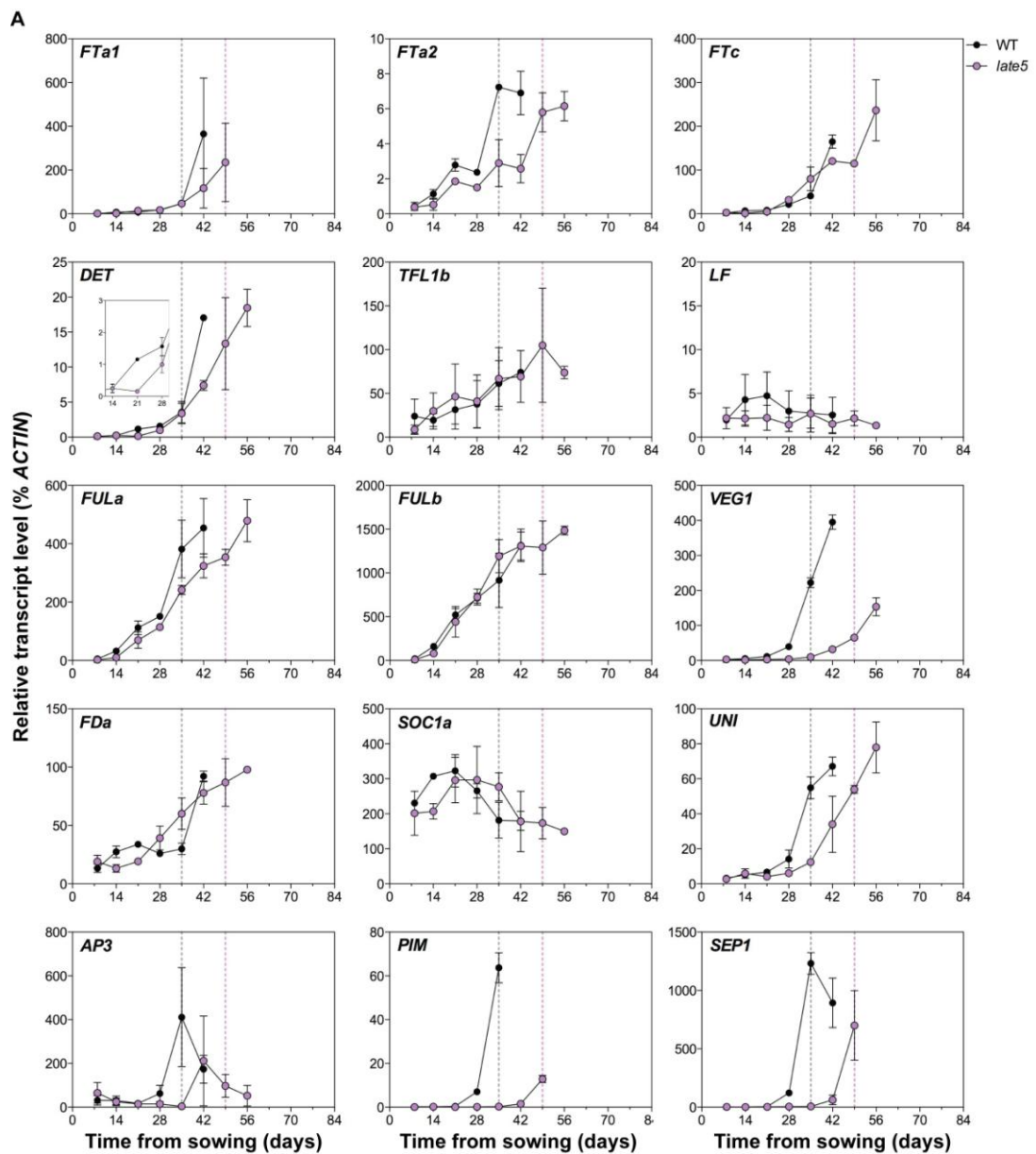
### 6.3.3 Identification of genes regulated by *LATE5*

In order to identify the flowering genes that are regulated directly or indirectly by *LATE5*, gene expression was investigated in the *late5* mutant under LD conditions by qRT-PCR. As for the *veg2-2* mutant (Chapter 5, Figure 5.7), expression was investigated using a developmental series of apical and leaf tissue taken at time-points covering seedling emergence until after flowering in adult plants.

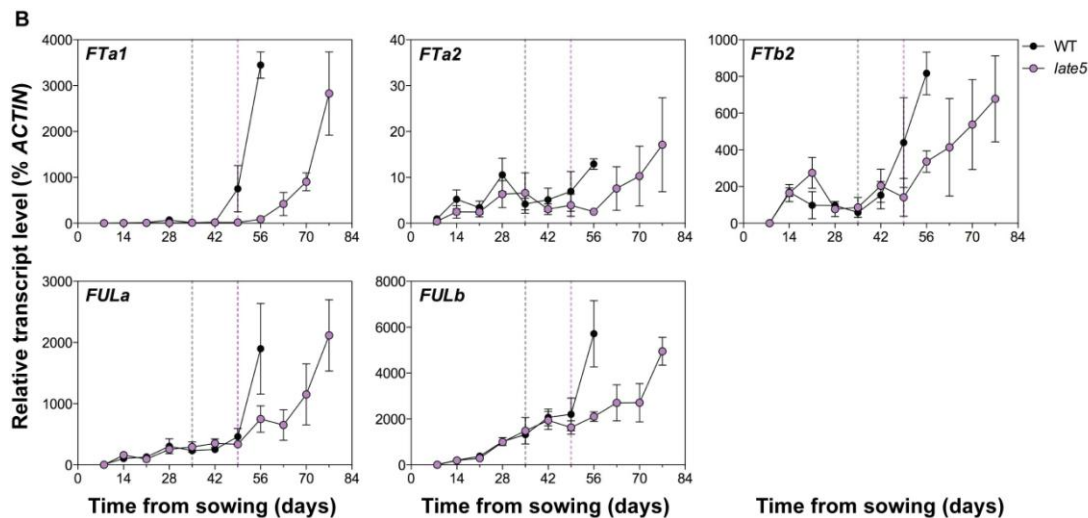
Floral buds were first macroscopically visible in the apex 35 days after sowing in wild-type and 49 days after sowing in *late5* (Figure 6.11). This was reflected by apical expression of floral identity genes *PIM* and *SEP1*, which were induced a week before developing floral buds were first visible in each genotype (Figure 6.11A). *VEG1*, *UNI* and *AP3* showed a similar one to two week delay in upregulation in the *late5* mutant (Figure 6.11A). Both the initial induction of *SOC1a* expression in the apex prior to flowering and its decrease at the onset of flowering were offset by approximately one week in the *late5* mutant, relative to wild-type (Figure 6.11A). The initial upregulation of *DET* was similarly delayed by one week in the *late5* mutant (Figure 6.11A). *FTa2* and *FULa* showed consistently lower expression in *late5* than in wild-type at most time-points, but showed similar timing of induction (Figure 6.11A). Curiously, expression of *FDa* was somewhat reduced in the *late5*

mutant at 14 to 21 days after sowing (Figure 6.11A), which is approximately the time at which the commitment to flowering occurs in wild-type (Hecht et al., 2011). The expression of *FTa1*, *FTc*, *TFL1b*, *LF* and *FULb* was comparable between *late5* and wild-type (Figure 6.11A).

In the leaf, the initial induction of *FTb2*, *FTa2*, *FULA* and *FULb* was comparable between *late5* and wild-type (Figure 6.11B). However, the increased expression of flowering genes including *FTa1*, *FTb2*, *FULA* and *FULb* during pod development, was delayed in accordance with flowering time in the *late5* mutant (Figure 6.11B).



**Figure 6.11.** Expression of flowering genes in the *late5* mutant. (continued next page)



**Figure 6.11. (continued)** Gene expression in wild-type (NGB5839; black circles) and the *late5* mutant (purple circles) in (A) the dissected shoot apex and (B) the uppermost fully expanded leaf, during plant development under a LD (24h) photoperiod. Values have been normalised to the transcript level of *ACTIN* and represent mean  $\pm$  standard error for  $n = 2$  biological replicates, each consisting of pooled material from two plants. Developing floral buds were first macroscopically visible in the wild-type apex 35 days after sowing (grey line) and first visible in the *late5* apex 49 days after sowing (purple line). For *DET*, initial time-points are magnified inset to show early induction which is masked by high expression levels during later floral development.

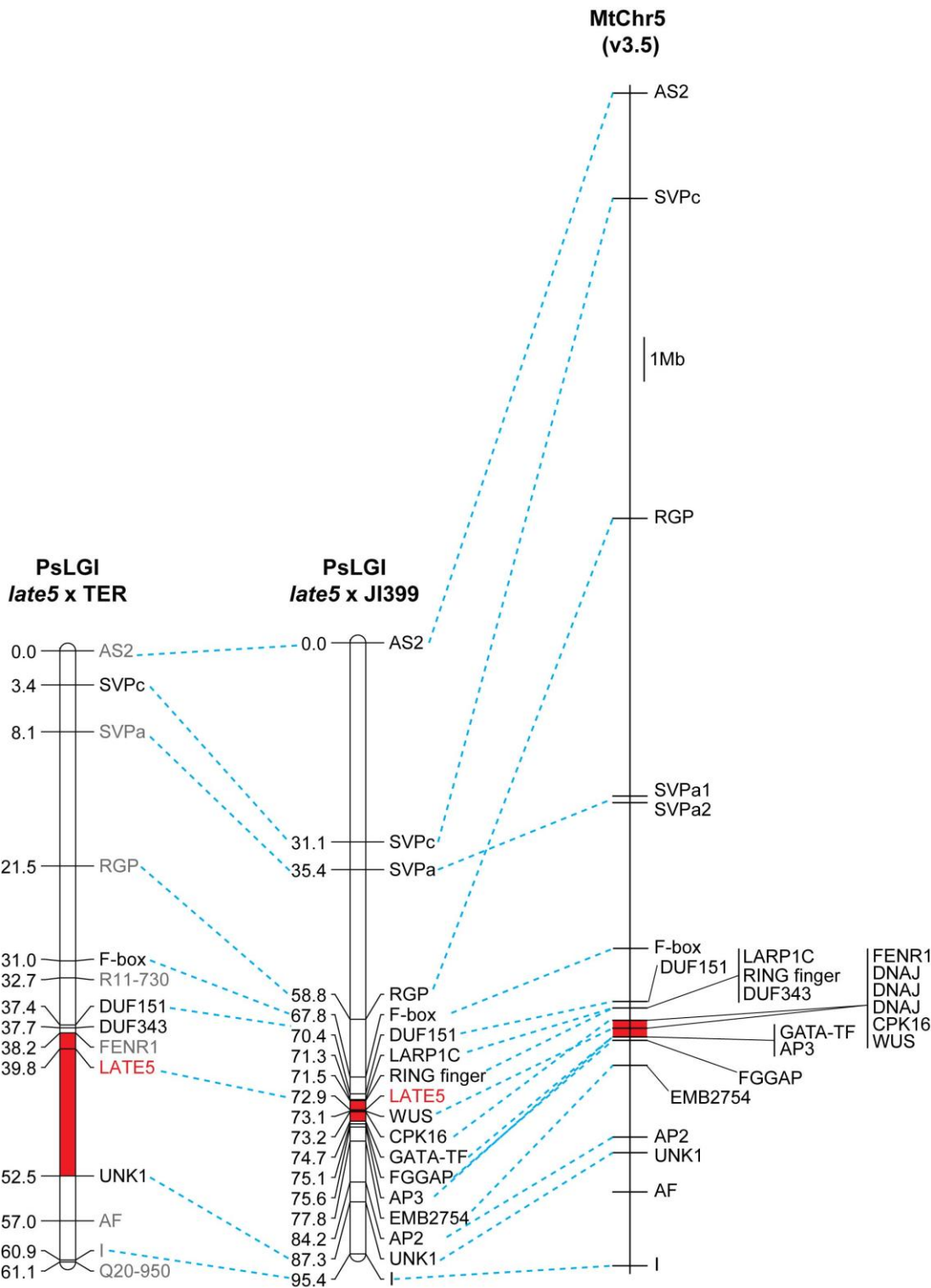
### 6.3.4 Fine-mapping of the *LATE5* locus

The *LATE5* locus was previously mapped to the base of pea linkage group I, close to *FDa* (Weller, 2007; Sussmilch, 2008). In this study, the map position of *LATE5* was refined in order to assist the identification of candidate genes. Initial mapping of *LATE5* utilised existing DNA of an  $F_2$  population comprising 79 plants from a narrow cross between *late5* (NGB5839 background) and cv. T  r  se (*late5* x TER population; Sussmilch, 2008). Putative marker loci were selected from the syntenic region of *Medicago* chromosome 5 that were located in the region of interest, contained introns and were single copy in *Medicago*. These loci were isolated in pea, sequenced in NGB5839 and T  r  se, and where polymorphisms were present, these were used for molecular marker design and scored in the population. In this manner, five new markers were designed and mapped in the existing *late5* x TER population (Figure 6.12). This narrowed the map position of *LATE5* down to a 14.3cM region on pea linkage group I, between the flanking markers *FENR1* and *UNK1* (Figure 6.12). Recombination events were detected between *LATE5* and both *FENR1* and *UNK1*. To expand this mapping population, more crosses were made between *late5* and T  r  se and 85 new  $F_2$  plants were grown. However, concurrent attempts to identify loci suitable for molecular marker design between *FENR1* and



*UNK1*, based on the position of corresponding genes in *Medicago*, revealed a surprising lack of polymorphisms between the parental lines in this region. More than 68 introns from 14 genes were isolated and sequenced but no polymorphisms were found between NGB5839 and Térèse (see Appendix 1, Table A1.5). A wide cross between *late5* and *Pisum elatius* var. *humile* line JI1794 was performed to improve the likelihood of polymorphisms for design of molecular markers. However, background segregation of other flowering loci, including *HR* (Weller et al., 2012), coupled with subtlety of the *late5* phenotype made it difficult to score *LATE5* as a morphological marker in the F<sub>2</sub> progeny with sufficient confidence. Finally, an intermediate cross between *late5* and JI399 (Cennia) was performed to generate an F<sub>2</sub> population comprising 184 individuals. This population was also segregating for *SN*, as JI399 is homozygous for the *sn-1* allele (Liew et al., 2014). *late5* segregants could be clearly distinguished in this F<sub>2</sub> population on the basis of flowering time (Figure 6.13). Genotyping for the *sn-1* allele confirmed that *late5* mutant phenotype was clear on an *sn* background (Figure 6.13).

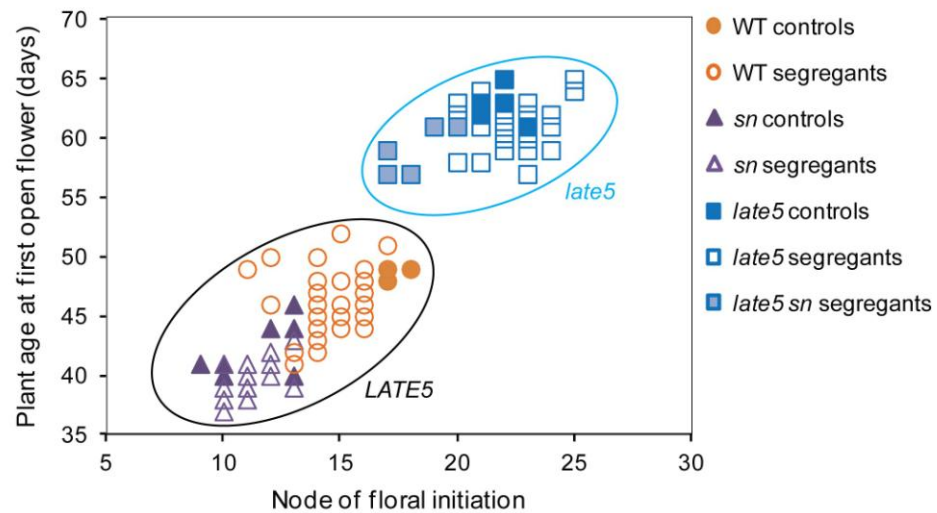
Many polymorphisms were identified between parental lines NGB5839 and JI399, in loci between *FENR1* and *UNK1*, which enabled design of new molecular markers for this population. Through repeated rounds of marker design and linkage analysis, the map position of the *LATE5* locus was refined to a 3.2cM region towards the base of pea linkage group I between the marker loci *RING finger* and *GATA-TF* (Figure 6.12). This corresponds to a syntenic region of 0.8Mb containing approximately 142 genes in *Medicago* (Figure 6.12). One marker, *CPK16*, was found to be closely linked to *LATE5*, with no recombinants obtained between these loci (Figure 6.12). As mapping in the *late5* x TER population indicated that *LATE5* is downstream of *FENR1*, with one recombinant present between *LATE5* and *FENR1*, the location of *LATE5* could be narrowed down to the region between *FENR1* and *GATA-TF* (Figure 6.12). The syntenic region between *Medicago FENR1* and *GATA-TF* comprises 0.6Mb, containing 95 annotated genes (Figure 6.12). BLAST searches of this genome region in *Medicago* and corresponding regions on soybean chromosome 16 and common bean chromosome 2 were conducted against *Arabidopsis*, to identify any non-annotated homologs of known *Arabidopsis* flowering genes, but none were present in the region between these flanking markers.



**Figure 6.12.** Comparative map between the region of linkage group I in pea containing *LATE5* and the corresponding region on chromosome 5 in *Medicago*.

Numbers in pea linkage maps indicate cM distances between loci, estimated from segregation data using JoinMap software (v4; Kyazma B.V., Wageningen, Netherlands). The physical map shown for *Medicago* is based on genome build Mt3.5 and details of *Medicago* loci are given in Table 6.2. Where possible, genes that correspond between maps are linked with broken blue lines. The region between the markers flanking the *LATE5* locus is highlighted in red in each map. Loci shown in grey in the *late5* x TER population were mapped in a previous study (Sussmilch, 2008).





**Figure 6.13.** Segregation at the *LATE5* locus in the  $F_2$  progeny of a cross between *late5* (NGB5839 background) and JI399.

Plants homozygous for the *late5* mutant allele were distinguished from plants homozygous for *LATE5* and plants heterozygous for this locus, based on flowering time. *late5* mutant phenotype was confirmed by inflorescence abnormalities (where present) and decreased  $I_2$  length (data not shown). Segregants homozygous for the *sn-1* allele were identified by genotyping. Plants were grown under 18h LD conditions. The  $F_2$  progeny comprised 184 plants. Data for 6-8 control plants for each genotype are also shown.

Homologs of *AP3* and *AP2*, which are both floral homeotic genes, were found to be present downstream of *GATA-TF* on *Medicago* chromosome 5. To determine if the pea orthologs of these genes were in the same position or slightly upstream, in the region of the *LATE5* locus, portions of these genes were isolated from pea and mapped. *AP3* and *AP2* both mapped downstream of *LATE5* towards the base of pea linkage group I (Figure 6.12). Recombination between *LATE5* and both *AP3* and *AP2* indicated that neither of these genes were suitable candidates for *LATE5*.

Although there were no clear candidates for *LATE5* in the syntenic region between *FENR1* and *GATA-TF* in *Medicago*, some interesting genes were identified (Table 6.4). The *Medicago* homolog of *WUS*, a gene with an important role in meristem maintenance, is present in this region (Table 6.4; Chen et al., 2009; Tadege et al., 2011). Partial sequence for the pea ortholog of this gene was identified in online resources (GenBank: FG529966), and intron sequence was isolated for molecular marker design. Mapping in the *late5* x JI399  $F_2$  population confirmed that *LATE5* is tightly linked to *WUS* and no recombinants were obtained between these loci (Figure 6.12). Other genes of interest in this region include three genes which show homology to *Arabidopsis DNA J PROTEIN C24* (*DJC24*) and *DJC23* (Table

6.4), which are part of the same J-domain protein family as J3, a protein that promotes flowering in *Arabidopsis* (Shen et al., 2011). Further progress towards identifying the *LATE5* gene could not be made within the time-frame of this study, but these genes are worth investigation as possible candidates in a future study.

**Table 6.4.** Genes of interest in the region of *Medicago* chromosome 5 syntenic to the location of *LATE5* on pea linkage group I. The top BLASTp hits of *Medicago* predicted protein sequence against *Arabidopsis* proteins at TAIR are given. Percentage identity at the amino acid level is indicated for each *Medicago* protein, compared to *Arabidopsis* BLASTp hits.

<b><i>Medicago</i> locus (Mt3.5)</b>	<b><i>Arabidopsis</i> locus (TAIR10)</b>	<b>Gene name</b>	<b>%</b>
Medtr5g022210	AT2G17880	DJC24	44
	AT4G36040	DJC23	43
Medtr5g022180	AT2G17880	DJC24	44
	AT4G36040	DJC23	41
Medtr5g022130	AT2G17880	DJC24	47
	AT4G36040	DJC23	44
Medtr5g021930	AT2G17950	WUS	35

## 6.4 Discussion

### 6.4.1 Further investigation of *late5* mutant morphology

Previously, details of the *late5* mutant phenotype were limited to basic observations. In this study, characteristics of the *late5* mutant were investigated in more detail, to examine the role of *LATE5* during pea inflorescence development.

The *late5* mutant is late flowering in both SD and LD photoperiods (Figures 6.2H and 6.3D), suggesting a delay in the V/I<sub>1</sub> transition, and this is supported by observations on other markers for this transition including *DET* expression and ontogenetic variation in internode length (Figures 6.1 and 6.11A). Expression of the most likely florigen gene, *Ftb2*, in the leaf is not affected in the *late5* mutant (Figure 6.11B), suggesting that *LATE5* does not influence the upstream signals that trigger the V/I<sub>1</sub> transition. Instead, these findings support a model wherein *LATE5* has an important role during the V/I<sub>1</sub> transition in pea.

Flowering time and *DET* induction can be compared directly between *veg2-2* and *late5*, as both genotypes are in the same background (NGB5839) and were included in (a) the same branching experiment that included flowering time measurements (Figures 3.7, 6.2H and 6.3D), and (b) the same developmental expression experiment that was used for qRT-PCR (Figures 5.7 and 6.11). Relative to *late5*, flowering is approximately two weeks later and *DET* induction is approximately two to three weeks later in the *veg2-2* mutant (Figures 3.7, 5.7A, 6.2H, 6.3D and 6.11A). A larger delay in *veg2-2* than *late5* could indicate one of two things: (i) *LATE5* may be less important than *FDa/VEG2* during the V/I<sub>1</sub> transition, or (ii) the mutation in the *late5* mutant may have a less severe impact on gene expression or protein function than the mutation in the *veg2-2* mutant. Future identification of the gene affected in the *late5* mutant would allow further insight into this difference in severity of phenotype.

The *late5* mutant exhibits I<sub>2</sub> identity defects that are similar to those observed in the *veg2-2* mutant, whereby partial I<sub>2</sub> identity is initially specified but then I<sub>1</sub> meristem identity is apparently acquired, and an indeterminate apex is retained (Figures 3.16 and 6.4). Unlike *veg2-2* mutants, these defects were found to be transient in *late5*, affecting only the first one or two reproductive nodes of each plant, and some *late5* mutants did not exhibit these defects at any node (Figure 6.4). These

observations suggest that *LATE5* has a similar role to *FDa/VEG2* in the specification and maintenance of  $I_2$  identity, but the role of *LATE5* may be transient and/or partially redundant.

Characterisation of floral defects in the *late5* mutant also revealed similarities with those observed in the *veg2-2* mutant, including fusion to leaf or petal tissue and displacement or a reduced number of floral organs, most commonly affecting the sepal and petal whorls, and exhibiting an acropetal decrease in severity (Figures 3.19 and 6.7). This indicates that *LATE5* has a similar role to *FDa/VEG2* in determining correct floral development at early reproductive nodes, possibly through regulation of the same MADS-box genes involved in specification of floral organs. It is interesting that the floral defects in both *late5* and *veg2-2* mutants only affect the first few floral nodes, and it is possible that there is an important age-related factor that affects floral development, with the result that *LATE5* and *FDa/VEG2* have only transient roles in this process.

As a morphological characteristic of interest,  $I_2$  length was examined in the *late5* mutant, as determinate  $I_2$  structures in *late5* appeared shorter than those in wild-type (Figure 6.4). Indeed, when compared across all reproductive nodes, mean  $I_2$  length was significantly less in *late5* (Figure 6.5A and C). However, the results of separate comparisons between  $I_2$  structures at corresponding stem nodes, indicated that length is similar between structures borne at the same stem node in wild-type and *late5* (Figure 6.5B and D). Thus it appears that the difference in total mean  $I_2$  length seen between wild-type and *late5* is a reflection of the fact that *late5* mutants normally bear  $I_2$  structures at later stem nodes than wild-type (Figure 6.6), and plant age may be influencing organ size. A similar phenomenon is also seen in plants homozygous for the *LF-d* allele, which flower late and have short  $I_2$  structures, and *lf* mutants, which flower early and exhibit increased  $I_2$  length (Murfet, 1985). As there is an acropetal decrease in  $I_2$  length observed with increased stem node in wild-type plants (Figure 6.5), it appears that *LATE5* and *LF* do not affect the natural system controlling  $I_2$  length in pea. This is not true for all genes within the flowering pathway, for example mutation to *PhyA* results in delayed flowering coupled with an increase in  $I_2$  length (Weller et al., 1997), suggesting that photoperiod may influence this system. In support of this theory, measurements in this study show that  $I_2$  length in wild-type is comparable between LD and SD grown plants, despite the delay in

flowering node under SD conditions (Figures 6.5 and 6.6). It would be interesting to investigate the possibility that signals downstream of the photoperiod pathway but upstream of genes that participate in the V/I<sub>1</sub> transition (e.g. *LATE5*) may have a role in controlling I<sub>2</sub> length in pea.

Lateral outgrowth was also examined in the *late5* mutant and found to be increased in *late5* relative to wild-type under LD conditions, but comparable between *late5* and wild-type under SD conditions (Figures 6.2 and 6.3). These results are similar to earlier findings for the *veg2-2* mutant, and as discussed in Chapter 3, this may reflect delayed pod development in the late-flowering mutants, which appear to have an influence on lateral outgrowth based on flower removal experiments in wild-type plants (Chapter 3, Section 3.3.2; Lockhart and Gottschall, 1961; Malik and Berrie, 1975).

## **6.4.2 Investigation of genetic interactions with *DET***

### **6.4.2.1 Variation in I<sub>2</sub> morphology observed in *det* and *lf det* mutants**

Examination of *det* and *lf det* mutant plants revealed variation in I<sub>2</sub> morphology that has not been reported previously. In *det* and *lf det*, the determinate end of each I<sub>2</sub> structure was seen to vary from a protruding stub to a subtle ring of tissue and there was incomplete leaf suppression at I<sub>2</sub> nodes resulting in bracts of varying sizes up to full compound leaves subtending flowers (Figure 6.8). On some terminal I<sub>2</sub> structures, a full compound leaf subtended an axillary flower and gave the appearance that the flower was terminating the main stem (Figure 6.8). The only sign that these plants terminated in an I<sub>2</sub>, was the stub or ring of tissue at the end of the terminal I<sub>2</sub> (Figure 6.8). Based on these observations, it is not surprising that there was initially some confusion over whether *det* plants terminate in a flower or an I<sub>2</sub>, during early characterisation of the *det* single mutant (Marx, 1986; Murfet, 1989b).

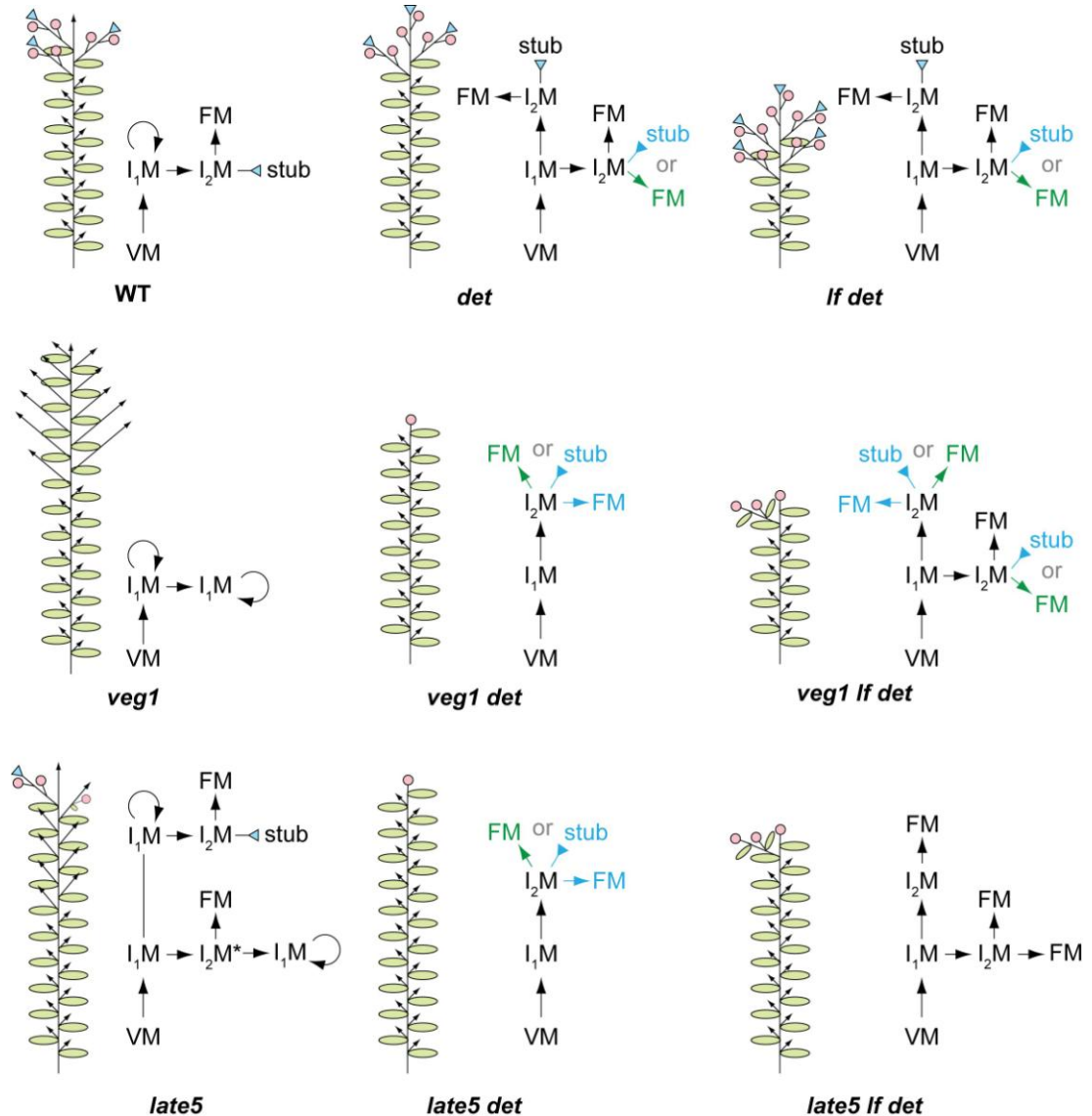
It is particularly intriguing that some axillary I<sub>2</sub> structures in *det* and *lf det* plants appeared to terminate in a flower with abnormal morphology rather than a stub or ring of tissue (Figure 6.8). This characteristic has also been observed previously in some *lf* single mutant plants (Taylor, 1998; Weller, 2007). It would be interesting to look at these structures in more detail, for example by examining longitudinal sections to compare tissue characteristics with wild-type I<sub>2</sub> structures and floral

pedicels, or by looking closely at these structures as they develop using scanning electron microscopy (SEM).

Overall these observations indicate that mutation to *DET* can influence  $I_2$  morphology, specifically the suppression of bracts/leaves, and exclusion of floral meristem genes from the  $I_2$ , in addition to the fate of the SAM (Figure 6.14). As *DET* expression is excluded from  $I_2$  structures (Berbel et al., 2012), and expression of *DET* within  $I_2$  meristems is associated with loss of  $I_2$  identity and meristem indeterminacy (Figure 3.16), it is not clear how *DET* could possibly have a role in wild-type  $I_2$  development. Instead, it seems more likely that the abnormalities in  $I_2$  morphology observed in the *det* mutant are an indirect effect of ectopic expression of MADS-box genes within the  $I_1$ . In this study, the aim was to present a brief overview of *det* and *lf det* phenotypes to provide a baseline for subsequent examination of mutant combinations with *veg1* and *late5*, but it would be interesting to do a more thorough survey of  $I_2$  morphology, including other *det* mutant alleles, to gauge the frequency and occurrence of these abnormalities in other backgrounds.

#### 6.4.2.2 Phenotypic characterisation of *veg1 det* and *veg1 lf det* mutants

The phenotype of the *veg1 det* mutant was revisited with knowledge of the possible range of  $I_2$  morphology in a *det* background. Previously, *veg1 det* was described as exhibiting a terminal flower phenotype, without any  $I_2$  structures, based on detailed investigation using SEM (Singer et al., 1999). Accordingly, in each *veg1 det* plant grown in this study, a flower appeared to terminate the main stem (Figures 6.9B and 6.14), and a similar phenotype was seen in *veg1 lf det* triple mutant plants (Figures 6.9H-S and 6.14). In some plants, there was no visible junction separating a floral pedicel from the main stem, as described previously for *veg1 det* (Figure 6.9H-I, N; Singer et al., 1999), but in other plants, the presence of a stub or ring of tissue indicated that these plants terminated in an  $I_2$  with an axillary flower subtended by a leaf, rather than with a terminal flower, similar to *det* single mutant plants (Figures 6.9C-G and 6.14). This interpretation is further supported by the presence of axillary  $I_2$  structures, with a similar appearance, at early reproductive nodes on the main stem of some *veg1 lf det* plants (Figure 6.9O-P). Using this interpretation, mutation to *VEG1* on a *det* or *lf det* mutant background appears to alter the severity but not the basic nature of the *det* inflorescence phenotype (Figure 6.14).



**Figure 6.14.** Summary diagrams illustrating the influence of the *det* mutation in various mutant backgrounds and schematics showing inferred defects in meristem identity.

For each genotype, the diagram on the left summarises basic mutant phenotype, based on observations shown in Figures 6.8-6.10, and the schematic on the right outlines the meristem identity transitions during inflorescence development, inferred from morphology. In diagrams: arrows indicate indeterminate growth, circles are flowers, triangles are terminal stubs, and ovals are leaves or bracts. In schematics, meristems are: vegetative meristem (VM), primary inflorescence meristem ( $I_1M$ ), secondary inflorescence meristem ( $I_2M$ ), floral meristem (FM). Placement of stubs is also indicated in schematics. Straight arrows indicate meristem transitions and products, circular arrows indicate meristem indeterminacy and straight lines lacking arrow heads indicate continued growth. The blue and green arrows and text indicate two alternative  $I_2M$  fates: termination in a stub or in a floral meristem.

The previous interpretation of the *veg1 det* mutant phenotype, coupled with an absence of  $I_2$  structures when *VEG1* is not induced in *veg1*, *gigas* and *veg2-1* mutants, led to the conclusion that *VEG1* is critical for  $I_2$  specification (Berbel et al., 2012). However, the observations from this study indicate that there are terminal  $I_2$  structures in *veg1 det* and *veg1 lf det* plants. Furthermore, some relatively normal axillary  $I_2$  structures were observed on branches of *veg1 lf det* plants (Figure 6.9L-M). These observations indicate that *VEG1* is not actually critical for  $I_2$  specification in the absence of *DET*, but it is clear that *VEG1* is important for correct  $I_2$  morphology. In *det* and *lf det* mutants, several normal  $I_2$  structures that exhibit complete suppression of leaves and terminate in a stub or ring of tissue are usually borne from the main stem before the  $I_1$  terminates in an ectopic  $I_2$  (Figure 6.14; Murfet, 1989a). Although variation in  $I_2$  morphology was observed in this study (Figure 6.9), extreme  $I_2$  defects including full compound leaves at  $I_2$  nodes, or replacement of an  $I_2$  stub with a terminal flower, were rare, occurring in less than approximately 10% of *det* and *lf det* mutant plants examined. In contrast, nearly all  $I_2$  structures seen in *veg1 det* and *veg1 lf det* mutants bore full compound leaves, and approximately half appeared to terminate in flowers (Figure 6.9). Thus loss of *VEG1* function has a clear influence on the severity of  $I_2$  defects seen in a *det* or *lf det* background.

The full details of how *VEG1* acts to ensure correct  $I_2$  specification have not yet been determined. As the key features that distinguish a wild-type  $I_2$  from an  $I_1$  are the presence of axillary flowers, suppression of leaves and determinacy, it is likely that *VEG1* regulates genes that control each of these characteristics.  $I_2$  specification, and subsequent specification of floral meristems, is blocked in the *veg1* mutant and in *gigas* and *veg2-1* mutants, which fail to induce *VEG1* (Chapter 3; Berbel et al., 2012). The occurrence of flowering in the *veg1 det* and *veg1 lf det* mutants indicates that *DET* is preventing expression of floral meristem identity genes in the absence of *VEG1*, as recently suggested (Berbel et al., 2012). Thus in the absence of functional *DET*, induction of *VEG1* is no longer a critical step for subsequent specification of floral meristems. This indicates that the most important function of *VEG1* is to exclude *DET* from the  $I_2$  meristem to maintain  $I_2$  determinacy and allow expression of floral meristem identity genes. The increased severity in  $I_2$  morphology defects in the *veg1 det* and *veg1 lf det* mutants relative to *det* and *lf det* indicate that *VEG1* has a



role beyond repression of *DET* (Figures 6.8 and 6.9). The investigation of other targets of *VEG1* remains an interesting area for future research.

The results from this study have also provided some additional insight into the relationship between *VEG1* and *LF*. In an earlier study, no phenotypic differences were detected between *veg1* and *veg1 lf* plants and it was concluded that *veg1* is epistatic to *lf* (Reid and Murfet, 1984). As flowering occurs earlier than wild-type in the *lf* mutant (Murfet, 1975), and timing of the V/I<sub>1</sub> transition occurs at the same time as wild-type in the *veg1* mutant (see Chapter 3), true epistasis of *veg1* over *lf* would be seen if the V/I<sub>1</sub> transition occurred at the same time as wild-type in the *veg1 lf* mutant. Although Reid and Murfet (1984) characterised ontogenetic variation in internode length in *veg1* and *lf* single mutants and report that *veg1 lf* plants could not be distinguished from *veg1* plants, no data is shown for the *veg1 lf* double mutant. In this study, additional mutation to *DET* enabled flowering time to be used as an easy measure to compare the influence of *lf* on a *veg1* background. Flowering time in the *veg1 lf det* mutant was earlier than *veg1 det* (Figure 6.9A), indicating an additive relationship between *VEG1* and *LF*, rather than the epistatic relationship reported previously. In light of this, it would be worth re-examining the *veg1 lf* double mutant, as it seems most probable that the timing of the V/I<sub>1</sub> transition is earlier in *veg1 lf* than in *veg1*.

Curiously, flowering occurred later in *veg1 det*, than in wild-type or *det*, and later in *veg1 lf det* than in *lf* or *lf det* (Figure 6.9A). As *VEG1* does not appear to have a role in the V/I<sub>1</sub> transition in a wild-type background (Chapter 3; Berbel et al., 2012), this suggests that there is some delay between the occurrence of the V/I<sub>1</sub> transition and production of flowers in the absence of *VEG1*. It is possible that the pathway that activates MADS-box genes for floral specification takes longer to induce a sufficient level of floral gene expression, in the absence of *VEG1*. The *veg2-1 det* mutant is non-flowering, which suggests that *FDa/VEG2* is critical for activation of MADS-box genes for floral meristem specification (Reid et al., 1996). In contrast, *FTa1/GIGAS* is not essential for this role, as *gi det* double mutants flower under both LD and SD photoperiods (Taylor, 1998). It is most probable that the activation of MADS-box genes for floral specification in the *veg1 det* and *veg1 lf det* mutants is dependent on *FDa/VEG2* function, but this could be tested directly by combining *veg2* mutations into these mutant backgrounds.

#### 6.4.2.3 *Phenotypic characterisation of late5 det and late5 lf det mutants*

The influence of *late5* in a *det* or *lf det* background was similar to that of *veg1* (Figure 6.14). *late5 det* plants appeared to terminate in a flower, but a ring of tissue was sometimes present at the junction between the stem and floral pedicel, indicating that the stem terminated in an I<sub>2</sub> with an axillary flower, and some normal I<sub>2</sub> structures were borne on branches (Figure 6.10B-K). In *late5 lf det* triple mutants, several I<sub>2</sub> structures were borne at reproductive nodes on the main stem, which each bore axillary flowers and appeared to terminate in a flower (Figure 6.10M-Q). The main stem of *late5 lf det* plants appeared to terminate in a similar I<sub>2</sub> structure (Figure 6.10L-M). These observations indicate that similar to *veg1*, *late5* affects the severity but not the fundamental nature of the *det* mutant phenotype. The increase in severity of I<sub>2</sub> morphology defects when *LATE5* function is affected in a *det* or *lf det* background, suggests that *LATE5* has a role in ensuring correct I<sub>2</sub> morphology. This is consistent with *late5* single mutant phenotype, where I<sub>2</sub> structures at early reproductive nodes may exhibit morphological defects (Figure 6.4). The mechanism of *LATE5* action will be an interesting subject to investigate further once the *LATE5* gene is characterised at a molecular level.

#### 6.4.3 *Identification of genes regulated by LATE5*

The results of qRT-PCR analysis conducted in this study, are useful as a first step towards understanding the relative location of *LATE5* within the flowering pathway. Altered expression patterns in the *late5* mutant relative to wild-type indicate that *LATE5* acts transcriptionally upstream of *FDa*, *SOC1a*, *DET*, *VEG1*, *UNI*, *AP3*, *PIM*, *SEP1* and possibly *FTa2* and *FULa*, in the apex (Figure 6.11A). This suggests that *LATE5* could be a transcription factor or promote transcription factor function by some mechanism, for example as a co-factor, or by disruption/breakdown of antagonistic proteins.

The finding that *FDa* is misregulated in the *late5* mutant is especially interesting, given the similarity in phenotype between *late5* and *veg2-2* mutants (Chapter 3; Section 6.3.1; Figure 6.11A). In particular, the *late5* mutant exhibits decreased *FDa* expression during the approximate window of time when wild-type plants commit to flowering (Figure 6.11A; Hecht et al., 2011). This may account for the apparent delay in the V/I<sub>1</sub> transition in the *late5* mutant (Figures 6.1 and 6.11A).

However, this does not adequately explain the inflorescence and floral defects in the *late5* mutant (Figures 6.4 and 6.7), as *Fda* expression levels would have increased by the time affected I<sub>2</sub> and floral meristems were specified (Figure 6.11A), suggesting that *LATE5* may act through an alternative mechanism. Some genes are misregulated in both *late5* and *veg2* mutants including *VEG1*, *UNI*, *AP3*, *PIM* and *SEP1*, but other genes appear to be affected in *late5* and not *veg2* mutants, or vice versa indicating that *LATE5* does not act simply through regulation of *Fda/VEG2* (Figures 5.7A and 6.11A). In particular, *FTc* induction was delayed in the apex of *veg2-2* plants, but not *late5* plants, and the increase in *SOC1a* expression, which occurs prior to wild-type flowering, was unaffected in *veg2-2* plants, but was delayed by approximately one week in the *late5* mutant (Figures 5.7A and 6.11A). In addition, expression of *LF* was slightly higher in the *veg2-2* mutant than in wild-type but was comparable between *late5* and wild-type (Figures 5.7A, 5.8 and 6.11A).

Delayed upregulation of the I<sub>2</sub> meristem identity gene *VEG1*, and the floral meristem identity genes *PIM* and *UNI*, could account for the inflorescence and floral defects seen in the *late5* mutant (Figure 6.11A). In addition, misregulation of *SOC1a* may affect the timing of the V/I<sub>1</sub> transition and/or floral development. The role of *SOC1a* has not previously been characterised in pea, but the expression pattern, with upregulation prior to flowering and downregulation coinciding with *PIM* induction (Figure 6.11A), suggests a role for *SOC1a* in the promotion of flowering and subsequent repression by *PIM* during floral development, similar to *SOC1* in *Arabidopsis* (Samach et al., 2000; Liu et al., 2007). The exact mechanism of *LATE5* action during inflorescence development remains to be investigated, once the molecular identity of *LATE5* is determined.

#### **6.4.4 Fine-mapping of the *LATE5* locus**

The location of *LATE5* on pea linkage group I was investigated in this study through the use of two F<sub>2</sub> populations. The *late5* x TER population, was used to narrow the location of *LATE5* down to a 14.3cM region on pea linkage group I, between the flanking markers *FENR1* and *UNK1* (Figure 6.12). However, despite isolating and sequencing more than 68 introns from 14 genes predicted to be present in this region based on synteny with *Medicago*, no polymorphisms were identified between NGB5839 and T  r  se, preventing further marker design (see Appendix 1,

Table A1.5). This population was produced by a narrow cross between *late5* (NGB5839 background) and cv. T  r  se; two lines that are highly similar at a genetic level. Performing a narrow cross can be an advantage for ease of detection of subtle mutant phenotypes, as any background variation in flowering loci can create uncertainty when identifying mutant segregants (Weller et al., 2013). However, this also has the disadvantage that the frequency of polymorphisms is reduced between these cultivars. Crosses between mutant loci on NGB5839 background and T  r  se have proved useful for mapping and subsequent identification of mutant loci in previous studies (e.g. Hecht et al., 2007). However, it appears that there may be an island of homozygosity between NGB5839 and T  r  se in the genomic region of interest for this study. The main disadvantage of using a wider cross was also seen in this study, when *late5* phenotype could not be determined with confidence in the F<sub>2</sub> progeny of a cross between *late5* and JI1794. Fortunately, *late5* phenotype was clear in the F<sub>2</sub> progeny of an intermediate cross between *late5* and JI399 and there were sufficient polymorphisms between parental lines for further molecular marker design.

Using the *late5* x JI399 F<sub>2</sub> population, the map position of the *LATE5* locus was refined to a region of 3.2cM towards the base of pea linkage group I, between the marker loci *RING finger* and *GATA-TF* (Figure 6.12). The location of *LATE5* was further refined to the region between *FENRI* and *GATA-TF*, using segregation data from the *late5* x TER population, which indicated that *LATE5* is downstream of *FENRI* (Figure 6.12). Although no obvious candidates for *LATE5* could be identified in the syntenic regions of *Medicago* chromosome 5, soybean chromosome 16, or common bean chromosome 2, some genes of interest were identified, including *WUS*, and three genes which show homology to *DJC24* and *DJC23* (Table 6.4).

The pea *WUS* homolog was found to be closely linked to *LATE5*, with no recombinants obtained between these loci (Figure 6.12). Across vascular plants, ranging from ferns to angiosperms, *WUS* has an important role in maintaining the populations of undifferentiated stem cells within indeterminate meristems (Nardmann and Werr, 2012). In *Arabidopsis*, *wus* mutant plants exhibit delayed bolting and floral defects, in addition to premature termination of meristems and severe defects in organisation of shoot tissues (Laux et al., 1996). Similarly in *Antirrhinum*, shoot meristems and individual leaves are initiated from the SAM in a

disorganised manner in *wus* mutant plants, but these plants are non-flowering (Kieffer et al., 2006). In soybean, accumulation of the two *WUS* homeologs in floral meristems during the floral transition has led to the suggestion that *WUS* genes may have a novel role during the floral transition in soybean (Wong et al., 2011). However, over-expression of *GmWUSa* in *Arabidopsis* leads to a delay in flowering, which suggests soybean *WUS* genes may actually inhibit the floral transition (Wong et al., 2011). Without characterisation of mutant phenotype for these genes in soybean, the exact role(s) of soybean *WUS* genes during flowering remains unclear. The *WUS* locus is an unlikely candidate for *late5*, as *late5* does not exhibit premature termination of meristems or defects in shoot organisation. However, it is worth retaining *WUS* as a gene of interest until it can be ruled out as a positional candidate for *LATE5* by further fine-mapping.

Three *DJC24/DJC23*-like genes that are present between *FENR1* and *GATA-TF* on *Medicago* chromosome 5, could not be isolated from pea during the time-frame of this study, but warrant further investigation (Table 6.4). J-domain proteins act as co-factors to provide functional specificity to the ubiquitous 70-kDa heat shock protein (HSP70) family (see Kampinga and Craig, 2010). The HSP70 molecular chaperone machinery aids in the folding of proteins as they are synthesised, directs protein transport across membranes and modulates protein interactions by controlling protein conformation (Mayer and Bukau, 2005). As there is little divergence in sequence between HSP70 proteins, J-domain proteins are important for specificity in binding to target proteins (Boorstein et al., 1994; Misselwitz et al., 1998). In *Arabidopsis*, *DJC24* is expressed predominantly in flowers (Chiu et al., 2013). Another J-domain protein, J3, has been found to promote flowering by antagonising *SVP*-mediated repression of *SOC1* and *FT* (Shen et al., 2011). Based on this knowledge, the three *DJC24/DJC23*-like genes appear to be interesting candidates for the *LATE5* locus.

The molecular identity of the *LATE5* locus could not be determined within the time-frame of this study, but significant progress was made in refining the map position of this locus. Suggestions for future steps that could be taken in order to identify *LATE5* are outlined below. Firstly, the *DJC24/DJC23*-like genes should be isolated and mapped in pea. If these genes are linked to *LATE5*, they should be further investigated as candidates for the *LATE5* locus. Secondly, seed should be

grown from plants that showed a wild-type *LATE5* phenotype, from the *late5* x JI399 F<sub>2</sub> population, to discriminate plants that were homozygous for the wild-type *LATE5* allele, from those that were heterozygous for that locus. Due to the proximity of the flanking markers *RING finger* and *GATA-TF*, it is relatively unlikely that two recombination events would occur between them in the one individual. Thus, to limit the F<sub>3</sub> population size, this could be restricted to plants which showed recombination between *RING finger* and *GATA-TF*, because segregation data would be most informative for *LATE5* position in these individuals. Thirdly, more F<sub>2</sub> seed from the cross between *late5* and JI399 could be grown to expand the mapping population to increase resolution, and more markers could be designed within this region. Alternatively, and more economically, DNA could be extracted from the F<sub>3</sub> population produced from plants exhibiting recombination between *RING finger* and *GATA-TF*, and new markers could be mapped in this population to further refine the *LATE5* map position.

#### 6.4.5 Conclusions

Although the molecular nature of the *LATE5* locus was not identified during the course of this study, the results presented in this chapter represent a significant advancement in the knowledge of the role of *LATE5* during inflorescence development. In particular, the high similarity between *late5* and *veg2-2* mutant phenotypes suggests that *LATE5* may be associated with *FDa/VEG2* function. The absence of known flowering genes within the regions of *Medicago*, soybean and common bean genomes that are syntenic with the region containing *LATE5* in pea, suggests that identification of *LATE5* will provide a new insight into the mechanisms regulating flowering. Overall, the results of this study provide a strong foundation for future identification of the *LATE5* locus.

# CHAPTER 7: Characterisation of *SVP*-like genes in pea

## 7.1 Introduction

MADS-box transcription factors are key regulators of many developmental processes in plants. These transcription factors may act as homo- or hetero-dimers or as part of ternary transcription factor complexes to regulate transcription of target genes (see Kaufmann et al., 2005). The MIKC<sup>c</sup> group of type II MADS-box genes play important roles during the transition to flowering, and in the specification of identity for floral meristems and floral organs (see Gramzow and Theissen, 2010). Thus far in this study, two genes from this group, *VEG1* and *PIM*, have been discussed in some depth for their respective roles in I<sub>2</sub> and floral meristem identity in pea (Chapter 3). In addition, expression of other MADS-box genes, including *FULA*, *FULb*, *SOC1a*, *AP3* and *SEPI* have been investigated in inflorescence mutants and found to be regulated to varying extents by *Fda/VEG2* and/or *LATE5* (Chapters 5 and 6). This chapter, will focus on another clade of MIKC<sup>c</sup> MADS-box genes, known as the *StMADS11* subfamily, after the first isolated member of the clade (Carmona et al., 1998), or alternatively called *SVP*-like genes for their similarity to the better known *Arabidopsis* gene *SVP* (Hartmann et al., 2000).

Genes belonging to the *StMADS11* subfamily have been identified in diverse angiosperm species. In *Arabidopsis*, this subfamily of MADS-box genes has two closely related members, *SVP* itself and *AGL24*, which have opposite roles in the control of flowering time but act redundantly to control floral meristem identity (Hartmann et al., 2000; Yu et al., 2002; Gregis et al., 2006). Some *SVP*-like genes in other species have been identified as repressors of the transition to flowering, similar to *SVP*, including *INCOMPOSITA (INCO)* in *Antirrhinum*, *SVPI* and *SVP3* in kiwifruit, and *VEGETATIVE TO REPRODUCTIVE TRANSITION 2 (TaVRT2)* in wheat (Masiero et al., 2004; Kane et al., 2005; Wu et al., 2012). Others have been found to promote the floral transition, sharing some similarities with *AGL24* function, including *J* in tomato and capsicum (Quinet et al., 2006; Cohen et al., 2012; Thouet et al., 2012). A role in floral meristem identity similar to that of *SVP* and *AGL24*, is also seen for *SVP*-like genes in both eudicot and monocot species

including *Antirrhinum* and barley (Masiero et al., 2004; Trevaskis et al., 2007). In barley, overexpression of *SVP*-like genes results in increased panicle branching (Trevaskis et al., 2007), indicating that *SVP*-like genes could potentially function in compound inflorescence development. Also, the tomato *J* gene has been found to have an important role in development of the compound inflorescence, suggesting that related genes may be important for the genetic mechanisms underlying the diversity in inflorescence architecture (Thouet et al., 2012).

In legume species, one *SVP*-like gene has been isolated in pea, three have been described in *Medicago*, and nine have been identified in soybean (Hecht et al., 2005; Sussmilch, 2008; Jung et al., 2012; Jaudal et al., 2013; Shu et al., 2013). The presence of multiple *SVP*-like genes in *Medicago*, suggests that other *SVP*-like genes may be present in pea that have not previously been identified. The roles of *StMADS11* subfamily members in inflorescence and floral identity highlights these genes as possible regulators of compound inflorescence development in pea, and for this reason, *SVP*-like genes in pea were investigated in this study.

### 7.1.1 Chapter aims

The aim of this chapter was to identify additional *SVP*-like genes in pea and begin to investigate the characteristics of each gene. Firstly, other members of the *StMADS11* subfamily were identified among sequenced legume species and used to identify transcript sequence for corresponding pea *SVP*-like genes. Phylogenetic analysis was conducted to investigate the relationships between the legume *SVP*-like genes relative to *Arabidopsis* MIKC<sup>c</sup> transcription factors and *StMADS11* subfamily members from diverse angiosperm species. Newly isolated pea *SVP*-like genes were mapped and the expression of these genes was investigated in apex and leaf tissues in wild-type under LD and SD conditions, and in *gigas-2*, *veg2-2* and *late5* mutants in LD conditions.



## 7.2 Materials and methods

This section contains specific details of materials and methods for studies included in this chapter. General materials and methods also relevant to this chapter are described in Chapter 2. The details of sequences and alignments for this chapter are shown in Appendix 6.

### 7.2.1 Identification of legume SVP-like genes and gene isolation from pea

tBLASTn searches were conducted using AtSVP and AtAGL24 protein sequences as queries against genome sequence resources for *Medicago* (Mt3.5), soybean (v1.1), *Lotus* (build 2.5) and common bean (v1.0), and transcript resources for these species (DFCI TGI; GenBank dbEST; see Table 2.4 for details of online resources). BLAST hits were confirmed to be SVP-like genes in BLASTp searches against *Arabidopsis* (TAIR10). *Medicago* SVP-like genes were used as queries in BLASTn searches against available pea sequence resources (GenBank TSA sequence and nucleotide databases). Pea BLAST hits were confirmed with reciprocal BLAST searches against *Arabidopsis* and *Medicago*. Partial pea sequence was extended by RACE PCR to obtain full-length coding sequence.

### 7.2.2 Identification of SVP-like genes in other angiosperm species

BLASTp and tBLASTn searches using predicted protein sequence for *PsSVPa*, *PsSVPb* and *PsSVPc* were conducted in collections of protein, nucleotide and transcript sequences at GenBank. In addition tBLASTn searches were conducted against genome resources for selected members of the rosoid I lineage including peach (v1.0), cucumber (v1) and poplar (v3.0; see Table 2.4 for genome resource details; see Figure 1.2 for taxonomic relationships). BLAST hits were confirmed to be SVP-like genes by reciprocal BLAST searches against *Arabidopsis*. For an ancestral *StMADS11* subfamily gene, full coding sequence for *GGM12* was obtained by performing a BLASTn search using partial *GGM12* sequence (AJ132218; Winter et al., 1999; Becker et al., 2000) as a query against the UC Davis Putative Unique transcripts for *Gnetum gnemon* (UCD.454) at ConGenIE.

### 7.2.3 Mapping of pea *SVP*-like genes

Intron regions of *SVPb* and *SVPc* were sequenced in the pea cultivars used as parental lines in mapping crosses and identified polymorphisms were used to design molecular markers for these genes. *SVPa* and *SVPc* were mapped in the *late5* x JI399 F<sub>2</sub> population, as described in Chapter 6 (Table 6.2). Details of genes on *Medicago* chromosome 5 that correspond to mapping loci on pea linkage group I are also given in Chapter 6 (Table 6.2). *SVPb* and surrounding markers on pea linkage group VII were mapped with the technical assistance of S. Ridge in the F<sub>2</sub> population of a cross between *late bloomer 2* (*late2*) and cv. T  r  se, comprising 80 individuals. Details of genes on *Medicago* chromosome 4, which correspond to mapping loci on pea linkage group VII are shown in Table 7.1, below. Full details of molecular markers scored during this study are given in Appendix 1 (Table A1.4).

**Table 7.1.** Marker loci on pea linkage group VII used in this study. Primer details for molecular markers are given in Appendix 1 (Table A1.4).

Name	Corresponding <i>Medicago</i> locus (Mt3.5)	Population	Marker type	Source
<i>Aldo</i>	Medtr4g071860	<i>late2</i> x TER	Size	Aubert et al. (2006)
<i>Acetisom</i>	Medtr4g079780	<i>late2</i> x TER	CAPS	Deulvot et al (2010); Bordat et al. (2011)
<i>MitCySyn</i>	Medtr4g087520	<i>late2</i> x TER	Size	Bordat et al. (2011)
<i>SVPb</i>	Medtr4g093970	<i>late2</i> x TER	HRM	This study

### 7.2.4 qRT-PCR analysis

Ontogenetic expression patterns of each pea *SVP* gene were investigated in wild-type (NGB5839) under LD (16h) and SD (8h) conditions in two existing developmental series comprising apex and leaf tissue (Hecht et al., 2011). Plants for these experiments were grown concurrently. RNA for these developmental series was extracted by V. Hecht prior to this study. Subsequent synthesis of cDNA and qRT-PCR analysis was conducted within the time-frame of this study without technical assistance. Investigation of gene expression in the *gigas-2* mutant was performed on the same LD (16h) experiment with the same wild-type line (NGB5839). Gene expression patterns in *veg2-2* and *late5* mutants were investigated in the same experiment presented in Chapters 5 and 6, the details of which are outlined in Section 5.2.1. qRT-PCR primer details are given in Appendix 1.

## 7.3 Results

### 7.3.1 Characterisation of the legume *StMADS11* subfamily

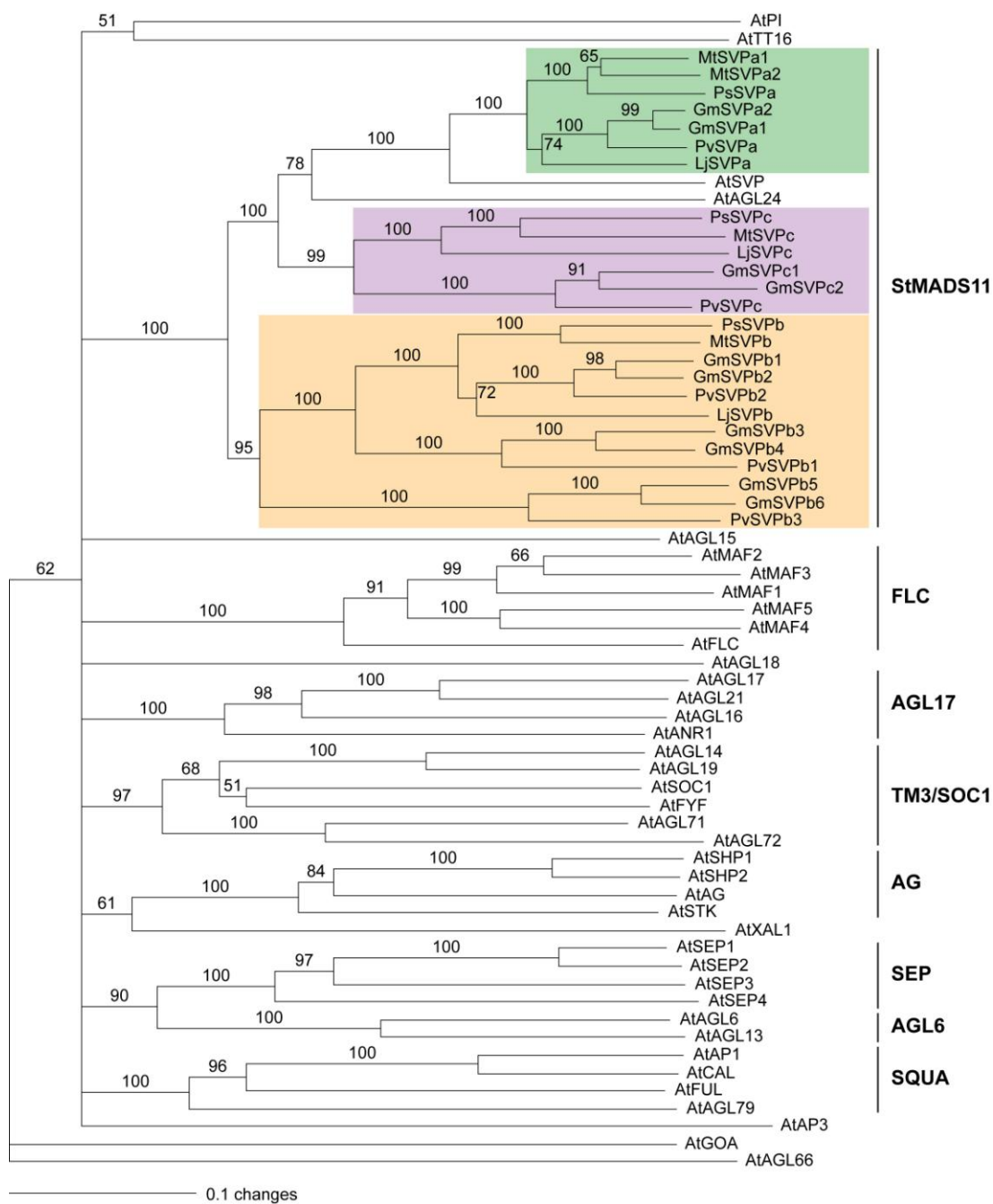
Prior to commencement of this study, partial sequence for a single *SVP*-like gene had been isolated from pea (*PsSVP*), extended with RACE PCR to obtain full-length coding sequence, and mapped to pea linkage group I (Hecht et al., 2005; Sussmilch, 2008). In addition, three *SVP*-like genes had been identified in *Medicago* and nine in soybean (Hecht et al., 2005; Jung et al., 2012; Jaudal et al., 2013; Shu et al., 2013). In this study, tBLASTn searches were conducted with AtSVP and AtAGL24 proteins as queries against *Medicago*, soybean, common bean and *Lotus* genomes. BLAST hits were confirmed by BLASTp searches of predicted protein sequence against *Arabidopsis* proteins. In this manner, four *SVP*-like genes were identified in *Medicago*, ten in soybean, five in common bean and three in *Lotus* (Table 7.2). Coding sequences for the genes identified in *Medicago* were used in BLASTn searches against available pea transcript resources, to identify the corresponding pea genes. Full-length coding sequence was identified for an additional pea gene and partial coding sequence was identified for a third and extended to full-length by RACE PCR (Table 7.2).

Phylogenetic analysis with identified legume *SVP*-like genes and *Arabidopsis* MIKC MADS-box transcription factors confirmed that the legume genes fell within the *StMADS11* clade with *AtSVP* and *AtAGL24* (Figure 7.1). Within this clade, legume genes clustered into three separate subclades: *SVPa*, *SVPb* and *SVPc* (Figure 7.1). All three subclades were represented in each legume species investigated in this study (Table 7.2, Figure 7.1). Legume genes were named or renamed according to subclade for clarity and consistency, providing a framework for the future naming of novel *SVP*-like genes in other legume species (Table 7.2, Figure 7.1).

**Table 7.2.** Legume *SVP*-like genes identified during this study. Locus details and chromosomal location are given for species with sequenced genomes, based on genome builds that were current at the time of this study (Mt3.5, *Lotus* build 2.5, Glyma v1.1, Phvul v1.0). Chromosomal location for pea is based on mapping data shown in Figure 7.3. For expressed sequences, source of sequence is indicated as (a) GenBank or (b) DFCI TGI, and coverage of coding sequence is indicated as full-length (FL) or partial (P) with percentage indicated. Asterisks indicate incorrect annotation in online resources, as determined by alignments with expressed sequences and/or between legume species. Previous gene names are indicated where applicable. Details of online resources are given in Chapter 2 (Table 2.4).

Species	Name	Locus ID	Chromosomal location	Expressed sequence	% Identity		Reference
					AtSVP	AtAGL24	
Pea ( <i>Pisum sativum</i> )	<i>PsSVPa</i> ( <i>PsSVP</i> )	-	LGI	AY830919 <sup>a</sup> (P:94%) JI901140 <sup>a</sup> (FL)	81	52	Hecht et al. (2005); Sussmilch (2008)
	<i>PsSVPb</i>	-	LGVII	JI898361 <sup>a</sup> (FL) JI909838 <sup>a</sup> (P:80%) JI910349 <sup>a</sup> (P:23%) This study (FL)	40	43	This study
	<i>PsSVPC</i>	-	LGI	JI972724 <sup>a</sup> (P:22%) This study (FL)	48	50	This study
<i>Medicago truncatula</i>	<i>MtSVPa1</i> ( <i>MtSVP</i> ; <i>MtSVP1</i> )	Medtr5g032520	chr5: 13532861 - 13539544	TC176819 <sup>b</sup> (FL)	70	56	Hecht et al. (2005); Jaudal (2011); Jaudal et al. (2013); Jung et al. (2012)
	<i>MtSVPa2</i> ( <i>MtSVP2</i> )	Medtr5g032150	chr5: 13382579 - 13385429	TC187641 <sup>b</sup> (FL)	67	55	Jaudal (2011); Jaudal et al. (2013); Jung et al. (2012)
	<i>MtSVPb</i>	Medtr4g093970*	chr4: 32381918 - 32391910	TC202150 <sup>b*</sup> (FL) TC182367 <sup>b</sup> (P:95%)	41	44	This study
	<i>MtSVPC</i> ( <i>MtAGL24</i> )	Medtr5g066180*	chr5: 26915911 - 26936912	TC183050 <sup>b</sup> (FL)	47	50	Jaudal (2011); Jaudal et al. (2013); Jung et al. (2012)
<i>Lotus japonicus</i>	<i>LjSVPa</i>	chr2.CM0008.1180.r2.d*	-	TC60537 <sup>b</sup> (FL)	66	55	This study
	<i>LjSVPb</i>	chr4.CM0100.30.r2.m*	-	TC81268 <sup>b</sup> (FL)	39	42	This study
	<i>LjSVPC</i>	-	-	TC62447 <sup>b</sup> (FL)	41	45	This study

Species	Name	Locus ID	Chromosomal location	Expressed sequence	% Identity		Reference
					AtSVP	AtAGL24	
Soybean ( <i>Glycine max</i> )	<i>GmSVPa1</i> ( <i>GmMADS1</i> )	Glyma01g02880	Gm01: 2360198 - 2366406	BI784874 <sup>a</sup> (P:79%)	85	56	Hecht et al.(2005); Jung et al. (2012); Shu et al. (2013)
	<i>GmSVPa2</i> ( <i>GmMADS2</i> )	Glyma02g04710	Gm02: 3850231 - 3856750	TC432917 <sup>b</sup> (FL)	71	56	Jung et al. (2012); Shu et al. (2013)
	<i>GmSVPb1</i> ( <i>GmMADS3</i> )	Glyma07g30040	Gm07: 35111248 - 35123164	TC429291 <sup>b</sup> (FL)	43	43	Jung et al. (2012); Shu et al. (2013)
	<i>GmSVPb2</i> ( <i>GmMADS4</i> )	Glyma08g07260	Gm08: 5215213 - 5225206	CD396853 <sup>a</sup> (P:62%)	43	45	Jung et al. (2012); Shu et al. (2013)
	<i>GmSVPb3</i>	Glyma13g33051	Gm13: 35009349 - 35016613	TC459586 <sup>b*</sup> (P:69%)	41	41	This study
	<i>GmSVPb4</i> ( <i>GmMADS7</i> )	Glyma15g06302	Gm15: 4454005 - 4461092	-	43	43	Jung et al. (2012); Shu et al. (2013)
	<i>GmSVPb5</i> ( <i>GmMADS6</i> )	Glyma13g33031*	Gm13: 34995931 - 35007295	AW432749 <sup>a</sup> (P:44%)	39	40	Shu et al. (2013)
	<i>GmSVPb6</i>	Glyma15g06314	Gm15: 4464584 - 4474957	-	38	38	This study
	<i>GmSVPC1</i> ( <i>GmMADS5</i> )	Glyma06g10020	Gm06: 7555760 - 7561695	TC437725 <sup>b</sup> (FL)	51	50	Jung et al. (2012); Shu et al. (2013)
	<i>GmSVPC2</i>	Glyma04g10015*	Gm04: 8282749- 8273776	-	47	45	This study
Common bean ( <i>Phaseolus vulgaris</i> )	<i>PvSVPa</i>	Phvul.002G147600	Chr02: 28542528 - 28547627	TC35086 <sup>b*</sup> (FL)	71	55	This study
	<i>PvSVPb1</i>	Phvul.006G202300	Chr06: 30647668 - 30653064	-	39	40	This study
	<i>PvSVPb2</i>	Phvul.002G212400*	Chr02: 37451699 - 37453782	-	42	45	This study
	<i>PvSVPb3</i>	Phvul.006G202200	Chr06: 30637568 - 30644312	-	53	51	This study
	<i>PvSVPC</i>	Phvul.009G037300	Chr09: 7765144 - 7768533	-	38	38	This study



**Figure 7.1.** Phylogenetic neighbour-joining tree of legume SVP-like proteins and *Arabidopsis* MIKC MADS-box transcription factors.

This phylogram was constructed from full-length predicted protein sequence for genes from *Arabidopsis* (At), common bean (Pv), *Lotus* (Lj), *Medicago* (Mt), pea (Ps) and soybean (Gm), rooted to the MIKC\* gene AtAGL66. Bootstrap values obtained from 1000 trees are indicated as a percentage above or next to each branch. Clades named after the first isolated clade member are indicated. Sequence and alignment details are given in Appendix 6.

Based on phylogenetic analysis between legume *SVP*-like genes and *Arabidopsis* MIKC MADS-box proteins, the *SVPa* subclade showed the highest similarity to *Arabidopsis SVP* (Figure 7.1). This was reflected in the similarity of deduced sequence for legume *SVPa* proteins when compared with *AtSVP* (66-85%; Table 7.2). Overall, there was a tendency for proteins within *SVPb* and *SVPc* subclades to share higher percentage identity with *AtAGL24* than with *AtSVP*, but the difference was small (2-4%), and the level of similarity with *AtAGL24* was lower for *SVPb* and *SVPc* proteins (38-51%) than for *SVPa* proteins (52-56%; Table 7.2). This is consistent with the results of phylogenetic analysis, which show *AtAGL24* as a sister group to *AtSVP* and the legume *SVPa* subclade (Figure 7.1). This indicates that no legume genes show specific similarity to *AGL24*.

Novel *SVP*-like genes were identified for each legume species investigated in this study: pea (*PsSVPb*, *PsSVPc*), *Medicago* (*MtSVPb*), *Lotus* (*LjSVPa*, *LjSVPb*, *LjSVPc*), soybean (*GmSVPb3*, *GmSVPb6*, *GmSVPc2*) and common bean (*PvSVPa*, *PvSVPb1*, *PvSVPb2*, *PvSVPb3*, *PvSVPc*; Table 7.2). Two of the eight soybean *SVP*-like genes (v1.0: Glyma15g06300 and Glyma15g06310) that were previously described by Jung et al. (2012) correspond to a single gene (*GmSVPb4*) in updated annotation of the soybean genome (v1.1: Glyma15g06302). Two of the nine soybean *SVP*-like genes (*GmMADS11* and *GmMADS16*) described by Shu et al. (2013) showed higher similarity to *Arabidopsis* genes *AGL18*, *AGL15* and *AGL16*, than to *SVP* or *AGL24*, in reciprocal BLAST searches. This was in agreement with the findings of Jung et al. (2012), so *GmMADS11* and *GmMADS16* were not included as *SVP*-like genes in this study.

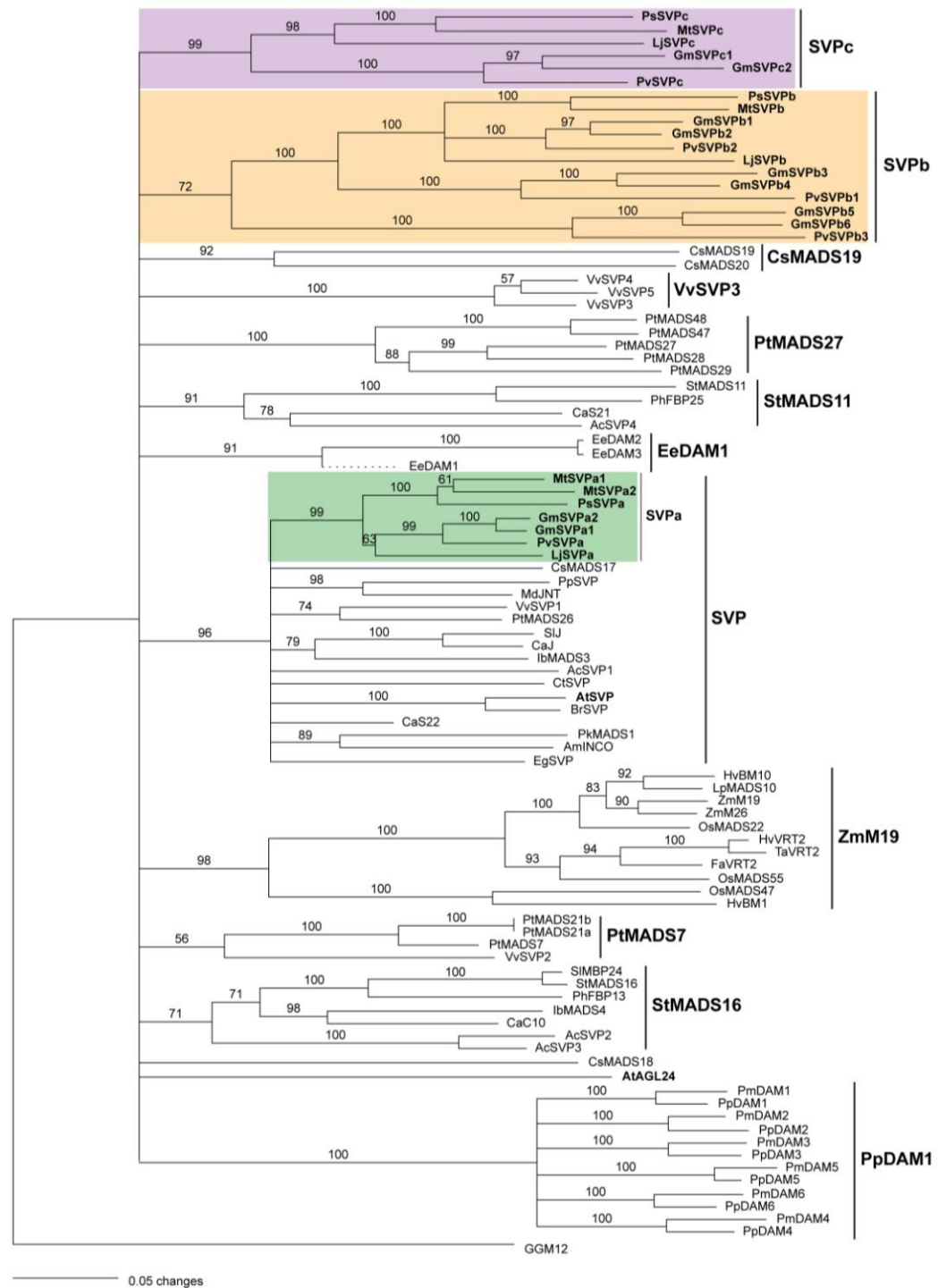
Two *SVPa* genes were identified in *Medicago*, but only one was identified in pea (Figure 7.1; Table 7.2). In terms of protein sequence, *Medicago SVPa1* and *SVPa2* shared a relatively equal level of similarity with pea *SVPa*, with 87% and 86% amino acid identity, respectively. In Figure 7.1, *PsSVPa* forms a sister group to both *MtSVPa1* and *MtSVPa2*, suggesting that the duplication event that gave rise to the two *Medicago SVPa* genes may have occurred after divergence of the Trifolieae and Viceae tribes. Each soybean *SVP*-like gene was a member of a homeologous gene pair (Figure 7.1), reflecting the tetraploid origin of the soybean genome (Shoemaker et al., 1996; Schmutz et al., 2010). Within the *SVPb* subclade, there were two extra groups of *SVPb* genes present in soybean and common bean, relative to

other legumes, indicating that expansion of this subclade has occurred within the Phaseoleae (Figure 7.1).

The results of initial phylogenetic analysis left open the possibility that *SVPb* and *SVPc* subclades may be present in other rosid I lineages, so the analysis was next expanded to explore the relationships between legume *SVP* subclades and other angiosperm *SVP*-like genes. Firstly, the three pea *SVP* sequences were used in BLAST searches against resources for other angiosperm species, including representatives of other orders within the rosid I clade (Rosales, Cucurbitales and Malpighiales; see Figure 1.2 for taxonomic relationships). *SVP*-like genes that were identified in BLAST searches and confirmed in reciprocal BLAST searches against *Arabidopsis* were included with *SVP*-like genes characterised in previous studies for phylogenetic analysis.

Figure 7.2 shows a number of clear subclades present within the *StMADS11* clade of *SVP*-like genes, but relationships among these subclades were poorly resolved, most likely due to overall similarity among *SVP*-like genes. No non-legume *SVP*-like genes fell within the *SVPb* or *SVPc* subclades, suggesting that these subclades may be limited to legumes and represent a divergence within the Fabales prior to separation of the galegoid and phaseoloid clades (Figure 7.2). Legume *SVPa* genes fell within the eudicot *SVP* subclade, which included representatives from diverse eudicot species, including *Arabidopsis SVP*, tomato *J*, *Antirrhinum INCO*, and a novel *SVP* homolog from peach, named here as *PpSVP* (Figure 7.2). This indicates that a member of this subclade was present in a common ancestor of the rosid and asterid lineages and that minimal sequence divergence has occurred between members of this subclade since relatively early in the evolution of the core eudicots. Curiously, *Arabidopsis AGL24* did not fall within any subgroup in this analysis, suggesting that this gene may have arisen from a duplication specific to brassicas (Figure 7.2). The legumes were not the only eudicot lineage with multiple subclades, as other species including poplar, grape and kiwifruit also exhibited divergence of subgroups of *SVP*-like genes (Figure 7.2). In contrast, monocot *SVP*-like genes formed a single subclade (*ZmM19*; Figure 7.2).



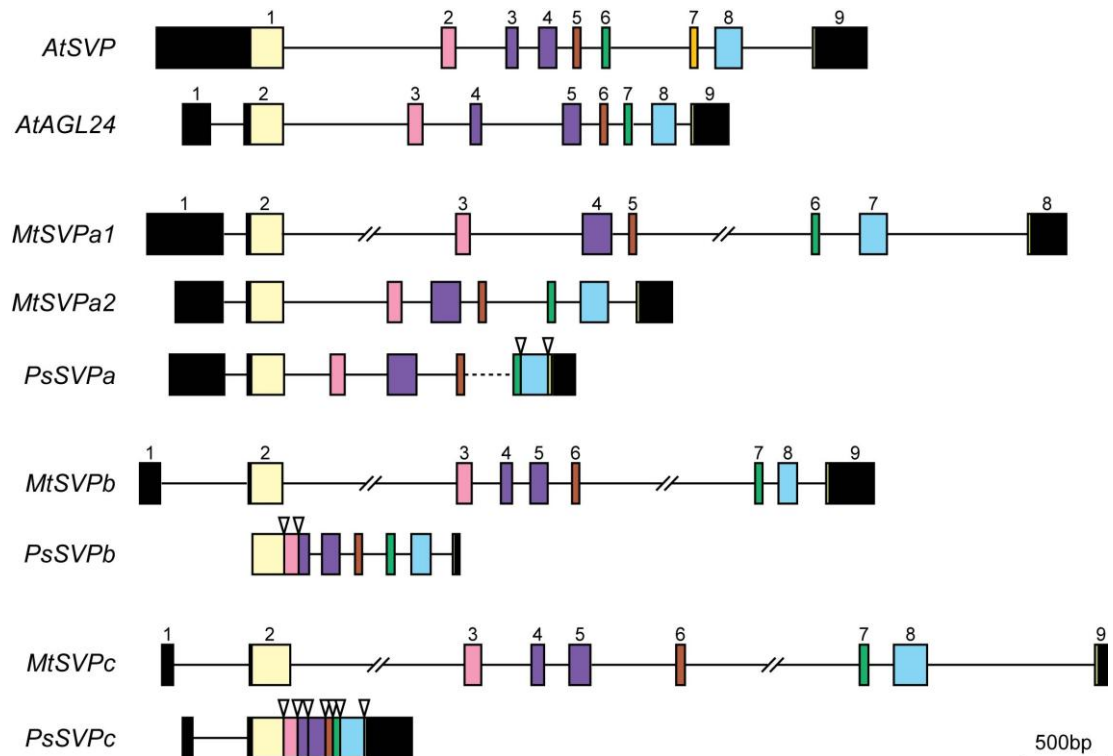


**Figure 7.2.** Phylogenetic neighbour-joining tree of the *StMADS11* subfamily of MADS-box transcription factors in diverse angiosperm species.

This phylogram was constructed from full-length sequence for proteins from *Antirrhinum* (Am), apple (Md), *Arabidopsis* (At), barley (Hv), capsicum (Ca), Chinese cabbage (Br), common bean (Pv), cucumber (Cs), *Eucalyptus* (Eg), grape (Vv), japanese apricot (Pm), kiwifruit (Ac), leafy spurge (Ee), *Lotus* (Lj), maize (Zm), *Medicago* (Mt), pea (Ps), peach (Pp), perennial ryegrass (Lp), petunia (Ph), poplar (Pt), potato (St), rice (Os), sapphire dragon tree (Pk), soybean (Gm), sweet potato (Ib), tomato (Sl), trifoliate orange (Ct) and wheat (Ta), and rooted to GGM12 from *Gnetum gnemon*. Subclades were named after the first identified subclade member. Bootstrap values obtained from 1000 trees are indicated as a percentage above each branch. Sequence and alignment details are given in Appendix 6.

### 7.3.2 Conserved structure of *SVP*-like genes

Gene structure was examined in pea and *Medicago* *SVP*-like genes and compared with *Arabidopsis* *SVP* and *AGL24*, in order to look further at the relationships between these genes. Only partial genomic sequence was obtained for each pea gene during the course of this study. However, intron placement is generally highly conserved between *Medicago* and pea, which allowed the placement of pea introns to be predicted from *Medicago* with a high level of confidence (Weller et al., 2013). Consistent with this, overall gene structure was found to be remarkably well conserved across *Arabidopsis*, *Medicago* and pea *SVP*-like genes (Figure 7.3). *AtAGL24* and legume genes shared a conserved non-coding exon in the 5' untranslated region (exon 1; Figure 7.3). Although this non-coding exon was not obtained for *PsSVPb* during the course of this study, it is predicted to also be present. No intron was annotated in the 5' region of *AtSVP*, but *AtSVP* contained an additional exon (exon 7), which was not present in the other genes and coded for a region of the C-domain that was not present in *AtAGL24* or the legume proteins (Figure 7.3; Appendix 6). The only other structural difference between these genes was a reduction in exon number in the legume *SVPa* genes relative to the other legume genes (Figure 7.3). Exon 4 in the *SVPa* genes corresponds to exons 4 and 5 in *AtAGL24*, *SVPb* and *SVPc* genes and exons 3 and 4 in *AtSVP*. This suggests that the reduced number of exons in the legume *SVPa* genes resulted from loss of an intron within exon 4 of the corresponding gene in a common ancestor of *Medicago* and pea. There is an intron conserved in this position in the *SVPa* genes of soybean and common bean (see annotation of Glyma01g02880, Glyma02g04710 and Phvul.002G147600 at Phytozome; <http://www.phytozome.net/>), suggesting that this intron was lost after divergence of the Phaseoleae lineage.



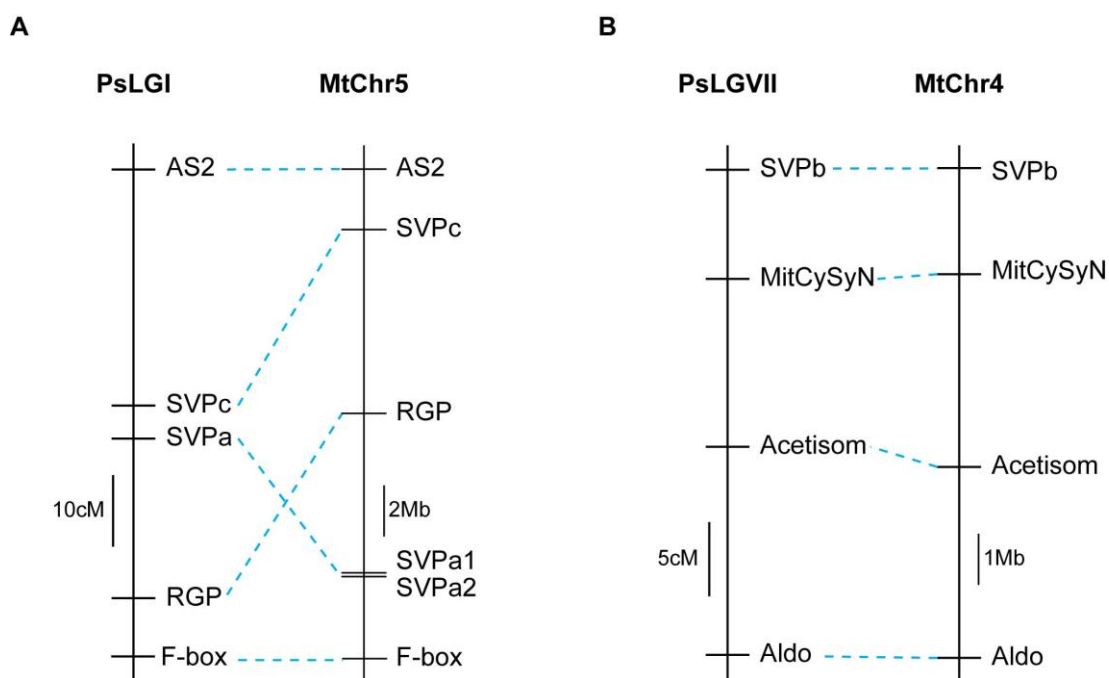
**Figure 7.3.** Diagram of gene structure for the *StMADS11* subfamily in *Arabidopsis*, *Medicago* and pea.

Exons are shown as boxes, with the untranslated regions shown in black and corresponding coding regions determined by protein alignment and coloured accordingly (see Appendix 6). The exons of the *Arabidopsis* genes and the first gene for each legume subclade are numbered. Introns are shown as lines. For introns longer than 1kb, only the first 1kb is shown with a 'break' symbol. Not all introns were isolated in pea. Triangles indicate predicted placement of non-isolated pea introns, based on structure of the corresponding *Medicago* gene(s). Where partial sequence was obtained for intron 5 in *PsSVPa*, the size of the isolated portion is shown with a broken line. Full-length coding sequence was isolated for all pea genes. Full-length *SVPa* was isolated in a previous study (Sussmilch, 2008).

### 7.3.3 Map positions for the pea SVP-like genes

Next, the relative locations of SVP-like genes were examined in pea and *Medicago*. *MtSVPa1* and *MtSVPa2* are both located close together on *Medicago* chromosome 5 and *MtSVPc* is slightly upstream on the same chromosome (Figure 7.4A). The results of a preliminary study indicated that *SVPa* was located on pea linkage group I, above *RGP* (Sussmilch, 2008). This position was confirmed and compared to the relative position of *PsSVPc* by mapping both genes in the F<sub>2</sub> population of a cross between *late5* and JI399, comprising 184 individuals. Both *SVPa* and *SVPc* were closely linked (<5cM) and mapped to a region on the lower half of pea linkage group I between the markers *AS2* and *RGP* (Figure 7.4A). Mapping results indicate that there has been a rearrangement in the region between the marker loci *RGP* and *AS2* in pea, relative to *Medicago*, as previously described

(Bordat et al., 2011), which has resulted in the close proximity of *PsSVPa* and *PsSVPc* (Figure 7.4A). In *Medicago*, *SVPb* is located on chromosome 4 (Figure 7.4B). *PsSVPb* was mapped in the F<sub>2</sub> population of a cross between *late2* and cv. T  r  se, comprising 80 individuals, to the syntenic region of linkage group VII above the markers *MitCySin*, *Acetisom* and *Aldo* (Figure 7.4B). The position of the *SVP* genes in pea, as determined in this study, does not qualify them as positional candidates for any known pea mutant loci.



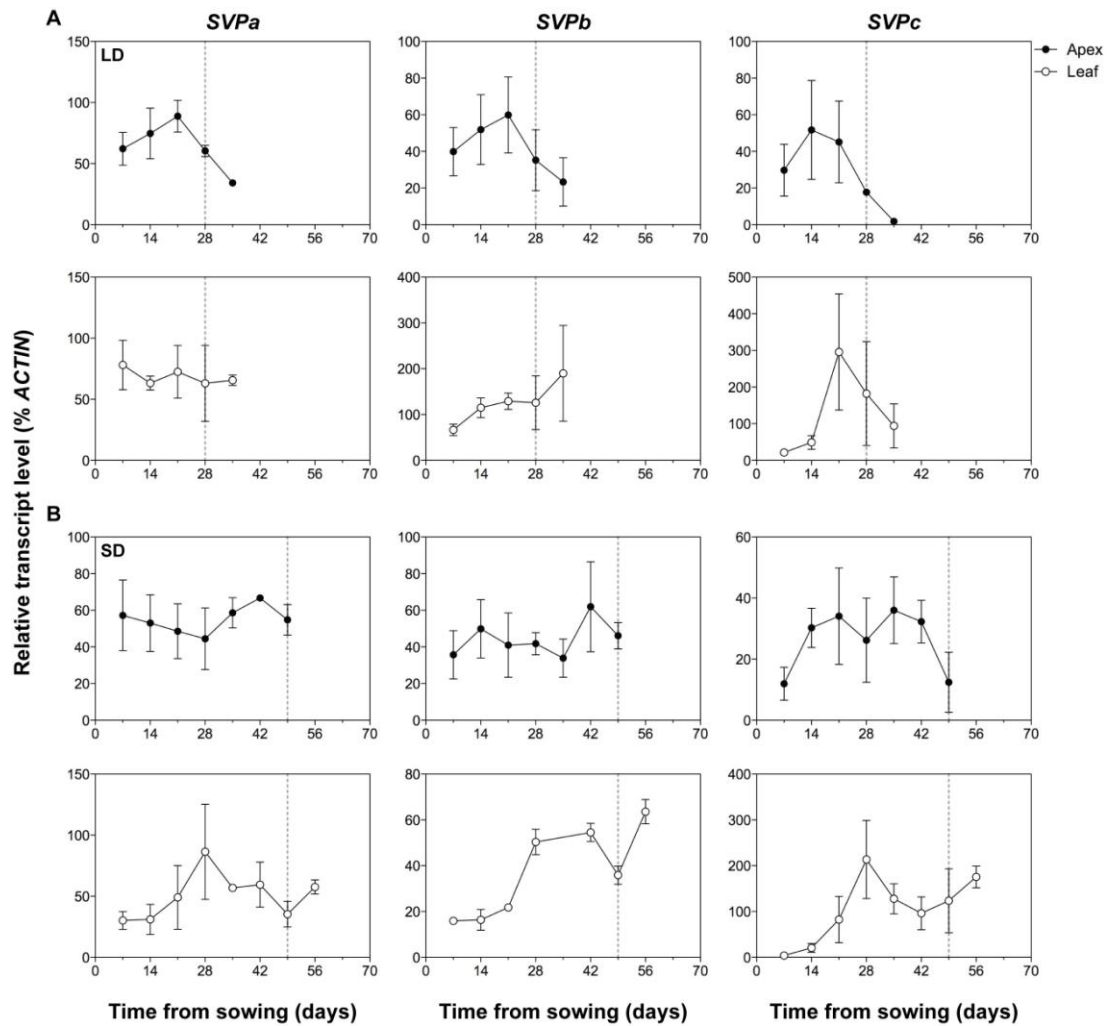
**Figure 7.4.** Comparative maps between syntenic regions containing *SVP*-like genes in pea and *Medicago*.

The physical maps shown for *Medicago* are based on genome build Mt3.5 and details of *Medicago* loci are given in Tables 6.2 and 7.1. Distances between pea loci were estimated from segregation data using JoinMap software (v4; Kyazma B.V., Wageningen, Netherlands). Genes that correspond between maps are linked with broken blue lines.

### 7.3.4 Ontogenetic expression patterns of pea *SVP*-like genes

Expression of each pea *SVP*-like gene was investigated by qRT-PCR analysis in apex and leaf tissue in wild-type plants throughout development under both LD and SD photoperiods. Under LD conditions, apical expression of *SVPa*, *SVPb* and *SVPc* followed a similar pattern of gradual upregulation with a peak in expression approximately 14-21 days after sowing, prior to the production of floral buds at day 28, and a decrease in expression thereafter (Figure 7.5A). In the leaf, *SVPa* was expressed at a relatively constant level throughout development, *SVPb* exhibited a

gradual increase in expression throughout development and *SVPc* was upregulated prior to flowering and downregulated thereafter (Figure 7.5A). Overall expression levels were similar between apex and leaf tissue for *SVPa*, whereas *SVPb* and *SVPc* exhibited relatively higher expression levels in the leaf (Figure 7.5A).

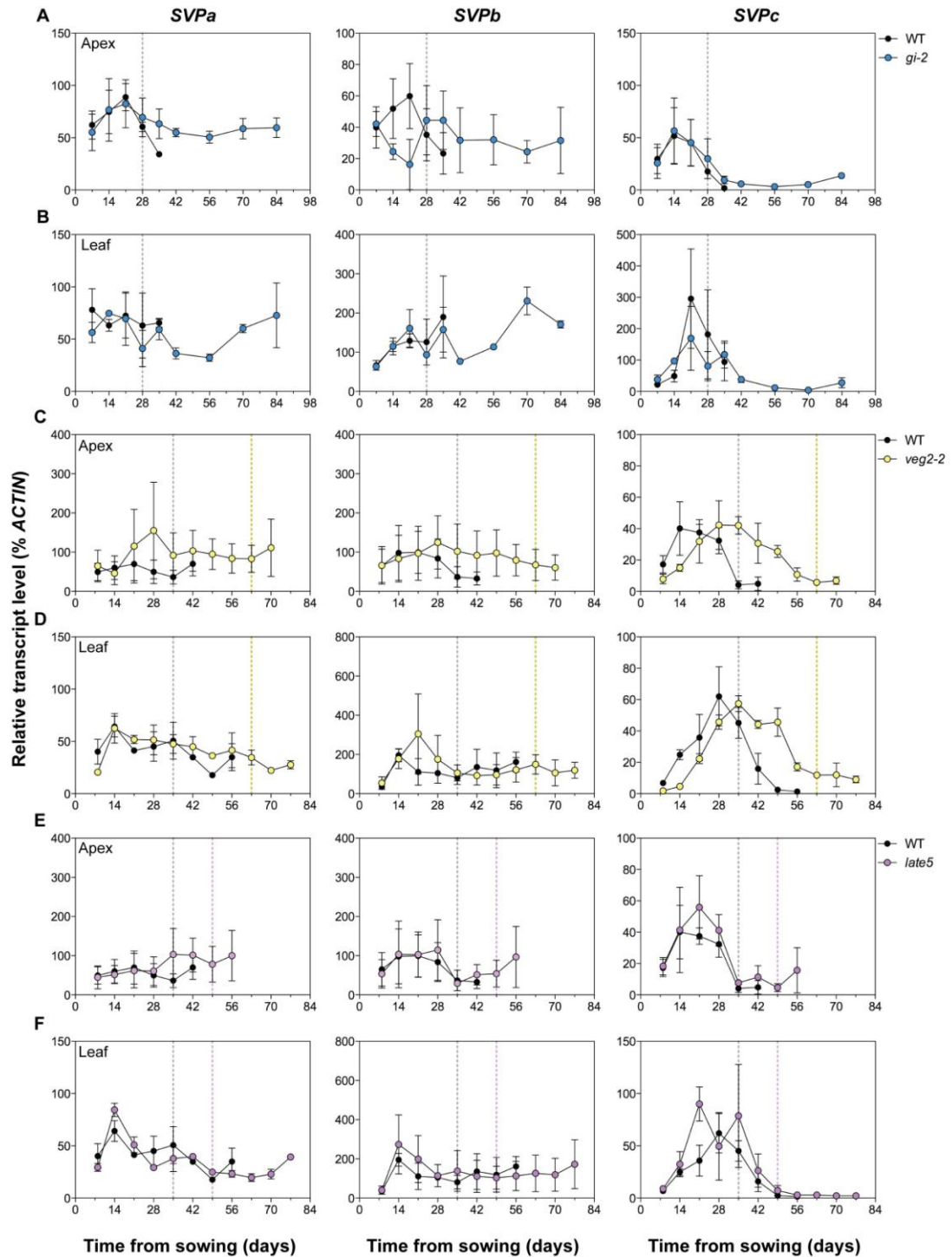


**Figure 7.5.** Ontogenetic expression patterns of pea *SVP*-like genes in wild-type plants.

Gene expression in wild-type (NGB5839) during plant development under (A) LD (16h) and (B) SD (8h) photoperiods, in dissected shoot apices (black circles), or the uppermost fully expanded leaf (open circles). Values have been normalised to the transcript level of *ACTIN* and represent mean  $\pm$  standard error for  $n = 2$ -3 biological replicates, each consisting of pooled material from two plants. Developing floral buds were first macroscopically visible in the apex 28 days after sowing in LD and 49 days after sowing in SD (grey line).

Under SD conditions, flowering occurred later in wild-type plants, with floral buds first visible in the apex 49 days after sowing (Figure 7.5B). Apical expression of *SVPa* and *SVPb* remained relatively constant throughout development, with a small increase in expression immediately prior to flowering and decrease in expression at the onset of flowering (Figure 7.5B). For *SVPc*, expression appeared to increase at approximately 14 days after sowing and decrease at the onset of flowering (Figure 7.5B). In the leaf, expression of *SVPa*, *SVPb* and *SVPc* increased from 14-21 days after sowing, peaked at around 28 days after sowing, decreased prior to flowering and appeared to increase again after flowering (Figure 7.5B). Overall expression level was comparable between apex and leaf tissues for *SVPa* and *SVPb*, but was higher in the leaf for *SVPc* (Figure 7.5B).

To determine if any of the pea *SVP*-like genes are direct or indirect targets of *FTa1/GIGAS*, *FDa/VEG2* or *LATE5*, expression was investigated in available developmental series containing the inflorescence mutants *gigas-2*, *veg2-2* and *late5*, grown under LD conditions. In the non-flowering *gigas-2* mutant, the expression patterns of *SVPa* and *SVPc* in both apex and leaf were similar to wild-type (Figure 7.6A-B). Expression of *SVPb* was similar to wild-type in the leaf, but the upregulation of *SVPb* in the apex prior to flowering appeared delayed in the *gigas-2* mutant, relative to wild-type (Figure 7.6A-B). In the *veg2-2* mutant, expression of *SVPa* and *SVPb* was comparable with wild-type, but the upregulation of *SVPc* was convincingly delayed in the *veg2-2* mutant, relative to wild-type, in both apex and leaf tissues (Figure 7.6C-D). In the *late5* mutant, expression of all three *SVP*-like genes was comparable to wild-type throughout development (Figure 7.6E-F). These results indicate that *SVPc* is directly or indirectly regulated by *FDa/VEG2*, and that *SVPb* could be transcriptionally downstream of *FTa1/GIGAS*.



**Figure 7.6.** Ontogenetic expression patterns of *SVP*-like genes in pea inflorescence mutants.

Gene expression in wild-type (NGB5839; black circles) and (A-B) *gigas-2* (blue circles), (C-D) *veg2-2* (yellow circles) and (E-F) *late5* mutants (purple circles) under LD conditions in (A, C, E) the dissected shoot apex and (B, D, F) the uppermost fully expanded leaf. For (A-B) plants were grown in LD (16h) conditions in the same experiment shown in Figure 7.5. For (C-F) *veg2-2* and *late5* mutants were grown under LD (24h) conditions in a separate experiment. Values have been normalised to the transcript level of *ACTIN* and represent mean  $\pm$  standard error for  $n = 2-3$  biological replicates, each consisting of pooled material from two plants. Broken vertical lines indicate the first macroscopic appearance of developing floral buds in the apex for wild-type (grey line), *veg2-2* (yellow line) and *late5* plants (purple line). *gigas-2* mutants did not flower under these conditions.

## 7.4 Discussion

### 7.4.1 Characterisation of the legume *StMADS11* subfamily

In this study, novel *SVP*-like genes were identified in pea (*PsSVPb*, *PsSVPc*), *Medicago* (*MtSVPb*), *Lotus* (*LjSVPa*, *LjSVPb*, *LjSVPc*), soybean (*GmSVPb3*, *GmSVPb6*, *GmSVPc2*) and common bean (*PvSVPa*, *PvSVPb1*, *PvSVPb2*, *PvSVPb3*, *PvSVPc*; Table 7.2). Phylogenetic analysis revealed three separate subclades of legume *SVP*-like genes, *SVPa*, *SVPb* and *SVPc*, which were represented in all legume species (Figure 7.1). Apart from the expected presence of homeologous gene pairs in soybean, some expansion within legume subclades has occurred in specific groups or species of legumes. Firstly, the presence of two extra groups of *SVPb* genes in soybean and common bean relative to the galegoid legumes, indicates that the *SVPb* subclade has expanded within the Phaseoleae (Figure 7.1; Table 7.2). Gene function has not yet been determined for any legume *SVPb* genes, so the significance of this expansion is not yet clear. Secondly, two *SVPa* genes were found close together in *Medicago*, but only one *SVPa* gene was identified in pea, *Lotus* and common bean (Figures 7.1 and 7.4; Table 7.2). The results of phylogenetic analysis indicate that *MtSVPa1* and *MtSVPa2* are paralogs with *PsSVPa* as a sister group (Figure 7.1), suggesting that the duplication that resulted in two *SVPa* genes in *Medicago* occurred after divergence of the Trifolieae and Viceae tribes. It remains a possibility that additional *SVP*-like genes may be present in pea, which were not represented in available pea sequence resources and were thus not identified during this study. This possibility could easily be investigated when pea genome sequence becomes available in the future.

Broader phylogenetic analysis also identified a number of distinct subclades within the angiosperm *StMADS11* subfamily (Figure 7.2). These included a large eudicot *SVP* subclade containing the legume *SVPa* genes, the *ZmM19* subclade comprising all monocot *SVP*-like genes, the *PpDAM1* subclade of dormancy-associated MADS-box (*DAM*) genes from peach and Japanese apricot, and two asterid subclades (*StMADS11* and *StMADS16*), in addition to the legume *SVPb* and *SVPc* subclades, which appeared to be legume-specific (Figure 7.2). The presence of multiple *SVP*-like genes in separate subclades is not unique to legumes, and it appears that other subclades have arisen independently in other rosid species (e.g.



poplar and grape) and in asterids (e.g. potato and kiwifruit; Figure 7.2). Some divergence in function between subclades has been found in kiwifruit, where *SVP*-like genes differ in their ability to complement the *Arabidopsis svp* mutant and show different overexpression phenotype in *Arabidopsis* (Wu et al., 2012). Similarly, *Medicago SVPa* and *SVPc* genes have different effects when overexpressed in *Arabidopsis* (Jaudal, 2011), indicating that there could also be divergence of function between legume *SVP* subclades.

The large eudicot *SVP* subclade included genes from diverse eudicot species representing both rosid and asterid lineages, with strong bootstrap support (96%; Figure 7.2). This indicates that a member of this subclade was present in a common ancestor of these lineages and that minimal sequence divergence has occurred between members of this subclade since relatively early in the evolution of the core eudicots. Accordingly, a novel *SVP*-like gene from this subclade was identified in the peach genome in this study (Figure 7.2). In peach, *DAM* genes within the *PpDAM1* subclade shown in Figure 7.2 have been found to have an important influence on the chilling requirement for bud break and flowering of low- and high-chill cultivars (Jiménez et al., 2010; Yamane et al., 2011). Given the relevance of the *DAM* genes for the peach industry, the function of *PpSVP* also warrants further investigation. In terms of the legume *SVP*-like genes, the clustering of legume *SVPa* genes in this conserved eudicot *SVP* subclade suggests that the legume subclades may have evolved from an *SVPa*-like gene in a legume ancestor. It would be interesting to investigate the level of microsynteny between the regions containing each legume *SVP*-like gene and *SVP*-like genes in non-legume eudicot species to see if this holds any further clues regarding the evolution of these genes.

A high level of similarity between genes within the eudicot *SVP* subclade suggests that functional constraints may have limited divergence as subsequent duplications gave rise to other subclades within specific lineages. Indeed, a number of genes are thought to act in a similar fashion to *AtSVP* as repressors of the floral transition, including *BrSVP*, *EgSVP*, *CtSVP*, *AcSVPI* and *AmINCO* (Brill and Watson, 2004; Masiero et al., 2004; Lee et al., 2007a; Li et al., 2010; Wu et al., 2012). However, flowering is delayed in the *j* mutants of tomato and capsicum, which also cluster within the eudicot *SVP* subclade, suggesting an opposite role for *J* genes in promoting inflorescence identity, somewhat similar to the role of *AGL24*

(Szymkowiak and Irish, 2006; Cohen et al., 2012; Thouet et al., 2012). Thus it appears that there is also limited functional divergence within the conserved eudicot *SVP* subclade.

The best prediction for the role of legume *SVPa* genes comes from functional studies of *Medicago* genes, but these do not give an especially clear indication. Phenotypes resulting from overexpression of *MtSVPa1* and *MtSVPa2* in *Arabidopsis* suggest that these genes can act as floral repressors and influence floral development in *Arabidopsis* (Jaudal et al., 2013), similar to *SVP* (Hartmann et al., 2000). However, overexpression of *MtSVPa1* in *Medicago* results in floral defects but does not affect flowering time (Jaudal et al., 2013). It is possible that the presence of *FLC* in *Arabidopsis*, but not in legume species, may allow *MtSVPa1* to function as a floral repressor in *Arabidopsis* but not in *Medicago* (Hecht et al., 2005; Jaudal et al., 2013). However, this does not offer a complete explanation, as in *Arabidopsis*, the effects of *svp* and *flc* mutations are additive indicating that *SVP* can also delay flowering independently of *FLC* (Li et al., 2008). An endogenous role for legume *SVP*-like genes as floral repressors should not be ruled out until a thorough investigation of gene function for all the *SVP*-like genes within a model legume species is conducted using mutants and/or transgenic legume plants.

In the phylogenetic analysis conducted in this study, *Arabidopsis AGL24* did not fall within any subgroup (Figure 7.2). Previously, *StMADS16* and *IbMADS4* (genes within the *StMADS11* subclade in Figure 7.2) have been shown in a subclade with *AGL24* (Becker and Theissen, 2003). However, this suggests the presence of an *AGL24* subclade conserved across rosoid and asterid lineages and no such subclade was visible when an increased number of species were included in this study (Figure 7.2). It seems more likely that *AGL24* is specific to *Arabidopsis* or the brassica lineage, possibly deriving from a whole genome duplication event in an ancestor of this group (The *Arabidopsis* Genome Initiative, 2000; Blanc et al., 2003; Ermolaeva et al., 2003). Previously, *MtSVPc* was named as *MtAGL24*, based on pairwise comparisons of protein sequence with *AGL24* and *SVP* (Jaudal, 2011). However, this is somewhat misleading, as *MtSVPa1* and *MtSVPa2* both show higher similarity to *AGL24* than *MtSVPc* does (Table 7.2). The conclusion from this study is that there does not appear to be any legume genes that show specific similarity to *AGL24* (Figure 7.2). This of course does not rule out the possibility of convergent

subfunctionalisation, as is seen to some extent in the *J* genes (Cohen et al., 2012; Thouet et al., 2012).

The expansion of legume *SVP* genes provides another example in an emerging picture that important flowering genes present as a gene pair in *Arabidopsis* (e.g. *SVP/AGL24*, *FT/TSF*, *FD/FDP*) are present in three clear subclades in legume species (Figure 7.1; Chapter 4; Hecht et al., 2011). For *SVP*-like genes, *FT* genes and *FD* genes, two of the subclades appear to be legume-specific, suggesting that the ancestral *SVP*, *FT* and *FD* genes each tripled in number during legume evolution. A whole genome duplication event prior to divergence of phaseoloid and galegoid legumes has been inferred (Schlueter et al., 2004; Mudge et al., 2005; Shoemaker et al., 2006), and it is possible that each of these separate families of flowering genes underwent further expansion from two to three members through local duplication events in an ancestral legume. It will be interesting to determine if this trend continues with other flowering genes as more legume gene families are characterised.

#### 7.4.2 Characteristics of pea *SVP*-like genes

The results of qRT-PCR analysis indicated that the three pea *SVP*-like genes are each expressed in both the leaf and apex under LD and SD conditions. In apex tissue under LD conditions and leaf tissue under SD conditions there was a clear pattern of developmental regulation seen for each *SVP*-like gene, with gradual upregulation leading to a peak in expression 14-28 days after sowing, followed by down-regulation of expression (Figure 7.5). In LD conditions, the peak occurred immediately prior to flowering, but under SD conditions flowering occurred several weeks later (Figure 7.5), suggesting the pattern of expression was not closely linked to flowering time. The patterns of each *SVP*-like gene were more divergent in the leaf under LD conditions and in the apex under SD conditions, but *SVPc* exhibited a clear developmental pattern of induction and downregulation under both conditions in both tissue types (Figure 7.5). Inconsistency between tissue types and photoperiods, coupled with variation between replicates makes it difficult to interpret the expression patterns of *SVPa* and *SVPb*. It is possible that these genes show different expression patterns under different conditions, in the apex and leaf, but this should be confirmed in a follow-up study.

The patterns of expression seen for the three pea genes do not give any convincing clues as to the possible roles of these genes in pea (Figure 7.5). It could be expected that a gene with a role in repressing flowering time would be expressed at a high level during vegetative growth, then decrease immediately prior to or during the transition to inflorescence development, as is seen for *SVP* in *Arabidopsis* (Hartmann et al., 2000; Liu et al., 2007; Jang et al., 2009). The decrease in *SVP* expression in *Arabidopsis* is dependent on photoperiod and occurs later in SD than LD conditions, to coincide with flowering time (Jang et al., 2009). While expression of all three pea genes does decrease prior to the appearance of floral buds, this decrease is not photoperiod-dependent and occurs up to two weeks before the appearance of floral buds under SD conditions (Figure 7.5). In *Arabidopsis*, *AGL24* is upregulated during the floral transition due to a positive feedback loop between *AGL24* and *SOC1* (Yu et al., 2002; Michaels et al., 2003; Liu et al., 2008). Each of the pea genes exhibits induction prior to flowering under certain conditions, which can be seen most clearly in *SVPc* (Figure 7.5). This could suggest a role in promoting the V/I<sub>1</sub> transition, but again the fact that this peak does not immediately precede flowering under SD conditions does not seem consistent with this theory. Expression of pea *SVP*-like genes in floral meristems was not investigated in this study, but this should be investigated in a future study by qRT-PCR and/or *in situ* hybridisation, as it is possible that these genes could have a role in floral development, similar to that seen in other species (e.g. Masiero et al., 2004; Gregis et al., 2006).

Overall, patterns of expression were largely similar between the three pea *SVP*-like genes in the tissues investigated in this study. In other angiosperm species, *SVP*-like genes show divergent expression patterns, but this is mainly associated with tissue type. In grape, *VvSVP2* and *VvSVP5* are expressed in shoots and leaves, respectively, while expression of the other *VvSVP* genes appears restricted to vegetative buds (Díaz-Riquelme et al., 2009). Similarly in petunia, *PhFBP13* is expressed in vegetative tissues only, whereas *PhFBP25* is expressed in floral tissues (Immink et al., 2003). Expression of all three pea genes in both leaves and apices could indicate some level of redundancy in function, although further investigation of these genes in floral tissue could reveal differences in tissue-specificity between these genes. In order to characterise the roles of the three pea *SVP*-like genes and level of redundancy between them, it will be necessary to investigate mutant

phenotypes. Unfortunately, the mapping positions of the pea genes does not qualify any of these genes as positional candidates for existing flowering mutant loci (Figure 7.4). Isolation of pea mutants should be attempted in the future using a Targeting Induced Local Lesions in Genomes (TILLING) or alternative approach (Dalmais et al., 2008; Triques et al., 2008).

Examination of the expression of the three pea *SVP*-like genes in inflorescence mutants revealed that *SVPc* is misregulated in both the apex and leaf of the *veg2-2* mutant, indicating that *SVPc* is transcriptionally downstream of *FDa/VEG2*. *SVPc* expression should also be further investigated in the *veg2-1* deletion mutant to determine if expression is further delayed, or even absent in this mutant. Differences in expression of *SVPb* were also observed in the *gigas* mutant, suggesting that *SVPb* could potentially be regulated by *FTa1/GIGAS*. However, the difference in *SVPb* expression between the *gigas* mutant and wild-type was relatively small and observed only in one tissue type (apex), so this result requires further confirmation. One previous study has speculated that the *FD/FT* complex may be an upstream regulator of *SVP* in floral meristems in *Arabidopsis* (Grandi et al., 2012), but prior to this study, no data had been reported for any species to indicate that this regulation occurs. Expression of *AGL24* and *SVP* in the *fd* mutant or lines overexpressing *FD* have not been reported in *Arabidopsis*. However, *SVP* expression is similar, or slightly higher in the *Arabidopsis ft* mutant than in wild-type, and *AGL24* expression is unchanged (Yu et al., 2002; Li et al., 2008). In addition, expression of *AGL24* remains unchanged in lines overexpressing *FT* or *TSF* (Yamaguchi et al., 2005). These results indicate that neither *AGL24* nor *SVP* is regulated by *FT* or *TSF* in *Arabidopsis*. The possibility that *SVP*-like genes may be regulated by the *FDa/FT* pathway in legume species is an exciting one that warrants further investigation.

### 7.4.3 Conclusions

The results of this chapter offer an insight into the evolution of the *StMADS11* subfamily in legumes, and represent the first step towards characterising the members of this subfamily in pea. Preliminary results suggest that of the three *SVP*-like genes, only *SVPc* exhibits a consistent pattern of strong developmental regulation and *SVPc* was also the only *SVP*-like gene found to be significantly misregulated in any of the inflorescence mutants examined. All three genes are expressed in the apex, and future *in situ* hybridisation studies are needed to examine more detailed expression patterns in floral meristems/primordia, as suggested by studies in other species. The possibility that *SVP*-like genes are downstream targets of the *FDA/FT* pathway in pea has not been reported in other species and it would be interesting in the future to investigate this possibility further and determine if this could reflect direct transcriptional regulation. Future isolation of appropriate pea mutants will offer insight into the degree of functional divergence between the three legume subclades of *SVP*-like genes.

## CHAPTER 8: General Discussion

### 8.1 Summary of main findings

In this thesis, the genetic control of inflorescence development in pea has been investigated through characterisation of pea mutant loci with inflorescence defects and of specific genes or gene families with a possible role in this process. The results suggest that *VEG2* is important for (a) the correct timing of the V/I<sub>1</sub> transition, (b) the initial specification and the maintenance of I<sub>2</sub> identity, and (c) the specification of floral meristems under both LD and SD conditions (Chapter 3). In comparison, mutation to *VEG1* did not affect the timing of the V/I<sub>1</sub> transition under either photoperiod and *FTa1/GIGAS* appears to be important for this transition under SD conditions only (Figures 3.2-3.5). Next, the legume *FD* gene family was characterised in detail for the first time and *FDa* was further investigated as a candidate for the *VEG2* locus (Chapter 4). The entire coding sequence for *FDa* was shown to be deleted in the *veg2-1* mutant and a SNP affecting a highly conserved amino acid was shown to co-segregate with the *veg2-2* mutant phenotype (Chapter 4). Based on the strength of this evidence, the *VEG2* locus was characterised as *FDa*. *FDa/VEG2* was found to be expressed within the vegetative SAM, I<sub>1</sub> meristem, I<sub>2</sub> meristems, young floral meristems, leaf primordia and vasculature in wild-type plants (Figure 5.1). *FDa/VEG2* was found to interact with each of the five pea FT proteins and with DET/TFL1a, but not with LF/TFL1c (Figure 5.6). *FDa/VEG2* was found to be transcriptionally upstream of specific pea *FT* and *TFL1* homologs, the *LFY* ortholog *UNI*, and a range of MADS-box genes (Figures 5.7 and 5.8).

A detailed phenotypic examination of the *late5* mutant, indicated that *LATE5* is important for the timing of the V/I<sub>1</sub> transition, and has roles in the specification and maintenance of I<sub>2</sub> identity and in determining correct floral development (Figures 6.1, 6.4 and 6.7). *LATE5* and *VEG1* were found to interact genetically with *DET* in a similar manner, with both *late5* and *veg1* mutations affecting the severity but not the fundamental nature of the *det* single mutant phenotype (Figure 6.14). *LATE5* was found to act transcriptionally upstream of *FDa*, *DET*, *UNI* and a range of MADS-box genes (Figure 6.11). Some differences were observed between the genes misregulated in the *late5* and *veg2* mutants, suggesting that *LATE5* may act through a slightly different mechanism to *FDa/VEG2* (Figures 5.7 and 6.11). Mapping studies

delimited *LATE5* to a region of 3.2cM towards the base of pea linkage group I, which combined with segregation data from a second F<sub>2</sub> population, indicated that *LATE5* is within a region of pea linkage group I that corresponds to a syntenic region of *Medicago* chromosome 5 comprising 0.6Mb and containing 95 annotated genes (Figure 6.12). In *Medicago*, this region includes the meristem maintenance gene *WUS*, and three genes which show homology to *DJC24* and *DJC23* (Table 6.4).

Lastly, the legume *StMADS11* subfamily of MADS-box genes was characterised and two additional *SVP*-like genes were isolated in pea (Figure 7.1). Phylogenetic analysis revealed three subclades of legume *SVP*-like genes, two of which were legume-specific (*SVPb* and *SVPc*) and one that fell into a conserved eudicot subclade that included *AtSVP* (*SVPa*; Figure 7.2). Preliminary steps were taken to characterise the three pea *SVP*-like genes in more detail, including map position, gene structure and expression in apex and leaf tissue in wild-type under LD and SD conditions (Figures 7.3-7.5). Expression patterns for each gene suggested developmental regulation in certain conditions and tissues, but only *SVPc* showed a consistent pattern of developmental regulation in apex and leaf under all conditions tested (Figure 7.5). Expression was further analysed in pea inflorescence mutants, and results suggest that *SVPc* may be regulated by *FDa/VEG2* (Figure 7.6).

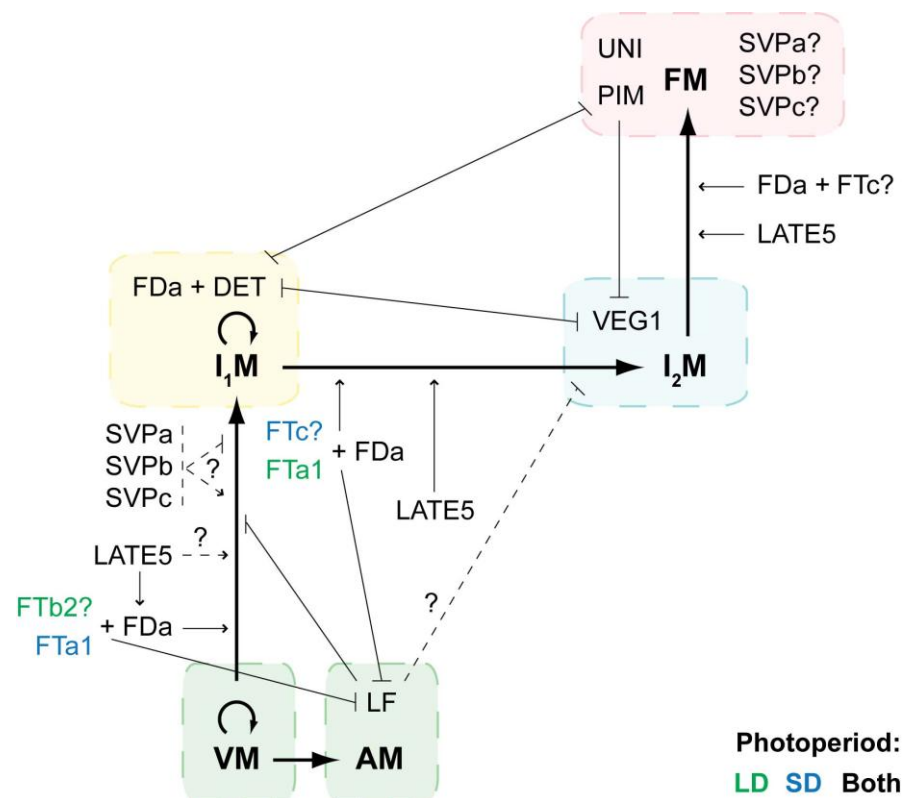
## 8.2 Revised model for inflorescence development in pea

Collectively, the results presented in this thesis make a significant contribution to understanding of pea inflorescence development and have allowed revision of the model for this process (Figure 8.1). The details of this model are discussed below.

During vegetative growth, *LF* is expressed in axillary meristems (A. Berbel, C. Ferrándiz and F. Madueño, unpublished data), where it delays their conversion to I<sub>2</sub> meristems by an unknown mechanism. It is possible that *LF* acts through repression of expression of *VEG1* in axillary meristems, as previously suggested (Beveridge et al., 2003). The V/I<sub>1</sub> transition appears to occur earlier in the *lf* mutant than wild-type (Murfet, 1975; Reid and Murfet, 1984), suggesting that *LF* normally represses this transition in wild-type plants (Figure 8.1). Roles in the V/I<sub>1</sub> transition are also apparent for *FDa* and *LATE5* (Figure 8.1). Results from this study suggest that *FDa/VEG2* acts to promote the V/I<sub>1</sub> transition in a photoperiod-independent fashion (Figures 3.2-3.5). All five FT proteins are candidates for acting with *FDa* to promote



**Figure 8.1.** A revised model for the genetic control of pea inflorescence development.  
(continued next page)



**Figure 8.1. (continued)** Coloured boxes surround each meristem, and include the key identity genes that specify and maintain meristem identity. Meristems are as follows: vegetative SAM (VM), vegetative axillary meristem (AM), primary inflorescence meristem (I<sub>1</sub>M), secondary inflorescence meristem (I<sub>2</sub>M), floral meristem (FM). Bold arrows between meristems indicate meristem transitions and circular arrows indicate meristem indeterminacy. The inferred roles are indicated for meristem identity genes as promoting (→) or repressing (—) each meristem transition and the expression or function of other meristem identity genes. Genes that act specifically under LD or SD conditions are shown as green or blue, respectively. Question marks and dashed lines indicate uncertainty.

Once the V/I<sub>1</sub> transition has occurred, DET/TFL1a most likely maintains indeterminate I<sub>1</sub> identity by acting in an anti-florigenic complex with FDa to repress expression of I<sub>2</sub> and floral meristem identity genes within the SAM (Figure 5.6C; Singer et al., 1990). Specifically, ectopic expression of *VEG1* within the SAM of *det* mutant plants and the expression of *PIM* in *veg1 det*, but not *veg1* mutant plants (Berbel et al., 2012), suggests that this complex represses expression of *VEG1* and *PIM* (Figure 8.1). Ectopic expression of *DET* in axillary meristems in the *veg1* mutant, suggest that *VEG1* may repress *DET* to specify and maintain I<sub>2</sub> identity (Berbel et al., 2012).

*FDa/VEG2* has a critical and photoperiod-independent role in the specification and maintenance of I<sub>2</sub> meristem identity, likely through regulation of *VEG1* expression (Figures 3.14-3.16). Once again, each of the five FT proteins in pea could potentially interact in a complex with FDa to promote I<sub>2</sub> identity (Figure 5.6B), but FTa1 and FTc stand out as clear candidates for this role (Figure 8.1). *FTa1/GIGAS* has an important role in promoting I<sub>2</sub> identity under LD but not SD photoperiods (Figures 3.14 and 3.15). Epistasis of *lf* and *det* over *gigas* suggest that the key roles of *FTa1/GIGAS* are to repress *LF* and *DET* function for specification of I<sub>2</sub> meristems (Taylor, 1998; Hecht et al., 2011). While competition for protein binding with FDa is the likely mechanism for antagonism with DET (Figure 5.6C), it is not yet clear how FTa1 represses *LF* function, but this could be via transcriptional regulation through interaction with FDa (Figure 8.1). *FTc* is expressed in the apex at higher levels than any of the other *FT* genes under SD conditions (Hecht et al., 2011), thus FTc could act with FDa to specify I<sub>2</sub> meristem identity under SD photoperiods. *LATE5* also has a role in promoting I<sub>2</sub> identity (Figure 6.4), through direct or indirect regulation of *VEG1* (Figure 6.11A).

It is likely that *PIM* functions as a transcriptional repressor during early stages of floral development in a similar manner to *Arabidopsis* (Kaufmann et al., 2010), to specify floral meristem identity by repressing *VEG1* and *DET* expression within floral meristems (Figure 8.1). In support of this theory, *VEG1* is ectopically expressed in the proliferating floral meristems of the *pim* mutant (Berbel et al., 2012). *FDa/VEG2* has an important role in the specification of floral meristems, in part through regulation of *PIM*, but potentially also through independent regulation of other floral genes including *UNI*, *AP3* and/or *SEP1* (Figures 3.14, 3.14, 5.7A and 5.8). The timing of induction indicates that *FTc* could act together with *FDa/VEG2* in this role (Figure 8.1). *LATE5* also appears to have an early role in controlling correct specification of floral meristems, likely through the regulation of the key floral meristem specification genes *PIM* and *UNI* (Figure 6.11A). Based on results from other angiosperm species, it is likely that *SVP*-like genes may also function during floral development (e.g. Gregis et al., 2006; Jaudal et al., 2013), but this remains to be investigated in pea.

### 8.3 Future directions

While the model presented above represents improved understanding of the genetic control of pea inflorescence development, there are many aspects of this process that remain to be addressed in future studies. First and foremost, the molecular identity of the *LATE5* locus and the functions of the three *SVP*-like genes present in pea are not yet known. Identification of the *LATE5* locus should now be a relatively straight forward task through fine-mapping and candidate gene analysis, with the availability of F<sub>3</sub> seed for a suitable mapping population (*late5* x JI399) and a few possible candidates in the area. Identification of *LATE5* is a particularly exciting prospect given that *LATE5* may represent a novel component of the flowering pathway. Similarly, future investigation of the *SVP*-like genes by isolation of mutants and further investigation of gene expression, as discussed in Chapter 7, would improve understanding of how these genes function during pea inflorescence development.

The mechanism of *LF* action remains an intriguing mystery, now that competition for protein binding with *FDa* can be ruled out as a possibility (Figure 5.6C). It seems likely that *LF* protein acts in a repression complex similar to *TFL1* in *Arabidopsis* (Hanano and Goto, 2011), but the remaining members of this putative complex remain to be determined. Novel proteins that bind to *LF* could be identified in a future study by co-immunoprecipitation. Testing of specific protein interactions between *LF* and pea *AREB3/DPBF4* homologs or other related class A bZIP transcription factors would also be worthwhile, given that wheat homologs of *AREB3/DPBF4* can bind to the promoters of MADS-box genes (Figure 4.4; Li and Dubcovsky, 2008).

The inherent differences in activity among pea *FT* proteins revealed by previous *Arabidopsis* complementation results (Hecht et al., 2011), and the photoperiod-based differences in the role of *FTa1/GIGAS* identified in this study also warrant further investigation. It is possible that a co-factor could show specificity in binding to the ligand-binding pocket present in *FT* proteins (Taoka et al., 2011), resulting in differences in activity and possibly even conferring photoperiod-specific differences in protein function. Co-immunoprecipitation could be used to identify proteins that bind to the pea *FT* proteins. The possibility that there may be differences in binding specificity between each *FT* protein and each 14-3-3 protein, and that this could affect complex formation and function, should also be investigated further.

This study made use of targeted qRT-PCR experiments to identify several genes regulated by *VEG2* and *LATE5* genes. However, the much broader approach using next-generation transcript sequencing will be helpful in future, both for discovery of additional targets and to gain a more comprehensive picture of the similarities and differences in the regulatory networks downstream of these genes. If a time-point were chosen at the time of wild-type flowering, novel genes that are differentially expressed in *late5*, *veg2-1* or *veg2-2* could be identified and then investigated in more detail by subsequent qRT-PCR and/or *in situ* hybridisation. Once mutants for *FTa2*, *FTb1*, *FTb2* and *FTc* are isolated, the targets of the five pea *FT* genes could also be investigated in the same manner. For the *FT* genes in particular, gene expression should be compared under different conditions, as results from this study suggest that these genes may function differently under LD and SD

photoperiods. As *FDa* is known to be a transcription factor that binds directly to target promoter sequence, it would be particularly interesting to examine which genes are direct targets of *FDa* by ChIP and/or transient assays. In particular, possible regulation of *SVP* and *TFL1* homologs by the *FD/FT* system in pea, which has not previously been reported in other species, should be investigated further to determine if this regulation is direct, and whether it could also be occurring in other legume and non-legume systems. Although also a novel finding, the regulation of *LFY* ortholog *UNI* by *FDa/VEG2* does not appear to be direct, as *UNI* is also downstream of *FDa* target *VEG1* (Appendix 5). The mechanism of *UNI* upregulation also warrants further investigation.

The finding that the V/I<sub>1</sub> transition eventually occurs in the three non-flowering mutants in pea raises the question of what alternative pathway promotes this transition when the *FDa/FT* pathway is blocked. This could be investigated through mutagenesis on a *veg2-2* mutant background to screen for mutations which further delay flowering time. Alternatively, a targeted approach to isolate and identify candidate genes based on knowledge in other systems could be adopted. In *Arabidopsis*, *LFY* functions independently of *FD/FT* to promote flowering (Ruiz-Garcia et al., 1997; Abe et al., 2005; Wigge et al., 2005), so the possibility that *UNI* could function prior to the V/I<sub>1</sub> transition redundantly with *FDa/VEG2*, should be investigated further. In addition, in *Arabidopsis* the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes define an endogenous age-dependent flowering pathway, regulated by microRNA156 (miR156), which acts in parallel with the *FT/FD* pathway and promotes flowering in the absence of photoperiodic cues (Wang et al., 2009). The possibility that a similar miR156/*SPL* pathway may operate to promote the V/I<sub>1</sub> transition in pea should be investigated further by gene isolation and functional analysis of the putative components of this pathway.

The exact mechanisms which lead to the development of a compound, rather than a simple inflorescence form in pea are not yet clear. The novel role of *VEG1* in specification of I<sub>2</sub> meristems may be a significant part of this mechanism (Berbel et al., 2012), but alteration of inflorescence architecture is likely to require a number of changes in the gene system controlling this process. It is an interesting exercise to imagine what combination of mutations in pea could potentially result in an inflorescence in the form of a simple raceme. As discussed earlier in Chapter 6,

conversion of the  $I_1$  meristem to an  $I_2$  meristem in a *det* background results in one or two axillary flowers borne directly from the main stem axis before termination of the SAM (Figure 6.8). Addition of *veg1* or *late5* mutations on to a *det* background prevents normal  $I_2$  structures from forming on the main stem prior to this, but the SAM still terminates prematurely in these plants (Figures 6.9 and 6.10). Perhaps addition of the *veg2-2* mutation on to a *veg1 det* or *late5 det* background would allow an indeterminate apex to be retained, similar to that seen in the  $I_2$  structures of *veg2-2* mutants (Figure 3.16). Further understanding of the mechanisms acting during pea compound inflorescence development, will allow investigation of differences that could account for the differences in inflorescence form seen between pea and other model angiosperms.

Lastly, there is considerable diversity in inflorescence form within the legume family itself, in terms of the number of flowers borne on inflorescence structures, arrangement of flowers and level of inflorescence branching (e.g. Tucker, 1987; Tucker, 2003). The genetic basis for this diversity has not yet been investigated, but knowledge of this would offer valuable insight into the evolution of inflorescence form, and may enable future optimisation of inflorescence structure for maximal yield and harvestability of crop legumes. With the ever-increasing availability of genomic and transcript resources for diverse legume species, it is becoming easier to isolate genes of interest from different legume species for subsequent functional characterisation, allowing comparison of gene function between species. A targeted focus on species with unusual inflorescence traits would contribute to progress in this area.

## 8.4 Concluding remarks

In conclusion, the results presented in this thesis improve understanding of the genetic control of inflorescence development in pea, particularly the important roles played by *FDa/VEG2*, and provide a strong foundation for future research in this area. Future characterisation of the *LATE5* locus and functions of the *SVP*-like genes should further contribute to knowledge of this process. The underlying genetic basis for differences in inflorescence form between angiosperm species remains an exciting area for future scientific discovery.

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## Appendix 1: Primer details

**Table A1.1.** Primers used on pea for all purposes except mapping. Primer names preceded by ‘Ps’ were designed from pea sequence and those preceded by ‘Mt’ were designed from *Medicago* sequence.

Gene	Purpose	Primer names	Primer sequences (5' to 3')	Tm (°C)	Source
<i>ACT</i>	qRT-PCR	PsACT-F PsACT-R	GTGTCTGGATTGGAGGATCAATC GGCCACGCTCATCATATTCA	59	Foo et al. (2005)
<i>AP3</i>	qRT-PCR	PsAP3-10F PsAP3-6R	TGGGGGATATTGATCTGTGG TCCAACTCCAAACCACCTTCACCT	60	This study
<i>DET (TFL1a)</i>	Full-length CDS for gateway entry construct	PsTFL1a-3F PsTFL1a-4R	AAAATGGCAAGAATGGCTCAA ATTCCCTTTCTGCAACACAAGT	60	This study
	qRT-PCR	PsTFL1a-1F PsTFL1a-2R	CGTTGGTAGAGTCATAGG AGGATCACTAGGGCCAGG	58	Hecht et al. (2011)
	Positive control PCR included with <i>FDa</i> PCRs on <i>veg2-1</i> template and <i>VEG1</i> PCRs on <i>veg1</i> template	PsTFL1a-1F PsTFL1a-Rev03	CGTTGGTAGAGTCATAGG CTTCTTGACGCGGTTTCTCT	55	V. Hecht, unpublished
<i>DUF343</i>	Determining the <i>veg2-1</i> deletion boundaries . Primers were used in various combinations; details are given in Section 4.2.3.	PsDUF-1F	AGATGAAGATGCATCAAAACCAGA	60	This study
		MtDUF-2F	TGGTTCCAAGAGATGGGAAG	60	
		MtDUF-R	CCAACAGCATGTTACAGAGA	60	
<i>FDa (VEG2)</i>	Genotyping for <i>veg2-1</i>	PsFD-7F PsFD-6R	ACCATGGAAGAGTTGTGGAAAG TCATTCACTTTCCGCATGAA	56	This study
	Genotyping for <i>veg2-2</i>	PsFD-4F PsFD-5R	GATCATTTTCCCTTCGACACTC GGGAAAGATTCAACACCACAA	60	Sussmilch (2008)
	Full-length CDS for gateway entry construct	PsFD-7F PsFD-5R	ACCATGGAAGAGTTGTGGAAAG GGGAAAGATTCAACACCACAA	60	This study
	qRT-PCR	PsFD-6F PsFD-3R	ATTTGATCCAAACGTCGGTGT ATCAACTTTTGTCTCCAGTTCCG	60	Sussmilch (2008)
	<i>in situ</i> probe (3')	PsFD-5F PsFD-6R	TTGCTGGAAGAGAATGCAAAG TCATTCACTTTCCGCATGAA	60	This study
	alternative <i>in situ</i> probe (5'; unsuitable)	PsFD-1F PsFD-2R	GACTCTCTCTCCCCAATCTCTT CTCATAATCATGGGTCGTCGTGTG	60	This study

Gene	Purpose	Primer names	Primer sequences (5' to 3')	Tm (°C)	Source
FDa ( VEG2) continued	Determining the <i>veg2-1</i> deletion boundaries . Primers were used in various combinations; details are given in Section 4.2.3. Primers are shown in order of location (5' → 3').	PsFD-1F	GACTCTCTCTCCCCCAATCTCTT	62	Sussmilch (2008); This study
		PsFD-GSP2R	TGTTTGGTGGGATAGGGCAATGTG	62	
		PsFD-7F	ACCATGGAAGAGTTGTGGAAAG	60	
		PsFD-GSP1R	TCATGGGTCGTCGTGTGTTTTGTT	62	
		PsFD-2R	CTCATAATCATGGGTCGTCGTGTG	62	
		PsFD-1R	GGCCGAGTGTTTAACTGAGAGC	62	
		PsFD-9R	GGTGTGGGATTTACGACATGG	62	
		FD-3R	ATCAACTTTTTGCTCCAGTTTCG	62	
		FD-5F	TTGCTGGAAGAGAATGCAAAG	60	
		FD-5R	GGGAAAGATTACAAACCACAA	60	
		PsFD-GSP1F	TGTGAATCTTTCCCGTTGTT	60	
		PsFD-GSP2F	ACACGCAGGAAAGTGAATGT	60	
		FD-6R	TCATTCACTTTCCGCATGAA	56	
		FD-7R	TGCACTCATTTGTCATAGTTGG	62	
		PsFD-8F	TGTTTTCTTCTCCTCGCGATA	56	
		PsFD-11R	CCGAAAATCTATTTCCAAACCA	58	
		PsFD-9F	CGCAACTCATATCCGAAAGT	58	
		PsFD-13R	GGCAACATGCAATGGGATGAACA	62	
		PsFD-12R	CATAAGGCAACATGCAATGG	58	
		PsFD-14R	TGCAAGTATGATTTTGGGCTACACC	62	
FDb (putative)	Attempted isolation of <i>PsFDb</i>	FD-1D	ATGGAAGAAGTDTGGAAWGAHATHAAYCTDKCTTC	60	Sussmilch (2008); This study
		FD-2D	CTAGCBCKKGAHCKWGCWGCAGAYTCYCKRTTCTTGATCA	60	
		FD-3D	MGRGARTCTGCWGCWMGDTMCMGVGCTAGRAARCAGG	60	
		MtFDP-1F	GACGACATAAGCGAATAATGAA	58	
		MtFDP-1R	AGCTCTTGATCGTGCAGCAG	62	
		MtFDP-2R	TGGAGCTGTTGATGTTTCGAT	58	
FTa1	Full-length CDS for gateway entry construct	PsFTLa-9F PsFTLa-4R	CCACATATGGCAGGTAGTAGC AGCGTCTCTGAGTGTAACGTGT	60	V. Hecht, unpublished
	qRT-PCR	PsFTLa-6F PsFTLa-2R	GCCCAAGCAACCCTACTTTT CCATCCTGGAGCGTAAACCC	60	Hecht et al. (2011)
FTa2	Full-length CDS for gateway entry construct	PsFTLb-5F PsFTLb-R4	ATGGCCTGTAGTAGCCGGAATCC AGCACCATTGATGCAGTACC	60	Hecht et al. (2011)
	qRT-PCR	PsFTLb-3F PsFTLb-5R	GGAAATGACCCCGTGATCTA TGAATCCCTAAGTTGGGTCG	60	
FTb1	Full-length CDS for gateway entry construct	PsFTLe-2FF PsFTLe-2R	ATGCGTATGAAATCATCGAATCC ATTATATGATCATCCTCCTTCCACC	62	Hecht et al. (2011)



Gene	Purpose	Primer names	Primer sequences (5' to 3')	Tm (°C)	Source
<i>FTb2</i>	Full-length CDS for gateway entry construct	PsFTLe-2FF PsFTLe2-1R	ATGCGTATGAAATCATCGAATCC GGTGCATGGATATGTTTAGAC	60	V. Hecht, unpublished
	qRT-PCR	PsFTLe2-F7 PsFTLe2-R7	CGACTACCGGGACAGCATTT CAGGTGAACCAAGGTTATAAAC	62	Hecht et al. (2011)
<i>FTc</i>	Full-length CDS for gateway entry construct	PsFTLc-9F PsFTLc-3R	ATGCCTAGGAATATGGTCGATCC GAACTAACCCCGCCCACTTGG	60	Hecht et al. (2011)
	qRT-PCR	PsFTLc-8F PsFTLc-7R	GATATTCCAGCCACAACAAGC TTATGACGCCACTCTGGAGCAA	62	
<i>FULa</i>	qRT-PCR	PsFULa-2F PsFULa-2R	GATTCTGCACTTGACCTCAACC TCATTTATAGGACGAAGCATCC	60	V. Hecht, unpublished
<i>FULb</i>	qRT-PCR	PsFULb-2F PsFULb-2R	GGAGGTGCAAAATAGCATGG TCTGCTCCGGTTTCTTCG	60	V. Hecht, unpublished
<i>PsGF14-1</i>	Full-length CDS for gateway entry construct	Ps14-3-3_1-1F Ps14-3-3_1-1R	ATGGCGGCCGCTCATACTCC CCCAGGACTCCAGAACTCAA	61	This study
	qRT-PCR	Ps14-3-3_1-2F Ps14-3-3_1-2R	CTGATCGTGCTGCAATCT TCCATAGGGTGAGATTATCACGA	60	
<i>PsGF14-2</i>	Full-length CDS for gateway entry construct	Ps14-3-3_2-1F Ps14-3-3_2-1R	ATGGCCACCGCACCAACAC CATTTTACTGTGGTTCATCATTGC	61	This study
	qRT-PCR	Ps14-3-3_2-2F Ps14-3-3_2-2R	TTCTGCTGCGCTAGGTGAC AGTTGGAGGCGAGCTCAGTGT	59	
<i>PsGF14-3</i>	Full-length CDS for gateway entry construct	Ps14-3-3_3-2F Ps14-3-3_3-1R	ATGGCGTATACCAAGGAACG AAGTTGCGAAAACCGCAGAC	62	This study
	qRT-PCR	Ps14-3-3_3-3F Ps14-3-3_3-2R	TTCCTTCATCCTCTGCTGGT TGGGATGTGTGGGAGCTAAC	59	
<i>PsGF14-4</i>	Full-length CDS for gateway entry construct	Ps14-3-3_4-1F Ps14-3-3_4-1R	ACCATGGCTTCTTCCACCAA TGTTACCGGATGATAACTACTTG	63	This study
	qRT-PCR	Ps14-3-3_4-1F Ps14-3-3_4-2R	ACCATGGCTTCTTCCACCAA CCATGAAGCTCTCCTTGACC	60	
<i>PsGF14-5</i>	Full-length CDS for gateway entry construct	Ps14-3-3_5-1F Ps14-3-3_5-1R	ATGGCTTCCACCAAGGATCG GTTCCCAACCAACACCAGTC	61	This study
	qRT-PCR	Ps14-3-3_5-2F Ps14-3-3_5-2R	AGCGGGTGAATCAACTGTGT GGGGTAATTTCAGCCTCTGCT	60	
<i>PsGF14-6</i>	Full-length CDS for gateway entry construct	Ps14-3-3_6-1F Ps14-3-3_6-1R	ATGTCCGCCGAGAAAGAGAG AGGGCCAGAGATCCAAAGAA	63	This study
	qRT-PCR	Ps14-3-3_6-1F Ps14-3-3_6-2R	ATGTCCGCCGAGAAAGAGAG TTCGTGCACCAATGACATTTT	60	
<i>PsGF14-7</i>	Full-length CDS for gateway entry construct	Ps14-3-3_7-1F Ps14-3-3_7-1R	ATGGATGTCACGGTTCCAGA TTCATAGCCACCTCCTTCA	63	This study
	qRT-PCR	Ps14-3-3_7-2F Ps14-3-3_7-2R	AAGATTGGGTTTGGCTCTCA AGGATTCTTCTCCCAACGTG	59	

Gene	Purpose	Primer names	Primer sequences (5' to 3')	Tm (°C)	Source
<i>LARP1C</i>	Initial isolation of partial sequence	MtHyp-F MtHyp-R	TAGAGGACGGTTTTGCTTGC TCAGCCACAAGAAAGCCATA	55	This study
	Isolation of full-length CDS	PsWiHe-1F PsHyp-1R	TCATCATCGGAGAGTCATCG TTCCCTCAGAGGCAGTAGGAAGC	55	
	Determination of <i>veg2-1</i> deletion boundaries. Primers were used in various combinations; details are given in Section 4.2.3.	PsHyp-1F	CCCGACTGTGCCCCAGTTGCT	62	
		PsHyp-GSP1F	CCGACTGTGCCCCAGTTGCTTTCT	62	
		PsHyp-GSP2F	GGTCAGCTTCAGCTTTCCACCAGT	62	
		PsHyp-GSP3F	TGACCATGTGCGGTCGATCA	62	
		PsHyp-GSP4F	CCAACATGAAGGAGTTGAATTGG	61	
<i>LATE1</i>	Positive control PCR included with <i>FDa</i> PCRs on <i>veg2-1</i> template	GI-L11F GI-L11R	AGGTCCTGGAATATCTCC CTTGCTTCCACTGAATTGG	55	Hecht et al. (2007)
<i>LF (TFL1c)</i>	Full-length CDS for gateway entry construct	PsLF-4F PsLF-5R	TTGTTCACTCTCTTGGGAAGTATG ATGATAATCGGCTTCTGATTGG	60	This study
	qRT-PCR	LF-CR2 LF-CR3	AAATAAGCAGCAGCAACAGGG CAGACATTCCAGGGACAACAG	60	Foucher et al. (2003)
<i>PIM</i>	Genotyping for <i>pim-2</i>	PsPIM-F1 PsPIM-R1	ACCTCTTAACATTCTTTGG AAGTGAAGAAAAGAGAATATA	50	V. Hecht, unpublished
	qRT-PCR	PsPIM-4F PsPIM-6R	GCTTCAGAGTTTGGAAACAGC GACTCCATGGTGGTTTGG	58	Hecht et al. (2011)
<i>RING finger</i>	Initial isolation of partial sequence	MtZnFin-F MtZnFin-R	GCAGAAACCCAAACAAACAA TCCCATTGAAAAACGACACA	62	This study
	Isolation of full-length CDS	PsZnFin-5F PsZnFin-3R	ACCACCCTTCCAAACCAAG CCATAGGCTGTGGTGGATT	58	
	Determination of <i>veg2-1</i> deletion boundaries. Primers were used in various combinations with primers in adjacent genes; details are given in Section 4.2.3.	PsZnFin-2F	TTAATTGGCCTGGAAGAGGA	58	
		PsZnFin-2R	CATGCGAACAAGGAAGAACA	58	
<i>SEP1</i>	qRT-PCR	PsPM6-7F PsPM6-8R	GATTGCCTGTACTGATTTG TTGTTGAGCTTGACTTGTGG	60	Hecht et al. (2011)
<i>SN (LUX)</i>	Genotyping for the <i>sn-1</i> allele	PsLUX-FLF PsLUX-sn1.2 PsLUX-7R PsLUX-8R	AACGGTTAGAAGAAGTGAATGG CGGATTCGGCGGTTAGACGC GTTGGAAAGACCTTGCATCC GGCCGTTTCAGCGTCGTTTC	58	Liew (2011)
<i>SOC1a</i>	qRT-PCR	PsSOC1a-Q1 PsSOC1a-Q2	GAGTGTAAAGCACTGTTAGAGCAAG TCTCGGATGATCATTTGGCGGTGG	65	V. Hecht, unpublished
<i>SVPa</i>	qRT-PCR	PsSVP-2F PsSVP-3R	CAACGGCGAGGCAAGTTACG TCTTCGAGTGCAAATGATGC	60	V. Hecht and S. Davidson, unpublished

Gene	Purpose	Primer names	Primer sequences (5' to 3')	Tm (°C)	Source
<i>SVPb</i>	qRT-PCR	PsSVPb-2F PsSVPb-5R	GAGTCCAGCGACACACTGC AAGGGTGTGATGCCTTGAA	57	This study
	Isolation of full-length CDS	PsSVPb-1F PsSVPb-4R	CTGGTTCGGTTTGTCTTGT CAGCGACATAGAAAGGAAGC	56	
<i>SVPc</i>	qRT-PCR	PsSVPc-1F PsSVPc-1R	TTCTTGCAAATTCAGCCACA ACCAACTTCAGCATCACACAA	59	This study
	3' RACE (gene specific primer)	PsSVPc-2F	CAATGGCTCGACAGAAGATAAAG	60	
	3' RACE (nested gene specific primer)	PsSVPc-3F	TGCAACAGCAAGACAAGTGACA	60	
<i>TFL1b</i>	qRT-PCR	PsTFL1b-3F PsTFL1b-4R	ACCAGGCACAACAGATGC AACAGCAGCCACAGGAGG	57	V. Hecht, unpublished
<i>UNI</i>	qRT-PCR	PsUNI-1F PsUNI-2R	CATCAGAGCTGAAAGAAGG GCTTCCTTTTCACGTTGC	55	Hecht et al. (2011)
<i>VEG1 (FULc)</i>	qRT-PCR and genotyping for <i>veg1</i>	PsFULc-2F PsFULc-2R	CGATGCCTTGAAACCATAGG AATTCCAATGACCCTCTTGC	58	Berbel et al. (2012)
	<i>in situ</i> probe	FULc-ISHF FULc-ISHR	TGGAGCTACCAATGAAACACAG ACCTGCTTCTTCAAATTCCAATG	58	Berbel et al. (2012)

**Table A1.2.** Primers used for isolation of full-length coding sequence for genes from species other than pea.

Species	Gene	Primer names	Primer Sequence	Tm	Source
<i>Arabidopsis</i>	<i>FD</i>	AtFD-1F AtFD-2R	TTTCCAATGTTGTCATCAGCTA TCCCCAAAAGAGAAACAAGG	58	This study
	<i>FT</i>	AtFT-1F AtFT-1R	AAACCACCTGTTTGTTCAGA CATCACCGTTCGTTACTCGT	63	This study
	<i>TFL1</i>	AtTFL-1F AtTFL-1R	AAGAAAATGGAGAATATGGGAAC AACTAGCGTTTGCGTGACAG	58	This study
Tomato	<i>SPGB</i>	SISPGb-1F SISPGb-1R	ATGTGGTCATCAAGCAGTGA TCAAAATGGAGCAGTTGACG	58	This study
	<i>SP</i>	SISP-1F SISP-1R	ATGGCTTCCAAAATGTGTGA CGATATATCAACGCCTTCTAGC	58	This study

**Table A1.3.** Vector-specific primers.

Vector	Source	Purpose	Primers	Primer Sequence	Tm	Source
pARC351	Derived from pRED-NLSa plasmid (P. Ouwerkerk) with modifications to introduce Gateway compatibility (R. Immink)	Yeast three-hybrid analysis	ADH1-1F ADH1-1R	CTCGTTCCCTTTCTTCCTTG CCTGAGAAAGCAACCTGACC	58	This study
pDEST22	Invitrogen ProQuest™ Two-Hybrid System	Yeast two- and three- hybrid prey	pDEST22-F Y2H-R	TGAAGATACCCACCAAACC CTCTGGCGAAGAAGTCCAAA	58	This study
pDEST32		Yeast two- and three- hybrid bait	pDEST32-F Y2H-R	CGCTACTCTCCCAAAACCAA CTCTGGCGAAGAAGTCCAAA	58	This study
pGEM-T Easy	Promega pGEM®-T Easy Vector System	Routine cloning	pGEMT-F pGEMT-R	GCCCGACGTCGCATGCTCC GAGCTCTCCCATATGGTCG	60	V. Hecht, unpublished
pYFN43	A. Ferrando (Belda-Palazón et al., 2012), derived from pMDC43 (Curtis and Grossniklaus, 2003)	BiFC analysis	BiFC-1F YFN-1R	TTTGAACGATCGGGGAAAT AGCTGAAGGGCATCGACTT	60	This study
pYFC43		BiFC analysis	BiFC-1F YFC-1R	TTTGAACGATCGGGGAAAT GTCCTGCTGGAGTTCGTGA	60	This study
TOPO	Invitrogen pCR®8/GW/TOPO® TA Cloning® Kit	Gateway entry	TOPO-1F TOPO-1R	CGCTAGCATGGATGTTTTCC AGGGGATATCAGCTGGATGG	65	V. Hecht, unpublished

**Table A1.4.** Primers used for molecular markers in various  $F_2$  mapping populations. Details of restriction enzymes or product melt temperatures are shown for CAPS and HRM markers, respectively. For details of plant lines see Section 2.1.

Locus	PsLG	Primer names	Primer Sequence	Tm	Population	Marker type	Source
<i>Acetisom</i>	VII	PsACETISOM-F1 PsACETISOM-R2	GATGGCAGGGCTACTAATGTTGC GGGGAAATGCAGGCAGACCCTC	60	<i>late2</i> x TER	CAPS (MseI)	Bordat et al. (2011)
<i>Aldo</i>	VII	PsAldo-F PsAldo-R	GAGAATCCCCCTGCTGTCC CAGCATAATACTCAGTAGCACC	65	<i>late2</i> x TER	Size	Aubert et al. (2006)
<i>AP2</i>	I	PsAP2-5g-2F PsAP2-1R	TTGAGTATTGTTTTGGTTGTG ACCCAAATACACTTGTTTTCC	55	<i>late5</i> x JI399	HRM (74°C)	This study
<i>AP3</i>	I	PsAP3-10F PsAP3-12R	TGGGGGATATTGATCTGTGG GGAAGTGTGAAACAGAAAGCA	55	<i>late5</i> x JI399	CAPS (PstI)	This study
<i>AS2</i>	I	PsAS2-F PsAS2-R	CTAATCACACGTTTAGGACCGG CGAAATCCAAACCGAACCTAATCC	55	<i>late5</i> x TER	CAPS (RsaI)	Aubert et al. (2006)
		PsAS2-F PsAS2-2R	CTAATCACACGTTTAGGACCGG CTGTTGTCGCGAGTATCCAA	55	<i>late5</i> x JI399	HRM (77°C)	This study
<i>CPK16</i>	I	PsCPK16-4F PsCPK16-4R	TTGTCCCCAATTTAGATTTTT AAGACACATTGGCCTCAAGC	53	<i>late5</i> x JI399	HRM (76°C)	This study
<i>DUF151</i>	I	MtDUF151-2F MtDUF151-2R	GCGTTTTCAGAATCCACACA TGCAACATTAATGGCATCTG	55	<i>late5</i> x TER	CAPS (AseI)	This study
		PsDUF151-5F PsDUF151-5R	CAACACCCATGGCCAAATCA ACATGCAATAGACACCGATGC	55	<i>late5</i> x JI399	HRM (77°C)	This study
<i>DUF343</i>	I	PsDUF-1F PsDUF-1R	AGATGAAGATGCATCAAAACCAGA GGAAGAGTGAGGGGAGGGCAACA	55	<i>late5</i> x TER NGB5839 x JI1794	HRM (78°C)	This study
<i>EMB2754</i>	I	PsEMB-5F PsEMB-5R	TGGGTGGGTTCTTAGGAGTT CATTTTCCATTTCCACACCA	55	<i>late5</i> x JI399	HRM (77°C)	This study
<i>F-box</i>	I	MtFbox-4F MtFbox-4R	GCAGGGGCCTCAGTGATAC ATGGGGATCAGCAATACCTC	55	<i>late5</i> x TER	CAPS (HpyCH4IV)	This study
		PsFBOX-5F PsFBOX-5R	TGGAGCCCCGATTTTGTAAAG ATTATCTCCAAAAGGAAGAAAGC	55	NGB5839 x JI1794 <i>late5</i> x JI399	HRM (76°C)	This study
<i>FDa</i>	I	PsFD-10F PsFD-GSP2R	GCGGTAATCATTTCTGACTCA TGTTTGGTGGGATAGGGCAATGTG	55	NGB5839 x JI1794	HRM (80°C)	This study
<i>FENR1</i>	I	FENR1-HF FENR1-HR	ATGCTTATGCCAAAAGATCCTAATGC CTCTGCTTACAGCAAAGTCAAGCCTGAAGTT	60	<i>late5</i> x TER	CAPS (HpyCH4IV)	J. Hofer, unpublished
<i>FG-GAP</i>	I	PsFGGAP-5F PsFGGAP-5R	CATCTAATCTGATGATGTAGCTT TTCCCATTTCTCAAAGGAT	55	<i>late5</i> x JI399	HRM (74°C)	This study

Locus	PsLG	Primer names	Primer Sequence	Tm	Population	Marker type	Source
GATA-TF	I	PsGATA-1F PsGATA-2R	TCTTCCACCATTCCCCAAG TTCCAACGCACTCCATTTT	55	NGB5839 x JI1794	HRM (75°C)	This study
		PsGATA-dF PsGATA-1R	CCATTCCAAGTCCGCAAC CCGGTGCATTTTGTTATTTTTTATT	50	late5 x JI399	dCAPS (Swal)	This study
LARP1C	I	PsHyp-GSP1F PsHyp-2R	CCGACTGTGCCCCAGTTGCTTTCT CCATGCAACTTCTTCAGGTC	55	NGB5839 x JI1794	HRM (81°C)	This study
		PsWiHe-5F PsWiHe-4R	GCCGTTTCTTCATCGCCG CTCCGAAGCTGCACCATTGG	60	late5 x JI399	CAPS (Tfil)	This study
MitCySyn	VII	PsMITCYSYN-F2 PsMITCYSYN-R2	CAGGTCGGTTTCGGTTTCACACTG GAGCTGTGCAAAGGAGATACGTC	58	late2 x TER	Size	Bordat et al. (2011)
Q20-950	I	Q-20	TCGCCCAGTC	40	late5 x TER	RAPD	Laucou et al. (1998)
R11-730	I	R-11	GTAGCCGTCT	40	late5 x TER	RAPD	Laucou et al. (1998)
RGP	I	PsRGP-F2 PsRGP-R	AAGGTATATTAGTCATATCTCCC CCCAAATGGTCACAGATAACC	60	late5 x TER	Size	Aubert et al. (2006)
		PsRGP-3F PsRGP-2R	GGGAGTGAGTTTGACTTTGG TTCCTTTTGA AAACCAATTCTC	55	late5 x JI399	HRM (77°C)	This study
RING finger	I	PsZnFin-2F PsZnFin-2R	TTAATTGGCCTGGAAGAGGA CATGCGAACAAGGAAGAACA	55	NGB5839 x JI1794	HRM (79°C)	This study
		PsZnFin-6F PsZnFin-4R	TTTCTTGTTTTCATCGTCCAA TAAGCGAGTGATTGGTGTGG	55	late5 x JI399	HRM (75°C)	This study
SVPa	I	dSVP-TER PsSVP-3R	AGATTAGTACATAAATTTGTTGGTGTAGTCA TCTTCGAGTGCAAATGATGC	50	late5 x TER	dCAPS (Tsp45I)	Sussmilch (2008)
		PsSVPa-13F PsSVPa-13R	ACATGGACAATGGATGCAGA TCTATCTTCAATCTCAAACACA	55	late5 x JI399	HRM (74°C)	This study
SVPb	VII	PsSVPb-5F PsSVPb-6R	GGTAAAAATGCTAAACCAAAAACC GGGAGTAGCAGAAAGATGTGC	53	late2 x TER	HRM (77°C)	This study
SVPc	I	PsSVPc-1F PsSVPc-1R	TTCTTGCAAATTCAGCCACA ACCAACTTCAGCATCACACAA	57	late5 x TER	CAPS (HinfI)	This study
		PsSVPc-1F PsSVPc-2R	TTCTTGCAAATTCAGCCACA CGACCTAAATTTGACCAAAAACC	55	late5 x JI399	HRM (76°C)	This study
UNK1	I	PsUNK1-4F PsUNK1-4R	TTCTTCATTAAGCAACAAGCAA CCGAGCTAGGATCTGCAAAA	55	NGB5839 x JI1794	HRM (75°C)	This study
		PsUNK1-4F PsUNK1-dR	TTCTTCATTAAGCAACAAGCAA CTATCCCGAGCTAGGATCTGCAAAAACAAGA	55	late5 x TER late5 x JI399	dCAPS (MbolI)	This study
WUS	I	PsWUS2F PsWUS2F	AGAGAGAAAACACAAACAAGTGA AGTGAAGTGAAATCTTGTGG	55	late5 x JI399	HRM (75°C)	This study

**Table A1.5.** Details of regions sequenced in NGB5839 and T  r  se that contained no polymorphisms between the two lines. All primers in this table were designed during this study.

<i>Medicago</i> locus (Mt3.5)	Name	Primer name	Primer sequence	Tm	Gene region	Approximate size in pea (bp)
Medtr5g021580	COL3	PsCOL3-1F	CTGCACTCTGTCGTGCTTGT	55	Exon 1 - Exon 4	900
		PsCOL3-1R	AAGGTTGCGAAGACGGATT			
		PsCOL3-2F	GCCTCCGGTTCCAACCTTA	59	Exon 4 - Exon 5	600
Medtr5g021520	UNK3	PsCOL3-3R	CGTGCCTGAATAGCATTGTC			
		PsUNK3-8F	GATCTCCGTCCCGGTCTC	55	Exon 1 - Exon 2	500
		PsUNK3-8R	TTCACGTGCACCACATCA			
		MtUNK3-3F	TGAAGCATTTGCTCAGATTG	50	Exon 2 - Exon 3	500
		MtUNK3-1R	CCACGGATCAAAACGAAGA			
		PsUNK3-9F	CATTTGCAGGAACCCCTA	50	Exon 3 - Exon 4	1250
		PsUNK3-4R	GGCCAGAGACTCACCCAAT			
		MtUNK3-2F	TGTAAAGCGGCTTCAAATG	50	Exon 4 - Exon 8	1000
		MtUNK3-2R	GGATGCACCAAAAGGAATGT			
Medtr5g021430	TMCC	PsUNK3-5F	CAGTTTGCGTTTTTGTGTT	50	Intron 7 - Intron 9	950
		MtUNK3-5R	CCGAAGATCAGAGGCAGAA			
		MtUNK3-6F	CGGCCACGGACAACATATC	50	Exon 10 - Exon 13	700
		MtUNK3-6R	CAAATGGGTTACTGGCAGA			
		PsUNK3-10F	CTTGGGGATTGTGGGAGA	55	Exon 13 - Exon 15	500
		PsUNK3-9R	GCAACCACCCCTTGAAC			
		PsTMCC-3F	GAGAGTTGGGAGTGTGACG	58	Exon 6 - Exon 9	700
Medtr5g021430	TMCC	PsTMCC-3R	CAATCGACCAGGTGCTGTC			
		PsTMCC-2F	TGACTGGATGCTTGGAGTTG	58	Exon 14 - Exon 16	700
		PsTMCC-2R	GCCCACAAATAAGAAGAGTGAC			
Medtr5g021340	GATA-TF	PsGATA-1F	TCTTCCACCATTCCTCAAG	55	Exon 1 - Exon 3	600
		PsGATA-1R	CCATTCCAAGTCCGCAAC			
Medtr5g021270	AP3	PsAP3-12F	TGTGGCAGCTGTCCAATAAT	55	5' UTR - Exon 2	700
		PsAP3-8R	CGCCACAGATCAATATCCCCCAA			
		MtAP3-5F	AATGGGTCGTGGGAAGATTG	50	Exon 1 - Exon 6	950
		MtAP3-3R	ATTTCCATTCTGCTCCA			
		PsAP3-7F	TGGAGCAGATGAATGGAAACCTCCT	58	Exon 6 - 3' UTR	600
		PsAP3-11R	ATCCCACTGCCACAACACAT			

<i>Medicago</i> locus (Mt3.5)	Name	Primer name	Primer sequence	Tm	Gene region	Approximate size in pea (bp)
Medtr5g021240	<i>D2HD</i>	PsD2HD-1F PsD2HD-1R	TGGTTCAATGGGTGGAGTG AACAACGGCCAGGTTTCTG	55	Exon 1 - Exon 2	850
		PsD2HD-3F PsD2HD-3R	GAAGCTGGGTGCATATTGG GCAAGCAAGAAGAGCCACA	55	Exon 4 - Exon 7	1000
		PsD2HD-5F PsD2HD-5R	TCCGCATTGAATTTTTGG GCAAGAACACCATCCGCTAT	50	Exon 10 - Exon 12	500
		PsD2HD-7F PsD2HD-7R	TGTCGAAGAAATGCGCTCT GCATCAAATCAAACCCATGT	55	Exon 13 - Exon 16	650
Medtr5g021150	<i>FG-GAP</i>	PsFGGAP-1F PsFGGAP-1R	CCATAAAATTGGGCAACCAC GCACTCTTCGATGCTGCTC	55	Exon 3 - Exon 5	750
		PsFGGAP-2F PsFGGAP-2R	GACGCATCACAGTTAATTCCA TGGGCCAGCTTCAATAAAGT	55	Exon 7 - Exon 9	800
		PsFGGAP-3F PsFGGAP-3R	TTGGTGGGTTCTAATGTTG CAGTTGATTGTTGCCACTGC	55	Exon 12 - Exon 15	950
		PsGLUT-1F PsGLUT-1R	TTCAGTTTCCGCTTTTCGTTT TGATCCACCAATATGCTTCC	55	Exon 1 - Exon 3	450
Medtr5g020850	<i>APFIL</i>	PsAPFIL-1F PsAPFIL-1R	CCAAGTGCTTCCATTACCG ACCTTTCCAGGCAAATTAGA	55	Exon 2 - Exon 3	1500
Medtr5g020640	<i>APO</i>	PsAPO-1F PsAPO-1R	TGTTTCGTCTGATGCTGTTT TGTGCAAAGCAGTATTGAATG	55	Exon 1 - Exon 4	750
		PsAPO-3F PsAPO-3R	CCAGTTGAGGGTAGCGAAG GCCGTCCAAGTTTGTCTG	55	Exon 5 - Exon 8	1400
Medtr5g020440	<i>GTPBP</i>	PsGTPBP-1F PsGTPBP-1R	CGGTCCATTCCAAATCAAA ACCATCTCGCAACTCTTTCC	59	Exon 5 - Exon 8	850
Medtr5g020140	<i>EMB2754</i>	PsEMB-1F PsEMB-1R	TGTTGGGGATAACTTTGCATC GCCATCTCCCGAGCTCCA	59	Exon 6 - Exon 7	400
		PsEMB-2F PsEMB-2R	TGCCTGAGGACATTGTTGAG TGTTTGGAGAGTGCTGCTTC	59	Exon 7 - Exon 8	800
		PsEMB-3F PsEMB-3R	TCCATCCCAAATATTGAGC CAAAAGAGGCCCAAGAAATG	56	Exon 8 - Exon 11	1350
		PsTIF11B-1F PsTIF11B-1R	TGTTTCAGGCCATTAGAGAAG TGTGCGACATTAGCTGCAA	55	Exon 1 - Exon 2	450
Medtr5g019910	<i>TIFIIB</i>	PsTIF11B-2F PsTIF11B-2R	TGCAGCTAATGTGCGACAG AATTTTCGGCTGAGTTTTCC	59	Exon 2 - Exon 3	800
		PsSym10-1F PsSym10-1R	CCCCTCGAAGAATCAGTTGAG GCCGGTAAGCAACTCAATCAG	54	Exon 1 - Exon 1	850
Medtr5g019040	<i>Sym10</i>	PsSym10-2F PsSym10-2R	TTTTGGGGTGGTTCTGATTG CATTTTATGGGTGCCAAAGG	55	Exon 1 - 3' UTR	850



## Appendix 2: Sequences and sequence alignments for Chapter 4

**Table A2.1.** Details of sequences used for phylogenetic analyses in Chapter 4. Asterisks indicate an altered annotation was used; putative intron sequence was removed or annotation of exons was corrected based on transcript sequence or alignments between species. Source details including websites and relevant references are outlined in Chapter 2 (Table 2.4).

Species	Gene name	Sequence	Source	Reference (s)
Apple ( <i>Malus domestica</i> )	MdFD1	MDP0000169473*	<i>Malus domestica</i> genome v1.0	Tsuji et al. (2013b)
	MdFD2	MDP0000636541*		
<i>Aquilegia coerulea</i>	AqucaFD	Aquca_075_00022*	<i>Aquilegia coerulea</i> genome assembly v1.1	This study
<i>Arabidopsis thaliana</i>	AtABF1 (AtbZIP35)	NP_564551	GenBank	Jakoby et al. (2002)
	AtABF2 (AtbZIP36)	NP_849777		
	AtABF3 (AtbZIP37)	NP_567949		
	AtABF4 (AtbZIP38)	NP_566629		
	AtABI5 (AtbZIP39)	NP_565840		
	AtAHBP1 B (AtbZIP20)	NP_196312		
	AtAREB3 (AtbZIP66)	NP_191244		
	AtbZIP1	NP_199756		
	AtbZIP3	NP_197087		
	AtbZIP4	NP_176162		
	AtbZIP5	NP_566925		
	AtbZIP6	NP_179870		
	AtbZIP7	NP_195487		
	AtbZIP8	NP_177054		
	AtbZIP9	NP_568457		
	AtbZIP13	NP_199221		
	AtbZIP15	NP_199105		
	AtbZIP16	NP_850248		
	AtbZIP17	NP_565946		
	AtbZIP18	NP_181594		
	AtbZIP19	NP_567974		
	AtbZIP21	NP_563810		
	AtbZIP23	NP_179268		
	AtbZIP24	NP_190764		
	AtbZIP25	NP_567003		
	AtBZIP28	NP_187691		
	AtbZIP29	NP_849520		
	AtbZIP30	NP_179719		
	AtbZIP31	NP_178956		
	AtbZIP32	NP_178948		
	AtbZIP33	NP_565355		
	AtbZIP34	NP_565970		
	AtbZIP42	NP_189674		
	AtbZIP43	NP_198696		
	AtbZIP44	NP_177672		
	AtbZIP48	NP_178489		
	AtbZIP49	NP_191225		
	AtbZIP50	NP_565162		

Species	Gene name	Sequence	Source	Reference (s)
<i>Arabidopsis thaliana</i> (continued)	AtbZIP52	NP_172170	GenBank	Jakoby et al. (2002)
	AtbZIP53	NP_191801		
	AtbZIP58	NP_172817		
	AtbZIP60	NP_174998		
	AtbZIP61	NP_191371		
	AtbZIP62	NP_173381		
	AtbZIP65	NP_850784		
	AtbZIP68	NP_174494		
	AtbZIP69	NP_172097		
	AtbZIP70	NP_200891		
	AtbZIP71	NP_180011		
	AtbZIP72	NP_196333		
	AtbZIP74	NP_850010		
	AtbZIP75	NP_850791		
	AtbZIP76	AAU94427		
	AtbZIP77	AAM64869		
	AtbZIP78	NP_849319		
	AtBZO2H1 (AtbZIP10)	NP_192173		
	AtBZO2H3 (AtbZIP63)	NP_568508		
	AtDPBF2 (AtbZIP67)	NP_566870		
	AtDPBF4 (AtbZIP12)	NP_850341		
	AtFD (AtbZIP14)	BAC65864	GenBank	Abe et al. (2005; 2005); Wigge et al. (Wigge et al., 2005)
	AtFDP (AtbZIP27)	CAD29861		
	AtGBF1 (AtbZIP41)	NP_195391	GenBank	Jakoby et al. (2002)
	AtGBF2 (AtbZIP54)	NP_192021		
	AtGBF3 (AtbZIP55)	NP_182150		
	AtGBF4 (AtbZIP40)	NP_171893		
	AtGBF5 (AtbZIP2)	NP_179408		
	AtGBF6 (AtbZIP11)	NP_195185		
	AtHY5 (AtbZIP56)	NP_568246		
	AtHYH (AtbZIP64)	NP_850605		
	AtOBF (AtbZIP26)	NP_196313		
	AtPAN (AtbZIP46)	NP_177031		
	AtPosF21 (AtbZIP59)	NP_001031456		
	AtTGA1 (AtbZIP47)	NP_851273		
	AtTGA3 (AtbZIP22)	NP_564156		
	AtTGA4 (AtbZIP57)	NP_196565		
	AtTGA6 (AtbZIP45)	NP_566415		
	AtVIP1 (AtbZIP51)	NP_564486		
Banana ( <i>Musa acuminata</i> )	MaFD1	GSMUA_Achr1P02640_001*	The Banana Genome Hub	Tsuji et al. (2013b)
	MaFD2	GSMUA_Achr5P11470_001*		This study
	MaFD3	GSMUA_Achr9P24090_001*		
	MaFD4	GSMUA_Achr9P21040_001		
	MaFD5	GSMUA_Achr5P11220_001*		
Barley ( <i>Hordeum vulgare</i> )	HvFD1	BAK04622	GenBank	Tsuji et al. (2013b)
	HvFD2	AK249012		
	HvFD3	AK359958		

Species	Gene name	Sequence	Source	Reference (s)
<i>Brachypodium distachyon</i>	BdFD1	XP_003578537	GenBank	Tsuji et al. (2013b)
	BdFD2	XP_003563257		
	BdFD3	XP_003570743		
Cassava ( <i>Manihot esculenta</i> )	MeFD1	cassava4.1_021397m*	Cassava genome v4.1	This study
	MeFD2	cassava4.1_029555m*		
	MeFD3	cassava4.1_023228m*		
	MeFD4	cassava4.1_021412m*		
Common bean ( <i>Phaseolus vulgaris</i> )	PvFDa	Phvul.002G105700*	<i>Phaseolus vulgaris</i> genome v1.0	This study
	PvFDb	Phvul.003G266100		
	PvFDc	Phvul.009G018700		
Cotton ( <i>Gossypium raimondii</i> )	GrFD1	Gorai.003G007000	Cotton genome v2.1	This study
	GrFD2	Gorai.007G191800		
	GrFD3	Gorai.009G192500		
	GrFD4	Gorai.009G269300		
	GrFD5	Gorai.002G122200		
Cucumber ( <i>Cucumis sativus</i> )	CsFD	Cucsa.322810	<i>Cucumis sativis</i> genome v1	This study
Date palm ( <i>Phoenix dactylifera</i> )	PdFD1	PDK_30s1175071g003	Date palm draft genome v3	Tsuji et al. (2013b)
<i>Eucalyptus grandis</i>	EgFD	Eucgr.J02482*	<i>Eucalyptus grandis</i> genome assembly v1.1	This study
Foxtail millet ( <i>Setaria italica</i> )	SiFD1	Si031077m	<i>Setaria italica</i> genome v2.1	Tsuji et al. (2013b)
	SiFD2	Si007412m		
	SiFD3	Si023448m		
	SiFD4	Si014546m		
Grape ( <i>Vitis Vinifera</i> )	VvFD1	CBI19920	GenBank	Tsuji et al. (2013b)
	VvFD2	XP_003635259		
Kiwifruit ( <i>Actinidia chinensis</i> )	AcFD	AGK89941	GenBank	Varkonyi-Gasic et al. (2013)
<i>Lotus japonicus</i>	LjFDa	chr2.CM0272.780.r2.m	<i>Lotus japonicus</i> genome assembly build 2.5	This study
<i>Medicago truncatula</i>	MtFDa	Medtr5g022780*	<i>Medicago</i> genome v3.5	This study
	MtFDb	Medtr8g075130*	<i>Medicago</i> genome v3.5	
	Mt Group A bZIPs	Medtr1g098590		
		Medtr2g086340		
		Medtr2g086390		
		Medtr3g010660		
		Medtr3g101780		
		Medtr4g085910		
		Medtr5g088230		
		Medtr6g026800		
		Medtr7g088090		
		Medtr7g104480		
		Medtr8g043960		
Maize ( <i>Zea mays</i> )	ZmDLF1	NP_001105962	GenBank	Muszynski et al. (2006)
	ZmFD2	NP_001130586		Tsuji et al. (2013b)
	ZmFD3	NP_001147710		
Peach ( <i>Prunus persica</i> )	PpFD1	ppa018386m*	Peach genome v1.0	This study
	PpFD2	ppa021354m*		
Poplar ( <i>Populus trichocarpa</i> )	PtFD1	XP_002327643*	GenBank	Tsuji et al. (2013b)
	PtFD2	XP_002307742		This study
	PtFD3	XP_002300691		
Potato ( <i>Solanum tuberosum</i> )	StFD1	not annotated; Chr02:60345702..60350121	<i>Solanum tuberosum</i> genome annotation v3.4	Tsuji et al. (2013b)
	StFD2	PGSC0003DMP400041309		
Rice ( <i>Oryza sativa</i> )	OsAREB3	LOC_Os05g41070	<i>Oryza sativa</i> genome, MSU Release 7.0	Tsuji et al. (2013b)
	OsFD1	LOC_Os09g36910		
	OsFD2	LOC_Os06g50600		
	OsFD3	LOC_Os02g58670		
	OsFD4	LOC_Os08g43600		
	OsFD5	LOC_Os06g50830		
	OsFD6	CT843963	GenBank	

Species	Gene name	Sequence	Source	Reference (s)
<i>Sorghum bicolor</i>	SbFD1	XP_002460587	GenBank	Tsuji et al. (2013b)
	SbFD2	XP_002439039		
	SbFD3	XP_002453108		
Soybean ( <i>Glycine max</i> )	GmFDa1	Glyma01g36810*	Soybean genome v1.1	Jung et al. (2012)
	GmFDa2	not annotated; Chr11: 5997867-5996321; proposed Glyma11g08490		This study
	GmFDb1	Glyma02g05100*		
	GmFDb2	not annotated; Chr16: 26917990-26913770; proposed Glyma16g23200		
	GmFDc1	Glyma04g02420		Tsuji et al. (2013b)
	GmFDc2	Glyma06g02470		
Strawberry ( <i>Fragaria vesca</i> )	FvFD1	mrna14556.1-v1.0-hybrid	<i>Fragaria vesca</i> genome v1.1	Tsuji et al. (2013b)
	FvFD2	mrna08566.1-v1.0-hybrid		
Tomato ( <i>Solanum lycopersicum</i> )	SISPGB	Solyc02g083520	Tomato genome ITAG2.3	Pnueli et al.(2001); Tsuji et al. (2013b)
	SIFD2	Solyc02g061990*		
Wheat ( <i>Triticum aestivum</i> )	TaFDL1	CK206464	GenBank	Li and Dubcovsky (2008); Tsuji et al. (2013b)
	TaFDL2	ABZ91908		
	TaFDL3	ABZ91909		
	TaFDL6	ABZ91910		
	TaFDL13	ABZ91911		
	TaFDL15	ABZ91912		

### Sequence alignment for Figure 4.1

Alignment for Figure 4.1, a phylogenetic neighbour-joining tree of full-length protein sequence for *Medicago* and *Arabidopsis* group A bZIP transcription factors, including the previously identified MtFD (MtFDa) and also the pea gene PsFD (PsFDa; Sussmilch, 2008). Full-length predicted protein sequences were aligned with ClustalX (Thompson et al., 1997) and manually adjusted with GeneDoc (Nicholas and Nicholas, 1997). Shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Sequence details are given in Table A3.1. Partial names are given for *Medicago* loci, full names are as follows: Medtr1g098590, Medtr2g086340, Medtr2g086390, Medtr3g010660, Medtr3g0101780, Medtr4g085910, Medtr5g088230, Medtr6g026800, Medtr8g075130\*, Medtr7g088090, Medtr7g104480, Medtr8g043960.

			*	20	*	40	*	60	
PsFD	:	-----							:
MtFD	:	-----							:
Medtr8g075	:	-----					MSHQPLQEQT	PQQ--QHH	: 17
AtFD	:	-----							: 50
AtFDP	:	-----							: 30
Medtr3g101	:	-----							: 45
AtABF2	:	-----							: 39
AtABF1	:	-----							: 41
AtABF4	:	-----							: 52
AtABF3	:	-----							: 45
Medtr4g085	:	-----							: 36
AtbZIP15	:	-----							: 38
Medtr3g010	:	-----							: 38
Medtr8g043	:	-----							: 37
Medtr5g088	:	-----							: 35
Medtr7g088	:	-----							: 34
Medtr6g026	:	-----							: 34
AtDPBF4	:	-----							: 34
AtAREB3	:	-----							: 34
Medtr2g086	:	-----							: 23
Medtr2g086	:	-----							: 37
Medtr7g104	:	-----							: 57
AtAB15	:	-----							: 55
AtDPBF2	:	-----							: 50
AtbZIP13	:	-----							: 38
AtGBF4	:	-----							: 35
Medtr1g098	:	-----							: 26
AtbZIP61	:	-----							: 34
			*	80	*	100	*	120	
PsFD	:	-----							: 20
MtFD	:	-----							: 19
Medtr8g075	:	LR--	PNKPPKNT	EDVWKD	INLPSLT	NHM--			: 44
AtFD	:	QDSQAQ	KRSLVT	EEVWND	INLASI	HHLNRH	SPHPQ		: 86
AtFDP	:	---	LGHNKSQVT	EEVWKE	INLGS	LHYHRQLN			: 59
Medtr3g101	:	GG--	SGKDFGSMN	DELLKNI	WSAEVQ	TMG--			: 74
AtABF2	:	G---	KDFGSMN	DELLKNI	WSAETQ	AMASGV	VPVL--		: 72
AtABF1	:	GE--	PGKDFGSMN	DELLKNI	WTAEDT	QAFMTT	TS--		: 74
AtABF4	:	GG--	PGKDFGSMN	DELLKSI	WTAEAA	QAMAMT	SAPAA--		: 88
AtABF3	:	GGGIGK	DFGSMN	DELLKNI	WTAEES	HMMGNNT	SYTNIS	NGNSGNT	: 101
Medtr4g085	:	GG--	VGKDFGSMN	DELLKNI	WNVEET	QALTS	LTG--		: 69
AtbZIP15	:	G---	LDCGSMN	DELVKH	ISSAET	QEGS--			: 64
Medtr3g010	:	GD--	LGKPLSSMN	DELLKN	VWTV	VEANQS	SIRMENE--		: 71
Medtr8g043	:	GD--	LGKPLSSMN	DELLKN	VWTV	EVNQ	STNTDNE--		: 70
Medtr5g088	:	GD--	LGKPLSSMN	DELLQNV	WTAE	GNKV	VGMES--		: 68
Medtr7g088	:	GN--	LGKPLSSMN	DELLKSL	WTSEAT	QGSGL	DSG--		: 67
Medtr6g026	:	GN--	LGKPLSSMN	DELLKSV	WSVEA	GEVSD	FGGSDVA--		: 70
AtDPBF4	:	GS--	SGKPLSSMN	DELLKTVL					: 54
AtAREB3	:	GS--	SGKALGSMN	DELLKSV	CSVEAN	QPSSMA	VN--		: 67
Medtr2g086	:	CN--	NDKNFSSLN	DEFLASI	WSSNDE--	DTAQ--		THNNTES	: 63
Medtr2g086	:	CK--	SGKSFSSLN	DEFLASI	WSSNDE--	ATTH--		THN--TKNV	: 76
Medtr7g104	:	CD--	SGKNFGSMN	DEFLSSI	WNAEEN--	QQQAAS--		NNN--NSN	: 97
AtAB15	:	CE--	NGKNFGSMN	DEFLVSI	WNAEEN	NNNQQA	AAAAAG	SHSV	: 114
AtDPBF2	:	MK--	SGKSF	GAMN	DEFLAN	LWTVEE--		NDNEGGG	: 87
AtbZIP13	:	RDLT	SVGYGGR	NDGLYSS	NSMT	VEGILH	DTFASD	PPAPTE	: 81
AtGBF4	:	PNPHAD--	HSRIS	FAYGG--		GVNDYT	FASDS--		: 62
Medtr1g098	:	-----						FLDDQ--	: 31
AtbZIP61	:	ATAGP	QQNP	SWN	DEFL	DFSAT	RRGTHRR	SISDS--	: 68

		*	140	*	160	*	180	
PsFD	:	-----	PMIMSTRDSSTFGGVILQDFLARFL	---	NINPPKNIIDHHYSSNNSS	---		: 63
MtFD	:	-----	PMIMSTRNSTFGGVILQDFLTREL	---	TLDPTKSLD	---	YSSNNSS	: 60
Medtr8g075	:	-----	SNT	---	VSSPSLMT	---		: 55
AtFD	:	-----	HNHEPRFRGQNHHNQNPNSIFQDFLKGSL	---	NQEPAPTSQTGSA PNGD	---		: 133
AtFDP	:	-----	IGHEPMLKNQNPNN	---	SIFQDFLNMFL	---	NQPPPPP	: 96
Medtr3g101	:	-----	GEEAISNHLQROGSLTLP	---	RTLSQKTVDEVVKDI	---	SKD	: 110
AtABF2	:	-----	GGGQEGQLQROGSLTLP	---	RTLSQKTVDEVVKDI	---	SKVGS	: 110
AtABF1	:	-----	SVAAPGPSGFVPGGNLQROGSLTLP	---	RTLSQKTVDEVVKYI	---	NSK	: 118
AtABF4	:	-----	TAVAQPG-AGIPPPGNNLQROGSLTLP	---	RTISQKTVDEVVKCI	---	ITKDG	: 134
AtABF3	:	-----	GGLA VGVGESGGFFTGSLQROGSLTLP	---	RTISQKRVDDVVKEL	---	MKEDD	: 150
Medtr4g085	:	-----	GGVGEGPNPNPGGTLQKQSLTLP	---	RTLSQKRVDEVVRDI	---	IMKD	: 111
AtbZIP15	:	-----	QROGSTLP	---	PTLSKQNVGEVVKST	---	TEEKH	: 93
Medtr3g010	:	-----	NTAQAGEVVFORQPNLSLT	---	GPLSKKTVDEVVRDI	---	IQ	: 106
Medtr8g043	:	-----	GTAQSSEACLQROGSLTLP	---	AASFKKTVDEVVRDI	---	IQ	: 105
Medtr5g088	:	-----	QVSSSS--SLQROASMTLA	---	RALSGKTVDDVWRE	---	IQLG	: 103
Medtr7g088	:	-----	TTDGYMQHGQLASGSSMNP	---	GDLSKKTIDEVVRDI	---	IQ	: 106
Medtr6g026	:	-----	ATAGGNMQHNQLGGFNSQESLTLS	---	GDLSKKTIDEVVKDI	---	MQG	: 111
AtDPBF4	:	-----	PPAEGLV--RQGSLTLP	---	RDLSKKTIDEVVRDI	---	IQ	: 87
AtAREB3	:	-----	GGAAAEGLS--RQGSLTLP	---	RDLSKKTIDEVVKDI	---	IQ	: 102
Medtr2g086	:	-----	EHTISQQLGNSSSV	---	PPICKKTSDEVVSE	---	THKN	: 96
Medtr2g086	:	-----	QHTISQQFGNSFSVP	---	PPICKKTVDEVVSE	---	THKN	: 109
Medtr7g104	:	---	LSAAQKG-----ISKQASLPRONSLSIP	---	APLCRKTVDEVVSE	---	THKE	: 138
AtABI5	:		VGVFSGGSRGNEDANNKRGIANESSLP	---	APLCRKTVDEVVSE	---	THRG	: 169
AtDPBF2	:	-----	EKPAVLPRQGSLSLP	---	VPLCKKTVDEVVLE	---	IQNG	: 120
AtbZIP13	:	-----	LDASINLMDASAPMEITTTASDVVDHGGGTETTRGGKS	---	VDIWR	---	IVSG	: 132
AtGBF4	:	-----	KPFEMAIDVRSIGDR	---	NSVNNGKSVDDVVKEL	---	IVSG	: 97
Medtr1g098	:	-----	ISLADAVATF	---	RTVDDVWRE	---	IVAG	: 54
AtbZIP61	:	-----	IAFLEPPSSGVG	---	NHHFDRFDE	---	IQFMS	: 100
		*	200	*	220	*	240	
PsFD	:	-----	SSVASDQN--PSFFCPTVSTAPPPLVTALS	---	LNTPDHFPFDTLIR	---		: 106
MtFD	:	-----	SSVASDQNNNNASFYCP-ISTTPPPLVTALS	---	LNTPD-FLYDPLIR	---		: 104
Medtr8g075	:	-----	PSSLHSTINLNSLPE-FHFDPLAH	---		---		: 78
AtFD	:	-----	STTVTVLY-----SSFPFPATVLSLNSGAG	---	FEFLDNQD	---		: 167
AtFDP	:	-----	STIVTALYG-----SLPLPPPATVLSLNSGVG	---	FEFLDTTE	---		: 131
Medtr3g101	:	-----	YGGPNL-----AAMPTQ-RQPTLGEMTLEEFFLRAGVVRED	---	DAKPN	---	DGVFL	: 154
AtABF2	:	SG-----	VGGSNLSQVAQAQSQSQSQ-RQOTLGEMTLEEFFLRAGVVRE	---	EAQVAARAQI	---		: 163
AtABF1	:	-----	EGSNGNT-----GTDALE--RQOTLGEMTLEDFLLRAGVVRED	---	NTQQ	---		: 158
AtABF4	:	NM-----	EGSSGGG-----GESNVPPGRQOTLGEMTLEEFFLRAGVVRED	---	NCVQMGQV	---		: 183
AtABF3	:	IG-----	NGVVNG-----GTSGIPQ-RQOTLGEMTLEEFFLRAGVVRE	---	EPQPVES	---		: 194
Medtr4g085	:	-----	SGSSMPQ-RQPTLGEMTLEEFFLRAGVVRED	---	TPNHAQQIE	---		: 150
AtbZIP15	:	TN-----	NNGGVTN-----ITHLQG--QOTLGEMTLEEFFLRAGAR	---		---		: 127
Medtr3g010	:	-----	QSNDH-----EEVKSQEIQSTLGEMTLEDFLVKAGVVS	---	AASSNRK	---		: 146
Medtr8g043	:	-----	QKKDS-----EEKKSRE	---	QOTLGEMTLEDFLVKAGIVAE	---	AASSNKT	: 145
Medtr5g088	:	-----	QKKQYG-----DDVKVEDREMSLG	---	GTLEDFLVQAGLFAGASTSP	---		: 143
Medtr7g088	:	-----	QKKSAS-----PDRRT--ATLGEMTLEDFLMKAGVATES	---	FFPS	---		: 141
Medtr6g026	:	-----	KKRQVD-----RDRKSREKQOTLGEMTLEDFLVKAGVVGES	---	FFHG	---		: 150
AtDPBF4	:	-----	DKNGNG-----TSTTTTHKQPTLGEMTLEDFLLRAGVVTE	---	TVVP	---		: 126
AtAREB3	:	-----	NKNG-G-----SAHERRDKQPTLGEMTLEDFLLRAGVVTE	---	TIPGSN	---		: 142
Medtr2g086	:	-----	QPQFKEANNLKRN--ETLKKQETPGEMTLEDFLVKAGVVQK	---	S	---		: 136
Medtr2g086	:	-----	QQQFKETNNLKRS--ETLKKQOTLGEMTLEDFLVKAGVVQK	---	S	---		: 149
Medtr7g104	:	-----	QQNHHNINNV AQN-TESTPRQPTFGEMTLEDFLVKAGVVRE	---	QQSGMPVAIA	---		: 188
AtABI5	:		SGNGGDSNGRSSSSNGQNN AQNGGETAARQPTFGEMTLEDFLVKAGVVRE	---	H	---		: 220
AtDPBF2	:	-----	VQQHPPSSNSGQNSAENIRQOTLGEMTLEDFLVKAGVVQEP	---		---		: 162
AtbZIP13	:	-----	EGK-----GMKEETSEEIMTLEDFLAKA-AVEDE	---	TAVT	---		: 164
AtGBF4	:	-----	EQKT-----IMMKEEPE	---	DIMTLEDFLAKA-EMDEG	---		: 127
Medtr1g098	:	-----	DAISGD-----RECKEEISDEM	---	TLEDFLVKAGAVEDE	---		: 87
AtbZIP61	:	HN-----	NNHHHHHHSINGNVGPTRSSSNTSTPS	---	SDHNSLSDDDNNKE	---	APPSPD	: 148

		*	260	*	280	*	300
PsFD	:	-----	HNKDNNSQLLFQQQHQ-----				: 122
MtFD	:	-----	HNKHNNSQLLLQQQ-----				: 118
Medtr8g075	:	-----	NDLQLEQNHH-----				: 88
AtFD	:	-----	PLVTSNSNLHTHHLS-----				: 183
AtFDP	:	-----	NLLASNP-----				: 138
Medtr3g101	:	DLGN----	VGNNGNLGLAFQAQGMN----	KVAGFMGNGNRINGNDDPLVGLQSPTNLPLNV			: 207
AtABF2	:	AENNKGGYFGNDANTGFSVEFQQPSPRVVAAGVMGN----	LGAEATANSLQVQG--SSLPLNV				: 219
AtABF1	:	NENSSSGFY--ANNGAAG-LEFGFG-----	QPNQNSISFNGNNSMIMNQA-PGLGLKV				: 208
AtABF4	:	NGNNNGFY--GNSTAAGGLGFGFG-----	QPNQNSITFNGTNDSMILNQ-PGLGLKM				: 234
AtABF3	:	VTNFNGGFYGFSGNGLGTASNGFV-----	ANQPQDLSGNGVAVRQDLLTA-QTQPLQM				: 247
Medtr4g085	:	RPNNNEWFS-DFSRSNNTNLLGFQ-----	QPNGN----NGDMSDNNNLVP-KHVPLPP				: 198
AtbZIP15	:	GGNTNGGSIHDSSSSISGNPHTSLG-----	VQIQPKAMVSDFMNMMVPRSHDSYLHQNV				: 181
Medtr3g010	:	-NTNGPTPKVSVVESNVALPQFSPHG-----	PWIIQYAPPHYQH-----				: 183
Medtr8g043	:	-NTD----TTAADSNNVAVSQFPSQG-----	QWIIQYPPQYQH-----				: 178
Medtr5g088	:	-----	TVGLDAMDTAIPQS-----				: 157
Medtr7g088	:	-EDNAMSGRVDSQQQ--QNTSQHG-----	HWMQYQVPVSVQQPQQQHQQ				: 183
Medtr6g026	:	-KESGLL-RVDSNEDS--RQKVSHGL-----	HWMQYPVHSVQQ--QQHQYE				: 190
AtDPBF4	:	-QENVV-----	NIASNG-----QWVEYHHQP-----				: 146
AtAREB3	:	-HDGPVGGGSAGSGAGLGQNTITQVG-----	PWIIQYHQLPS-----				: 176
Medtr2g086	:	-----	SSLSFQNH-----				: 145
Medtr2g086	:	-----	SALPFKNHN-----				: 158
Medtr7g104	:	PPPTAAAVSSHPPQQHYAAVYPNNNSTMAQAASFAIGGGNLLNVVAPPYQTVAQGGGAV					: 248
AtABI5	:	--PTNPKPNPNPNQNPSSVIP----	AAAQQQLYGVFQG----TGDPSPFGQAMG---V				: 267
AtDPBF2	:	-----	LKTTRMRSSSDFG-----				: 175
AtbZIP13	:	-----	ASAEDLDVKIPVT-----				: 177
AtGBF4	:	-----	ASDEIDVKIPTERLNN-----				: 143
Medtr1g098	:	-----	EAGEDVKMTIPLS-----				: 100
AtbZIP61	:	-----	HDHMDNNVANQNNAAAG-----				: 165

		*	320	*	340	*	360
PsFD	:	-----	QQRNITVSKVS-HVVNPTPFDPNVGV				: 147
MtFD	:	-----	QHNIGVSNVSPCFVNASPCDQNVGV				: 143
Medtr8g075	:	-----	HTTTLKVEALLSNS-----				: 103
AtFD	:	-----	NAHAFNTSFEALVPSSS-----				: 200
AtFDP	:	-----	RSFEESAKFGC-----				: 149
Medtr3g101	:	NGIRSTNQQQQMNSQSQAQQQHQNQQLQQLQQQQQQQQIFPKQPLNYATQMPLSNNQG					: 267
AtABF2	:	NGARTTYQQS-----	QQQQPIMPQKQFGFYGTQMGQLNSPG				: 255
AtABF1	:	GGTMQQQQQP-----	HQQQLQPH--QRLPPTIFPKQANVTFAAPVNMVNRG-				: 253
AtABF4	:	GGTMQQQQQQQ--QLLQQQQQMQQLNQPHQPQLPQTIFPKQANVAFSAPVNITNKG-					: 290
AtABF3	:	Q-QPQMVQQP-----	QMVQQPQLIQTQERFPFKQTTFASFNTVDVNNRSQ				: 292
Medtr4g085	:	SSINLNHSQR-----	PPPLFPKPTTVAFASPMHLLNNA-				: 231
AtbZIP15	:	NGSMSTYQPQ-----	QSIMSMPNGYSYGKQIRFSNGS-				: 213
Medtr3g010	:	-----	PQQSVMATYVPSQIIAQLHMA--AGAP				: 209
Medtr8g043	:	-----	LQQSSMGIYMPQSMAQLPHMG--SGV-				: 203
Medtr5g088	:	-----	FQPNTSLVSSSSISLS----				: 174
Medtr7g088	:	NHQNNMTGFAG-----	YMAGHVQQPVLDAGYTEAMVSLSPSSLMA				: 225
Medtr6g026	:	KHT---MPGFA-----	AVHAIQQPFQVAGNQALDAAISPSSLMV				: 226
AtDPBF4	:	-----	QQQGGFMTYPVCEMQDMV----MMG				: 167
AtAREB3	:	-----	MPQPQAFMPYPVSDMQAMVSQSSLMG				: 202
Medtr2g086	:	-----	GNVSNMPEPLNIASS-GLRPS-----				: 165
Medtr2g086	:	-----	GNVSNMRPLNIASCYGLRPS-----				: 179
Medtr7g104	:	GEPSSSGYVGNG-----	KTRDSIGTGYP PPPPAICYGGRVNGAAGG				: 290
AtABI5	:	GDP--SGYAK-----	RTGGGGYQQAPPVQAGVCYGGGVGFGAGG-				: 304
AtDPBF2	:	-----	YNPEFGVGLHCQNQNNYGDNRS-----				: 197
AtbZIP13	:	-----	NYGFDHSAPPHNPFQMDKVEGSIVAF				: 204
AtGBF4	:	-----	DGSYTFDFPMQRHSSFMVEGSMG-----				: 167
Medtr1g098	:	-----	ETLSGSGMFLDSSSQGIENVDGSGVIGF				: 128
AtbZIP61	:	-----	NNYNESDEVQSCKTEPQDGPFSAN--				: 189

		*	380	*	400	*	420
PsFD	:	-----	-----	-----	VSNAFTC-----	-----	: 154
MtFD	:	P-----	-----	-----	ASSSFTC-----	-----	: 151
Medtr8g075	:	-----	-----	-----	-----	-----	: -
AtFD	:	-----	-----	-----	-----	-----	:
AtFDP	:	-----	-----	-----	-----	-----	:
Medtr3g101	:	MRGG-IVGLSPDHGMNGN-LVQGGGIGMVGLAPGAVQIGAVSPANQI-SSDKMGKSN	GD	T	:		324
AtABF2	:	IRGGGLVGLGDQSLTNNVGFVQG----	ASAAIPGALGVGAVSPVTPL-SSEGIGKSN	GD	S	:	310
AtABF1	:	-----LFETSADGPANSN-----	MGGAGGTVTATSPG-----	TSSAENN	:		287
AtABF4	:	-----FAGAANNINNNNG--	LASYGGTGVTVAAATSPG-----	TSSAENN	:		328
AtABF3	:	PATQCQEVKPSILGIHNHPMNNLL--	QAVDFKTGVTVAAVSPGSQM-SPDLTPKSALDA	:			349
Medtr4g085	:	-----QLGNNGRSVGPVG--	TLGLSASNITAPVASPGSKM-SPDLITKRNLDP	:			277
AtbZIP15	:	-----LGSGNQSLQDTKRSLVPSVATIPSEAITCSPVTPFPPTLNGKQKINGES	:				261
Medtr3g010	:	-----SDSVPYTDGQVALASPVIGNLSDTQKSAR-----	:				238
Medtr8g043	:	-----SMEIPFADSHMAL-----	DTQMPGR-----	:			223
Medtr5g088	:	-----	DAKPGR-----	:			180
Medtr7g088	:	-----	TSSDT-QTQGR-----	:			235
Medtr6g026	:	DKLK-----	LMQFEESLNLLYKAINSFLGTLSDT-QTLGR-----	:			260
AtDPBF4	:	-----	GLSDTPQAPGR-----	:			178
AtAREB3	:	-----	GLSDT-QTPGR-----	:			212
Medtr2g086	:	-----	MEVGFPTQCVTSNSSATYQMTSG-----	:			188
Medtr2g086	:	-----	MGMGFSTQCVSRNGLATYQMLSHNNNLGV	:			208
Medtr7g104	:	YGVA-----	VAQTMGMGGPVPVSSDGIGN--	ENSGGQFG-	:		323
AtABI5	:	-----	QQMGMVGPLSPVSSDGLGHGQVDNIGGQYG-	:			334
AtDPBF2	:	-----	VYSENRPFYSVLGESSSCMTGNGRSNQYL-	:			226
AtbZIP13	:	GNG-----	LDVYGGGARGK-----	:			218
AtGBF4	:	-----	GGVTRGK-----	:			174
Medtr1g098	:	GNGN-----	VNVNGVEMVEGGGRGK-----	:			148
AtbZIP61	:	-----	-----	:			

		*	440	*	460	*	480	
PsFD	:	-----	FGKRFGEPPDVSPGERRNKRMIKNRESAARSARKQAYTTTEL	:	196			
MtFD	:	-----	FGKRFGEAPDISPGERRNKRMIKNRESAARSARKQAYTNEL	:	193			
Medtr8g075	:	-----	IERRHKRIMKNRESAARSARKQAYIFEL	:	132			
AtFD	:	-----	FGKKRGQDSNIGSGNRHHRMIKNRESAARSARKQAYTNEL	:	242			
AtFDP	:	-----	LGKKRGQSDDTRGDRYKRMKNRESAARSARKQAYTNEL	:	191			
Medtr3g101	:	SSVSPVPYVFNG----	GMRGRKNGNAVKEVIERORRMKNRESAARSARKQAYTMEL	:	379			
AtABF2	:	SSLSPSPYMFNG----	GVRGRK-SGTVEKVVERORRMKNRESAARSARKQAYTVEL	:	364			
AtABF1	:	TWSSPVPYVFG----	RGRRSNTGLEKVVERORRMKNRESAARSARKQAYTLEL	:	339			
AtABF4	:	SLS-PVPYVLN-----	RGRSNTGLEKVIERORRMKNRESAARSARKQAYTLEL	:	379			
AtABF3	:	SLS-PVPYMFNG-----	FVRKTGAVLEKVIERORRMKNRESAARSARKQAYTMEL	:	400			
Medtr4g085	:	SLLSPVPYAIN-----	RGRK-CVPVEKGVERORRMKNRESAARSARKQAYTVEL	:	328			
AtbZIP15	:	SLLSPSPYISNGSTSTRGCKINSEITAEKQFVDKKLRRKIKNRESAARSARKQAYTMEV	:	321				
Medtr3g010	:	-----	KRG-PEDMIERTVERROKRMKNRESAARSARKQAYTTTEL	:	278			
Medtr8g043	:	-----	KRSTPEDMVEKTVERROKRMKNRESAARSARKQAYTNEL	:	264			
Medtr5g088	:	-----	KRD-APDAYEKALERRLRRKIKNRESAARSARKQAYHNEL	:	220			
Medtr7g088	:	-----	KRVASGVVVEKTVERROKRMKNRESAARSARKQAYTOEL	:	276			
Medtr6g026	:	-----	KRVASGIVVEKTVERROKRMKNRESAARSARKQAYTOEL	:	301			
AtDPBF4	:	-----	KRVA-GEIVEKTVERROKRMKNRESAARSARKQAYTHEL	:	218			
AtAREB3	:	-----	KRVASGEVVEKTVERROKRMKNRESAARSARKQAYTHEL	:	253			
Medtr2g086	:	-----	AESSGAANRKRIIDGPPVLLDRKORRMKNRESAARSARKQAYTIEL	:	237			
Medtr2g086	:	KDFAVEKQCQLTESSGCSNRKRIVEGPPVVERORRMKNRESAARSARKQAYTVEL	:	268				
Medtr7g104	:	-----	IDMNGLRGRKRMVDGPEVVERORRMKNRESAARSARKQAYTVEL	:	372			
AtABI5	:	-----	VDMGGLRGKRKRVVDGPEKVVERORRMKNRESAARSARKQAYTVEL	:	383			
AtDPBF2	:	-----	TGLDAFRICKRIIDGPPHILMERORRMKNRESAARSARKQAYTVEL	:	275			
AtbZIP13	:	-----	RARVMVEPLDKAAARORRMKNRESAARSARKQAYQVEL	:	259			
AtGBF4	:	-----	RGRVMMEAMDKAAARORRMKNRESAARSARKQAYQVEL	:	215			
Medtr1g098	:	-----	RGRPVMEQLDKAAARORRMKNRESAARSARKQAYQVEL	:	189			
AtbZIP61	:	-----	QNSGGSSGNRIHDPKRVKRIILANRQSAQRSRVKLIQVISEL	:	230			



				*	500	*	520	*	540																																																					
PsFD	:	E	Q	K	V	D	F	L	---	E	E	N	A	K	L	K	R	Q	Q	---	Q	E	L	W	E	A	A	S	A	P	---	:	225																													
MtFD	:	E	Q	K	V	Q	L	L	Q	---	E	E	N	A	R	L	R	R	Q	Q	---	Q	E	L	W	E	A	E	S	G	G	Q	Q	K	---	:	224																									
Medtr8g075	:	K	K	K	V	K	S	L	E	---	E	E	N	A	R	L	K	R	Q	Q	---	H	V	L	C	D	T	A	S	N	H	K	Q	K	---	:	163																									
AtFD	:	E	L	E	V	A	H	L	Q	---	A	E	N	A	R	L	K	R	Q	Q	---	D	Q	L	K	M	A	A	A	I	Q	Q	P	---	:	272																										
AtFDP	:	E	L	E	V	A	H	L	Q	---	T	E	N	A	R	L	K	I	Q	Q	---	E	Q	L	K	I	A	E	A	T	Q	N	Q	---	:	221																										
Medtr3g101	:	E	A	E	V	A	K	L	K	---	E	E	N	E	E	L	Q	K	K	Q	E	E	I	M	E	L	Q	K	N	V	K	E	M	N	L	Q	R	---	:	416																						
AtABF2	:	E	A	E	V	A	K	L	K	---	E	E	N	D	E	L	Q	R	K	Q	A	E	I	M	Q	K	N	Q	E	T	E	M	R	N	L	L	Q	---	:	401																						
AtABF1	:	E	A	E	I	E	S	L	K	---	L	V	N	Q	D	L	Q	K	K	Q	A	E	I	M	K	T	H	N	S	E	L	K	E	F	S	K	Q	P	P	-L	:	377																				
AtABF4	:	E	A	E	I	E	K	L	K	---	K	T	N	Q	E	L	Q	K	K	Q	A	E	M	V	E	M	Q	K	N	E	L	K	E	T	S	K	R	P	-W	:	416																					
AtABF3	:	E	A	E	I	A	Q	L	K	---	E	L	N	E	E	L	Q	K	K	Q	V	E	I	M	E	K	Q	K	N	L	L	E	P	L	R	Q	P	W	G	M	:	439																				
Medtr4g085	:	E	A	E	V	A	K	L	K	---	E	V	N	E	E	L	Q	R	K	Q	A	E	F	M	E	M	Q	K	S	---	K	E	D	L	V	R	T	N	---	:	363																					
AtbZIP15	:	E	V	E	L	E	N	L	K	---	K	D	Y	E	E	L	L	K	H	V	E	L	R	K	R	Q	M	E	P	G	M	I	S	L	H	E	R	P	---	:	358																					
Medtr3g010	:	E	I	K	V	S	R	L	E	---	E	E	N	D	K	L	R	K	E	K	---	E	L	E	N	M	L	N	A	P	P	P	E	P	K	---	:	311																								
Medtr8g043	:	E	I	K	V	S	R	L	E	---	E	E	N	E	M	L	R	R	K	---	E	L	E	N	M	L	P	C	A	P	I	A	E	P	K	---	:	297																								
Medtr5g088	:	V	T	K	V	T	L	E	---	Q	Q	N	M	Q	L	K	K	E	K	---	E	F	E	Q	Q	L	Q	P	E	S	S	P	E	P	K	---	:	253																								
Medtr7g088	:	E	I	K	V	S	H	L	E	---	E	E	N	E	R	L	K	R	L	H	---	E	I	E	R	V	L	P	S	M	P	P	P	D	P	K	---	:	309																							
Medtr6g026	:	E	L	K	V	S	R	L	E	---	E	E	N	E	R	L	R	R	Q	N	---	E	M	E	K	E	V	P	T	A	P	P	E	P	K	---	:	334																								
AtDPBF4	:	E	I	K	V	S	R	L	E	---	E	E	N	E	K	L	R	R	L	K	---	E	V	E	K	I	L	P	S	E	P	P	P	D	P	K	---	:	251																							
AtAREB3	:	E	I	K	V	S	R	L	E	---	E	E	N	E	R	L	R	K	Q	K	---	E	V	E	K	I	L	P	S	V	P	P	P	D	P	K	---	:	286																							
Medtr2g086	:	E	A	E	I	N	L	L	Q	---	E	E	N	K	Q	L	K	Q	F	L	A	E	A	E	R	K	R	K	Q	---	E	L	L	Q	R	K	Q	S	A	:	273																					
Medtr2g086	:	E	A	E	I	N	L	L	K	---	E	E	N	E	K	L	Q	V	L	A	E	A	E	S	K	R	K	Q	---	E	L	L	Q	R	K	H	S	T	:	304																						
Medtr7g104	:	E	A	E	I	N	Q	L	R	---	E	E	N	S	Q	L	K	Q	A	L	A	E	L	E	R	R	R	Q	Q	C	S	E	E	T	N	V	R	V	Q	T	:	411																				
AtABI5	:	E	A	E	I	N	Q	L	K	---	E	E	N	A	Q	L	K	H	A	L	A	E	L	E	R	K	R	K	Q	Q	Y	F	E	S	L	K	S	R	A	Q	P	:	422																			
AtDPBF2	:	E	L	E	I	N	N	L	T	---	E	E	N	T	K	L	K	E	I	V	E	E	N	E	K	R	R	Q	---	E	I	I	S	R	S	K	Q	V	:	311																						
AtbZIP13	:	E	A	L	A	A	K	L	E	---	E	E	N	E	L	L	S	K	E	I	E	D	K	R	K	E	R	Y	Q	K	L	M	E	F	V	I	P	V	E	K	:	298																				
AtGBF4	:	E	T	L	A	A	K	L	E	---	E	E	N	E	Q	L	L	K	E	I	E	S	T	K	E	R	Y	K	K	L	M	E	V	L	I	P	V	D	E	K	:	254																				
Medtr1g098	:	E	S	L	A	V	K	L	E	---	E	E	N	D	K	L	M	K	E	K	A	E	R	K	K	E	R	F	K	Q	L	M	E	K	V	I	P	V	V	E	Q	:	228																			
AtbZIP61	:	E	R	S	V	T	S	L	Q	T	E	V	S	V	L	S	P	R	V	A	F	L	D	H	Q	R	L	L	N	V	D	N	S	A	I	K	Q	R	I	A	A	L	A	Q	D	K	I	F	K	D	A	H	Q	E	A	L	K	R	E	I	:	290

				*	560	*																																						
PsFD	:	---	---	K	K	N	S	L	H	R	T	L	A	P	E	---	:	238																										
MtFD	:	---	---	K	K	S	S	T	Y	R	---	T	S	S	E	---	:	235																										
Medtr8g075	:	---	---	R	K	G	N	L	Y	R	T	S	T	A	P	E	---	:	176																									
AtFD	:	---	---	K	K	N	T	L	Q	R	S	S	T	A	P	E	---	:	285																									
AtFDP	:	---	---	V	K	K	T	L	Q	R	S	S	T	A	P	E	---	:	234																									
Medtr3g101	:	E	V	---	---	K	R	K	C	L	R	R	T	Q	I	G	P	W	---	:	431																							
AtABF2	:	G	G	---	---	P	K	K	L	R	T	E	S	G	P	W	---	:	416																									
AtABF1	:	L	A	---	---	K	R	Q	C	L	R	R	T	L	I	G	P	W	---	:	392																							
AtABF4	:	G	S	---	---	K	R	Q	C	L	R	R	T	L	I	G	P	W	---	:	431																							
AtABF3	:	G	C	---	---	K	R	Q	C	L	R	R	T	L	I	G	P	W	---	:	454																							
Medtr4g085	:	---	---	K	I	K	Y	L	R	R	T	L	I	G	P	W	---	:	376																									
AtbZIP15	:	---	---	E	R	K	L	R	R	T	K	S	D	I	K	---	:	370																										
Medtr3g010	:	---	---	C	Q	L	R	F	V	S	S	A	S	E	---	:	322																											
Medtr8g043	:	---	---	Y	Q	L	R	F	I	A	S	C	P	E	---	:	308																											
Medtr5g088	:	---	---	Y	R	L	R	F	I	S	S	A	I	E	---	:	264																											
Medtr7g088	:	---	---	H	Q	L	R	R	T	S	S	A	P	L	---	:	320																											
Medtr6g026	:	---	---	N	Q	L	R	R	T	N	S	A	S	E	---	:	345																											
AtDPBF4	:	---	---	W	K	L	R	R	T	N	S	A	S	I	---	:	262																											
AtAREB3	:	---	---	R	Q	L	R	R	T	S	S	A	P	E	---	:	297																											
Medtr2g086	:	---	---	K	V	Q	K	G	T	E	K	S	---	S	S	T	T	W	---	:	288																							
Medtr2g086	:	---	---	K	A	Q	K	G	A	E	K	L	R	A	M	R	P	I	S	T	T	W	---	:	324																			
Medtr7g104	:	---	---	K	A	Q	K	A	K	E	K	L	R	G	L	R	N	T	S	C	H	L	---	:	431																			
AtABI5	:	---	---	K	L	P	K	S	N	G	R	L	R	T	M	F	N	P	S	C	P	L	---	:	442																			
AtDPBF2	:	---	---	T	K	E	K	S	G	D	K	L	R	K	I	R	M	A	S	A	G	W	---	:	331																			
AtbZIP13	:	---	---	P	K	Q	Q	---	P	P	R	F	L	R	I	R	S	L	E	W	---	:	315																					
AtGBF4	:	---	---	P	R	P	---	P	S	R	P	L	S	R	S	H	S	L	E	W	---	:	270																					
Medtr1g098	:	---	---	P	R	L	---	P	R	L	R	R	V	R	S	L	Q	W	---	:	243																							
AtbZIP61	:	---	---	E	R	L	R	Q	V	Y	H	Q	Q	S	L	K	K	M	E	N	N	V	S	D	Q	S	P	A	D	I	K	P	S	V	E	K	E	Q	L	L	N	V	:	329

### Sequence alignment for Figure 4.2

Alignment for phylogenetic neighbour-joining tree of the legume FD family (Figure 4.2). Full-length predicted protein sequences were aligned with ClustalX (Thompson et al., 1997) and manually adjusted with GeneDoc (Nicholas and Nicholas, 1997). Shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Full sequence details are given in Tables A3.1 and A3.2, above.

		*	20	*	40	*	60																															
AtFD	:	-----	MLSSAKHQ	RNHR	LSATN	KNQTL	TKVSS	IS	SSSSPSSSSS : 38																													
AtFDP	:	-----	MLSSAKHN	-----	-----	-----	KINN	HS	AFSISSSSS : 23																													
PsFDa	:	-----	-----	-----	-----	-----	-----	-----	: -																													
MtFDa	:	-----	-----	-----	-----	-----	-----	-----	: -																													
LjFDa	:	-----	-----	-----	-----	-----	-----	-----	: -																													
GmFDa1	:	-----	-----	-----	-----	-----	-----	-----	: -																													
GmFDa2	:	-----	-----	-----	-----	-----	-----	-----	: -																													
PvFDa	:	-----	-----	-----	-----	-----	-----	-----	: -																													
MtFDb	:	-----	-----	-----	-----	-----	-----	MSH	: 3																													
GmFDb1	:	-----	-----	MLSS	SSTST	TTTTTT	CHKR	NNLN	NHKALSP : 29																													
GmFDb2	:	-----	-----	MLSS	-----	TSTTT	SCHSR	NNLN	NHKALSP : 24																													
PvFDb	:	-----	-----	-----	-----	-----	-----	-----	: -																													
GmFdc1	:	-----	-----	-----	-----	MASWP	-----	PKPTE	IFC-VH : 15																													
GmFdc2	:	MGR	RYPGG	LIVWG	HEPGT	SLSTL	NVEI	PLSL	AKVEK	KEEGEAWPRGHPNLQRYFVRLS : 60																												
PvFdc	:	-----	-----	-----	-----	MASSP	CD	WCW	THLSQSLSSS : 19																													
AtDPBF4	:	-----	MGS	IRGN	IEEPI	SQSL	TRQNS	LYSL	KLHEV	QTHLGSSGKPLGSMNLDELLKTVL : 54																												
AtAREB3	:	-----	MDS	QRG	IVEQ	AKSQ	SLNR	QSS	LYSL	TLDEVQNHGSSGKALGSMNLDELLKSVC : 54																												
		* <th>80</th> <th>*</th> <th>100</th> <th>*</th> <th>120</th> <td></td> <td></td>	80	*	100	*	120																															
AtFD	:	SSS	-----	TSSSS	PLPSQ	DSQA	KRSL	VTME	EVW	ND--INLASTHHLNRHS : 82																												
AtFDP	:	SLS	-----	TSSS	-----	LGHN	KSQV	TME	EVW	KE--INLGSTHYHRQLN : 59																												
PsFDa	:	-----	-----	-----	-----	MEEL	WKD	-----	IN	MSSINEQNTRR : 20																												
MtFDa	:	-----	-----	-----	-----	MEEV	WKD	-----	IN	LSSINDQNTR- : 19																												
LjFDa	:	-----	-----	-----	-----	MEEV	WKD	-----	IN	LASTNDHNTH : 19																												
GmFDa1	:	-----	-----	-----	-----	MEEV	WKD	-----	IN	LATINEQSTI- : 19																												
GmFDa2	:	-----	-----	-----	-----	MEEV	WKD	-----	IN	----- : 9																												
PvFDa	:	-----	-----	-----	-----	MEEV	WKD	-----	IN	LATINDQVSS- : 19																												
MtFDb	:	QPL	-----	QEQT	PQQQ	HHLR	PNK	PKNT	ME	DVW	KD--INLPSTTNHMSN- : 46																											
GmFDb1	:	TTT	-----	KPSH	FHQ	TPIS	IS	SSSS	NKAM	EDV	EGININLTSTNDHNTNT : 75																											
GmFDb2	:	STT	-----	KPSH	FSLT	PRSS	HIST	NNK	DM	EVW	EG--INLTSTSDHNTNT : 68																											
PvFDb	:	-----	-----	-----	-----	ME	DVW	NG--	IN	STATSEHNTTH : 20																												
GmFdc1	:	ARE	-----	KAM	ASSP	CD	CWPH	LSP	SS--	IEH	VW	ND--IKLDSISNSPVDI : 57																										
GmFdc2	:	VRE	-----	KAM	ASSP	CD	CWPH	SSSS	SSS	IEH	VW	ND--IKLASTSNSSVDL : 104																										
PvFdc	:	SSS	-----	PSSS	SLP	QTL	LLPP	P	SSSS--	EH	VW	ND--IKLPSISNSPVDF : 60																										
AtDPBF4	:	-----	PPA	EGL	VRQ	GS	SLT	LP	DL	S	KKT	VDE	VWR	DIQ	DK	NG	GT	STTT : 99																				
AtAREB3	:	SVE	ANQ	PSS	MAV	NGG	AAQ	EGL	SRQ	GS	SLT	LP	DL	S	KKT	VDE	VW	KDIQ	QNK	NG-GSAHERR : 113																		
		* <th>140</th> <th>*</th> <th>160</th> <th>*</th> <th>180</th> <td></td> <td></td>	140	*	160	*	180																															
AtFD	:	PHP	QHN	HEP	RFR	GQ	NHHN	QNP	NSIF	QDF	LK	GS	LN	QEP	-----	APT	SQT	TG	SAP	NGD	ST : 135																	
AtFDP	:	---	IG	HEP	ML	KN	QNP	NN	---	SIF	QDF	LN	MP	LN	QPP	-----	PPP	-----	PPP	SS	ST : 98																	
PsFDa	:	-----	PM	IM	STR	S	TF	GG	VIL	QDF	L	AR	PL	NIN	PP	K	NID	H	YSS	NN	SSSSV	ASD	QN : 71															
MtFDa	:	-----	PM	IM	STR	N	S	TF	GG	VIL	QDF	L	TR	PL	T	L	D	P	T	K	S	L	D--YSS	NN	SSSSV	ASD	QNN : 69											
LjFDa	:	-----	P	ST	H	S	T	F	G	A	I	F	QDF	L	G	H	F	P	P	N	-----	T	T	V	SSS	L	S	A	S	P	Q	S : 57						
GmFDa1	:	-----	S	T	R	P	N	V	E	G	V	M	F	QDF	L	A	R	E	F	T	T	I	D	-----	P	P	N	T	T	L	S	S	A	S	E	T	A : 57	
GmFDa2	:	-----	---	V	F	Q	---	---	L	T	T	I	D	-----	---	---	S	P	N	---	---	I	I	L	S	S	A	S	E	T	G : 32							
PvFDa	:	-----	---	T	H	S	N	L	G	G	V	I	L	QDF	L	A	R	E	F	T	T	I	D	-----	---	---	P	N	A	T	L	S	S	Q	T	T : 51		
MtFDb	:	-----	---	T	V	S	S	P	S	I	M	T	P	S	S	L	H	S	-----	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	61		
GmFDb1	:	-----	---	S	K	G	A	K	F	QDF	L	S	R	P	F	T	N	F	S	-----	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	93		
GmFDb2	:	N	-----	---	T	S	K	G	A	N	F	QDF	L	S	R	P	F	T	N	F	S	-----	---	---	---	---	---	---	---	---	---	---	---	---	---	88		
PvFDb	:	-----	---	I	S	K	G	A	K	F	QDF	L	A	G	E	P	F	N	-----	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	36			
GmFdc1	:	DF	-----	---	N	N	N	---	H	S	V	S	D	S	S	E	L	N	-----	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	72			
GmFdc2	:	DL	-----	---	N	N	N	N	H	S	V	S	V	S	S	E	L	N	Q	E	L	S	T	F	L	-----	---	---	---	---	---	---	---	---	---	127		
PvFdc	:	N	-----	---	P	S	S	S	H	S	S	L	L	S	-----	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	71		
AtDPBF4	:	HK	-----	---	Q	P	T	L	G	E	I	T	L	E	D	L	L	R	A	G	V	V	T	E	T	V	V	---	---	---	Q	E	N	V	V : 131			
AtAREB3	:	DK	-----	---	Q	P	T	L	G	E	M	T	L	E	D	L	L	K	A	G	V	V	T	E	T	I	P	G	---	---	---	N	H	D	G	P	V	G : 148

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          *          200          *          220          *          240
AtFD      : TVTVLY---SSPFPFPAIVLSLNSGAGFEFLDNQDPLVTSNINLHTHHHLSNAHAFNTS : 191
AtFDP     : IVTALYG---SLPLPPPAIVLSLNSGVGFELDTTENLLASNP-----RS : 140
PsFDa     : --PSFCPTVSTAPPPLVTALSLNTRPDHFFPD---TLIRHNKDNNSQLLFQQQHQQQRN : 126
MtFDa     : NNASFYCP-ISTTPPLVTALSLNSRPD-FLYD---PLIRHNKHNNSQLLLQQQ---QHN : 121
LjFDa     : --TSLYSP----APPLVTALSLNARPD-FAFD---PLRPNNKSQLPQLHHHH---HPP : 103
GmFDa1    : A-NSLFSP---ASPGPPLVTLSSLSSLPHHHFR---FEHPSS-----IP : 94
GmFDa2    : -----FS-----LPHFHFQ---SQPSS-----QH : 48
PvFDa     : --SSLYGP-PSSPAPSLLTALSLSSHPH-LLFD---HLTHKPSHHH-----H : 91
MtFDb     : -----TINLNSLPEFHFDP---LAHNDLQLEQN-----H : 87
GmFDb1    : ---TIASA---DPSPPVTLTLSTRSEFHFDP---LTHKDLQLGQP-----HH : 132
GmFDb2    : ---TIAS---DPSPPVTLTLSTRSEFHFDS---ATHKDLQLGQP-----HH : 126
PvFDb     : -----PSP-VTALTLSTRSSEYLP---LHKDLQL-----L : 62
GmFDb1    : -----LTSTSSSVFHKHDHDSLLS----- : 92
GmFDb2    : -----TLTSTSSSVFHKHDHDSLLS----- : 148
PvFDb     : -----LSSS---HNH-HSLLS----- : 83
AtDPBF4   : -----NIASNGQWVEYHHQP---QQQGGFTYPCVCEMDMV-----MM : 166
AtAREB3   : GGSAGSGAGLGQNITQVGPIQYHQLPSMPQPQAFMPYPVSDMQAMVSQS-----SLM : 201

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          *          260          *          280          *          300
AtFD      : FEALVPS-----SSFG--KKGQDSNEGS--GNRRHKRMKINRE : 226
AtFDP     : FEESAKF-----GCLG--KKGQDSDDTR--GDRYKRMKINRE : 175
PsFDa     : ITVSKVS-HVNPPTPFDPNVGV-VSNAFTCFG--KRFGEPPDVSP--GERRNKRMKINRE : 180
MtFDa     : IGVSNVSPCFVNASPCDQNVGVPASSSFTCFG--KRFGEAPDISP--GERRNKRMKINRE : 177
LjFDa     : SKPSSPCFPINPPPPFDN--HLPFTSPLPCFGSNKRFAPADYGL--GDRRNKRMKINRE : 159
GmFDa1    : PPPSNK-----RFAQPADHCSTIGDRRNKRMKINRE : 125
GmFDa2    : PPPSNK-----TSAQPP-----AADRRNKRMKINRE : 74
PvFDa     : PPPSNP-----SSLG-TKRFFE-PDCNL--GDRRNKRMKINRE : 125
MtFDb     : HHTTTLN-----KVEALLNSI--ERRHKRMKINRE : 116
GmFDb1    : KNGSKVEP-----FGKPSGKIRILQSG---DMRKARLMKINRE : 166
GmFDb2    : KNDKVEP-----FGNPFNMIRILPSG---DMRKARLMKINRE : 160
PvFDb     : HTASKTEP-----FAHPFSNERAPPASR---DMRNARLMKINRE : 97
GmFDb1    : --VSNIS-----FEASGSKKTTLLDQRHARIKINRE : 121
GmFDb2    : --VSDP-----NTLQDQRHTRVIKINRE : 168
PvFDb     : --VSNAS-----FPSN--QTTHQDQRHVRIMKINRE : 110
AtDPBF4   : GGLSDTP-----QAPGRKRVA-GEIVEKTVERROKRMKINRE : 202
AtAREB3   : GGLSDT-----QTPGRKRVASGEVVEKTVERROKRMKINRE : 237

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          *          320          *          340          *          360
AtFD      : SAARSARKQAYTNELELEVAHLQAEENARLKROQDOLKMAAAIQQ---PKKNTLQRSST : 282
AtFDP     : SAARSARKQAYTNELELEVAHLQAEENARLKROQDOLKMAAAIQQ---QVKKTLQRSST : 231
PsFDa     : SAARSARKQAYTNELELEVAHLQAEENARLKROQDOLKMAAAIQQ---KKNSLHRTLT : 235
MtFDa     : SAARSARKQAYTNELELEVAHLQAEENARLKROQDOLKMAAAIQQ---KKSSLYR--T : 232
LjFDa     : SAARSARKQAYTNELELEVAHLQAEENARLKROQDOLKMAAAIQQ---KKNSLYRSAT : 215
GmFDa1    : SAARSARKQAYTNELELEVAHLQAEENARLKROQDOLKMAAAIQQ---KKG-TLCRAST : 181
GmFDa2    : SAARSARKQAYTNELELEVAHLQAEENARLKROQDOLKMAAAIQQ---KKGTTLYRAST : 131
PvFDa     : SAARSARKQAYTNELELEVAHLQAEENARLKROQDOLKMAAAIQQ---KKGSLYRAST : 181
MtFDb     : SAARSARKQAYIFELKKVKSLLEENARLKROQHVLCDTASNHHQ---KKGSLYRTST : 173
GmFDb1    : SAARSARKQAYLFEIKQKLELQAEENARLKROQHVLCDTASNHHQ---KKGSLYRTYT : 221
GmFDb2    : SAARSARKQAYLFEIKQKLELQAEENARLKROQHVLCDTASNHHQ---KKGSLYRTYT : 210
PvFDb     : SAARSARKQAYLFEIKQKLELQAEENARLKROQHVLCDTASNHHQ---KKGSLYRTYT : 152
GmFDb1    : SAVRSARKQAYRKGLVEEVARLLEENARLKROQHVLCDTASNHHQ---KKGSLYRTYT : 181
GmFDb2    : SAVRSARKQAYRKGLVEEVARLLEENARLKROQHVLCDTASNHHQ---KKGSLYRTYT : 225
PvFDb     : SAVRSARKQAYRKGLVEEVARLLEENARLKROQHVLCDTASNHHQ---KKGSLYRTYT : 169
AtDPBF4   : SAARSARKQAYTHELEIKVSRLEENARLKROQHVLCDTASNHHQ---DPKWKLRRTSS : 259
AtAREB3   : SAARSARKQAYTHELEIKVSRLEENARLKROQHVLCDTASNHHQ---DPKWKLRRTSS : 294

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AtFD      : APF : 285
AtFDP     : APF : 234
PsFDa     : APF : 238
MtFDa     : SSF : 235
LjFDa     : APF : 218
GmFDa1    : APF : 184
GmFDa2    : APF : 134
PvFDa     : APF : 184
MtFDb     : APF : 176
GmFDb1    : APF : 224
GmFDb2    : APF : 213
PvFDb     : APF : 155
GmFDb1    : SPF : 184
GmFDb2    : SPF : 228
PvFDb     : SPF : 172
AtDPBF4   : ASL : 262
AtAREB3   : APF : 297

```

### Sequence alignment for Figure 4.4

Alignment for phylogenetic neighbour-joining tree of the angiosperm FD family (Figure 4.4). Full-length predicted protein sequences were aligned with ClustalX (Thompson et al., 1997) and manually adjusted with GeneDoc (Nicholas and Nicholas, 1997). Shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Full sequence details are given in Tables A3.1 and A3.2, above.

	*	20	*	40	*	60	
AtDPBF4	:	-----MGSIRGNIEEPISQSLTRQNSLYSLKLHEVQTHLGSSGKPLG	:	42			
AtAREB3	:	-----MDSQRGIVEQAQSLNRQSSLYSLTLDEVQNHLGSSGKALG	:	42			
OsAREB3	:	MIQAMASHAGGSGGGGGSGRDAGSAQRGPMQGLARQGSlyGLTLNEVQSQLG--EPLL	:	57			
TaFDL2	:	-----	:	-			
TaFDL3	:	-----	:	-			
TaFDL6	:	-----MSSEGGGTAITGKKRNRAQIQLVREGSLYNLTSEVESHLG--APLL	:	46			
GmFDc1	:	-----	:	-			
GmFDc2	:	-----MGRRYPGGGLIVWGHEPGTSLSTLNVEIPL	:	30			
PvFDc	:	-----	:	-			
GmFDb1	:	-----	:	-			
GmFDb2	:	-----	:	-			
PvFDb	:	-----	:	-			
MtFDb	:	-----	:	-			
PsFDa	:	-----	:	-			
MtFDa	:	-----	:	-			
PvFDa	:	-----	:	-			
LjFDa	:	-----	:	-			
GmFDa1	:	-----	:	-			
GmFDa2	:	-----	:	-			
SlSPGB	:	-----	:	-			
StFD1	:	-----	:	-			
SlFD2	:	-----	:	-			
StFD2	:	-----	:	-			
AqucaFD	:	-----	:	-			
AtFD	:	-----MLSSAKHQ	:	8			
AtFDP	:	-----MLSSAKHN	:	8			
AcFD	:	-----MLSTMGGE	:	8			
MeFD1	:	-----	:	-			
MeFD4	:	-----MLSSTAGE	:	8			
PtFD1	:	-----MLSPPNCE	:	8			
GrFD1	:	-----	:	-			
GrFD2	:	-----MWTIFSMLGIGER	:	13			
VvFD2	:	-----MLSSTGGE	:	8			
PtFD3	:	-----	:	-			
PtFD2	:	-----	:	-			
MeFD2	:	-----	:	-			
MeFD3	:	-----	:	-			
GrFD3	:	-----	:	-			
GrFD4	:	-----	:	-			
GrFD5	:	-----	:	-			
VvFD1	:	-----	:	-			
MdFD1	:	-----MLSSTG--	:	6			
PpFD1	:	-----MLSSTSTG	:	8			
FvFD1	:	-----	:	-			
EgFD	:	-----	:	-			
MaFD1	:	-----	:	-			
MaFD5	:	-----	:	-			
MaFD2	:	-----	:	-			
MaFD3	:	-----	:	-			
PdFD1	:	-----	:	-			
MaFD4	:	-----	:	-			
CsFD	:	-----	:	-			
MdFD2	:	-----	:	-			
PpFD2	:	-----	:	-			
FvFD2	:	-----	:	-			
ZmDLF1	:	-----	:	-			
SbFD1	:	-----	:	-			
SiFD1	:	-----	:	-			
TaFD1	:	-----	:	-			
HvFD1	:	-----	:	-			
BdFD1	:	-----	:	-			
OsFD1	:	-----	:	-			
OsFD4	:	-----	:	-			
SiFD4	:	-----	:	-			
OsFD2	:	-----	:	-			

OsFD5	:	-----	:	-	
OsFD6	:	-----	:	-	
ZmFD2	:	-----	:	-	
SbFD2	:	-----	:	-	
SiFD2	:	-----	:	-	
BdFD2	:	-----	:	-	
HvFD2	:	-----	:	-	
ZmFD3	:	-----	:	-	
SbFD3	:	-----	:	-	
SiFD3	:	-----	:	-	
OsFD3	:	-----	:	-	
TaFDL15	:	-----	:	-	
HvFD3	:	-----	:	-	
TaFDL13	:	-----	:	-	
BdFD3	:	-----	:	-	
		*                  80                  *                  100                  *                  120			
AtDPBF4	:	SMNLDELLKTVL-----PPAEEGLVRQGS-LTLPRDLSKKT	VDEVWR	: 83	
AtAREB3	:	SMNLDELLKSVCSEANQP---SSMAVNGGAAQGLSRQGS-LTLPRDLSKKT	VDEVWK	: 98	
OsAREB3	:	SMNLDELLKSVFPDGADLDGGGGGGGIAGQSQPALGLQRQGS-ITMPPELSKKT	VDEVWK	: 116	
TaFDL2	:	-----		-	
TaFDL3	:	-----		-	
TaFDL6	:	SMNLDDFVRVSLPDEKNLPLPNGAGNSGSQSTSAFGLERQGS	SITVPLPLSKKT	VDEIWR	: 106
GmFDc1	:	-----MASWP---PKPTEIFC-VHAREKAMASSPCDCWPHLSPPSS---	IEHVWN	: 43	
GmFDc2	:	SLAKVEKKEEGGEAWPRGHPNLQRYFVRLSVREKAMASSPCDCWPHSSSSSSSS	IEHVWN	: 90	
PvFDc	:	-----MASSPCDCWTHLSQSLSSSSSSSPSSSLPQTLPLPPSPSSSS---	EHVWN	: 46	
GmFDb1	:	-MLSSSTSTTTTTTCHKRNNLNHKALSPSTTKPSHFHQTPISISSSSSNKAMEDVWE		: 59	
GmFDb2	:	-MLSS-----TSTTTTCHSRNNLNHKALSPSTTKPSHFHSLTPRSSHISTNNKDMEDVWE		: 54	
PvFDb	:	-----	MEDVWN	: 6	
MtFDb	:	-----MSHQPLQEQTTPQ-QQHHLRP---NKPPKNTMEDVWK		: 33	
PsFDa	:	-----	MEELWK	: 6	
MtFDa	:	-----	MEEVWK	: 6	
PvFDa	:	-----	MEEVWK	: 6	
LjFDa	:	-----	MEEVWK	: 6	
GmFDa1	:	-----	MEEVWK	: 6	
GmFDa2	:	-----	MEEVWK	: 6	
SlSPGB	:	--MWSSSD-----NRGLSASSSSSSSSSHSPFSPRL-----	KTMEEVWK	: 38	
StFD1	:	--MWSSS-----RSSSSSSSSSHSPFSPRL-----	KTMEEVWK	: 30	
SlFD2	:	--MWSSSSEHHQNHQNLATNSS---SSSSSCSYNHPINPILSITS---KTMEEVWK		: 49	
StFD2	:	--MWSSSNEEHQGLNKNLATNSSKSSSSSSSTSCSYNHPINPIL-INS---KTMEEVWK		: 53	
AqucaFD	:	--MWSSRGEE---IFNTRTNRRRSTSSSSSYQSQSPFLATPPFQIPSSRKAMEEVWK		: 55	
AtFD	:	RNHRLSATNKNQTLTKVSSISSSSPSSSSSSSTSSSSPLSQDSQAQKRLVTMEEVWN		: 68	
AtFDP	:	-----KINNHSAFSISSSSSSLSTSSS-----LGHNKSQVTMEEVWK		: 45	
AcFD	:	EESLKNYSTQGHNRPSLASSTPFPSPFSPFLPHASP--NQNLNRNPGPSPTMEEVWN		: 66	
MeFD1	:	-----MPFPPSNLL-----HQNR--KPSTMEEVWK		: 23	
MeFD4	:	AT---AQTKTLNRVSS---SSSTSSSPSPFSPSDIH-----HKG---KTMEEVWK		: 49	
PtFD1	:	GTSYNNKTKSLRKVSSSISKSSSTSSSPSIFSPSNL-----HCQAQQAETMEEVWK		: 62	
GrFD1	:	-----MLSPSNKPQITPLKPPSPSP-----	HRPKTMEEVWN	: 32	
GrFD2	:	RSDTCCLNTLSSVSSSSSSSSSSSSSSSSPLSTPSHL-----HNQT-HTPKTMEEVWK		: 66	
VvFD2	:	EANLSSGNNNNNRVSSS--SSKSSSTSSSPSPFPASSV-----LPTP--RGKNMEEVWK		: 59	
PtFD3	:	-----	MEEVWN	: 6	
PtFD2	:	-----	MNGASMEEVWD	: 11	
MeFD2	:	-----MRSFPERNDYLSRNSSSPSKSSSTSSSPSSQ-----LLNPSSARQAMEDVWK		: 47	
MeFD3	:	-----	MEDVWK	: 6	
GrFD3	:	-----	MEEVWK	: 6	
GrFD4	:	-----	MEEVGK	: 6	
GrFD5	:	-----	MEEVWG	: 6	
VvFD1	:	-----	MDEVWK	: 6	
MdFD1	:	---SDQTNHNTTS-----TSSWSSSSSPSPFS-QPSSFQTPQRTMEEVWK		: 47	
PpFD1	:	RGSSDQTNYNNTSNNSTNTPKRVASSSTNSSWSSSS-PSPFSHQPFSLQTPQRTMEEVWK		: 67	
FvFD1	:	-----	MEEVWK	: 6	
EgFD	:	-----	MEEVWK	: 6	
MaFD1	:	-----	MEEVWR	: 6	
MaFD5	:	-----MWRSEQGNHHGSTNNRVASSSSSSSSSRSPSSTTSSLLPQAPKRRTMEEVWK		: 54	
MaFD2	:	-----	MEEVWK	: 6	
MaFD3	:	-----MTPYSSMLSKNTRIMEEVWK		: 20	
PdFD1	:	-----	MEEVWK	: 6	
MaFD4	:	-----MPGRN---MEEVWK		: 11	
CsFD	:	-----	MEEVWK	: 6	
MdFD2	:	-----	MEDVWK	: 6	
PpFD2	:	-----MIPTTTTSPRSIKSMEDVWK		: 20	
FvFD2	:	-----	MTMEDVWK	: 8	
ZmDLF1	:	-----	MEDDEDIWA	: 9	
SbFD1	:	-----	MAAIDMEDDDDIWA	: 14	
SiFD1	:	-----	MAAMDLEDDEDIWG	: 14	

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TaFD1 : -----MAMEADDDDLWG : 12
HvFD1 : -----MSMEADDDVWG : 12
BdFD1 : -----MAAMEDDEDMW- : 11
OsFD1 : -----MAMEDDEDMWA : 11
OsFD4 : -----MERAAG----- : 6
SiFD4 : -----MYFAEGQRREVK : 12
OsFD2 : -----MANYHHQEY : 9
OsFD5 : -----MANYHHQEY : 9
OsFD6 : -----MANYHHQEY : 9
ZmFD2 : -----MAANYHHYQM : 10
SbFD2 : -----MAANYHHYQM : 10
SiFD2 : -----MAANYHHYQM : 10
BdFD2 : -----MASF----- : 4
HvFD2 : -----MANYRLGGG : 9
ZmFD3 : ----- : -
SbFD3 : ----- : -
SiFD3 : ----- : -
OsFD3 : ----- : -
TaFDL15 : ----- : -
HvFD3 : ----- : -
TaFDL13 : ----- : -
BdFD3 : ----- : -

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                *      140      *      160      *      180
AtDPBF4 : D--IQQ--DKNGNGTSTTTT-----HKQPTLGEITLEDLLR : 116
AtAREB3 : D--IQQ--NKNG-GSAHERR-----DKQPTLGEMTLEDLLK : 130
OsAREB3 : G--IQDVPKRGAEEGGRWRR-----ERQPTLGEMTLEDFLVK : 151
TaFDL2 : ----- : -
TaFDL3 : ----- : -
TaFDL6 : D--IQQEESSDDEKRSSGC-----DAQMSFGEITLLEEFQR : 141
GmFDc1 : D--IKLDSLNSPVDIDF-----NNN-HSVSDSSFLN- : 72
GmFDc2 : D--IKLASLSNSSVDLDL-----NNNNHVSVSFLNQ : 121
PvFDc : D--IKLPSSLNSPVDEN-----PSSSHSSLS- : 71
GmFDb1 : GININLTSLNDHNTNT-----SKGAKFQDFLSR : 87
GmFDb2 : G--INLTSLSDHNTNTN-----TSKGANFQDFLSS : 82
PvFDb : G--INSTALSEHNTTH-----ISKGAKFQDFLAG : 33
MtFDb : D--INLPSTLNHMSNT-----VSSPSLMT- : 55
PsFDa : D--INMSSLNEQNTRPMIM-----STRDSSTFGGVILQDFLAR : 43
MtFDa : D--INLSSLNDQNTR-PMIM-----STRNSTFGGGVILQDFLTR : 42
PvFDa : D--INLATLNDQ-----VSSTHSNLGGVILQDFLAR : 35
LjFDa : D--INLASLNDHNTHT-----PSTTHSTFGGAIFQDFLGG : 38
GmFDa1 : D--INLATLNEQSTI-----STRPNVEGVMFQDFLAR : 36
GmFDa2 : D--IN-----VFQD----- : 13
SlSPGB : D--INLSSLQDHTTNYSRDH-----HHLHDHNNHQAANFGGMILQDFLAR : 80
StFD1 : D--INLSSLQDHTTNYSKDHQ-----HHLHDHNNHQAANFGGMILQDFLAR : 73
SlFD2 : D--INLS-----CSRDA-----QNTS--GMVLQDLLAR : 73
StFD2 : D--INLS-----CSRDP-----QNTTAGGIILQDLLAR : 79
AqucaFD : D--INPSSSLQ--QSTREEQG-----TVPLPPSTNTCHHHPHHKTSLRGINLQDFLAR : 105
AtFD : D--INLASIHHLNRHSPHP-----QHNHEPRFRGQ-----NHHNQNPNSIFQDFLKG : 113
AtFDP : E--INLGSLHYHRQLN-----IGHEPMLKNQ-----NPNN--SIFQDFLNM : 82
AcFD : D--VSLASLHDQPTRD-----DPTRNPTFRG-----LTFLDLFSR : 99
MeFD1 : D--INLASLHDHSSGDQDI-----AVAPTRHN-----QHRN--PNFILQDFEAR : 63
MeFD4 : D--INLASLHDHPSGDQDL-----SFTPRLHN-----PHHN--PNFILQDFEAR : 89
PtFD1 : D--ISLASLHDHTSTQEL-----SMTPLRHNISHHHRRHHHHHHNNSPNFILQDFLAR : 114
GrFD1 : D--ITLASLHDHSSSSSSS-----REFFS-SS-----PHLILQDFLAA : 67
GrFD2 : E--INLASLHDN---SSS-----REGLNPHN-----PHFILQDFLAR : 98
VvFD2 : D--INLASLHDHPSR-EDL-----SVLPRPQN-----PHAS--FRGVILQDFLAR : 99
PtFD3 : D--INLASLHEHPNS-----HTGSNN-----NTDDHVFGMMFQDLLAR : 43
PtFD2 : D--INLASLHDHSNT-----NTSSN-----TNHHSFNGMVQDFLAR : 46
MeFD2 : D--INLTCLQDCPSG-----ANHAN-----HPAFPMILQDFLAR : 81
MeFD3 : D--INLTCLQCEP-----ISNPN-----HHR-PVGMILQDFLVR : 37
GrFD3 : D--ISLASLSDHPVD-----SILSTRK-----NPNFPMILLNFLAT : 42
GrFD4 : D--ISLSSLNGISLQKGHG-----TLISTAAT-----NPAFPMSMILQDFLAT : 46
GrFD5 : D--IGLASLNDHPAV-----TPATAN-----PSPFSPVILQDFLAI : 39
VvFD1 : D--LSLSSLHHHSIS-----TVNNATTN-----THHAFRSTLLQDFLAR : 43
MdFD1 : D--FNLASLTDPTTQIRSSSSTSPLLQINLPNGPHQG-----HHHHPNFRNMTLQDFLAR : 100
PpFD1 : D--INLASLSETTPNRRSS---LLHHINLPHG-----THDPNFRN--LQDFLAR : 109
FvFD1 : D--INLASLSNAN-----NSPNFRNMTLQDFLAR : 33
EgFD : D--ITLSSLQPHHHHHH-----HHHHSPSS-----VGPSLRGNGLQDFLGR : 45
MaFD1 : D--ISLCSLHHQERQLT-----PMNHHQRHSPA-----TSPSFRATMLQDFLAG : 48
MaFD5 : D--IGLRSLHHQERLLT-----PLN-HQRHSPT-----ASPSFRAIILQDFLAG : 95
MaFD2 : N--VTLNSLYQ-DKPMT-----PVDYRHDHPT-----SSPSFRGMILQDFPAG : 47
MaFD3 : D--ISLSSLQDMPSPISLPNHLERLCGVSSS-----TTTSFKSIILQDFLTE : 67
PdFD1 : D--ISLSTIHEGT-----EANRT-----TTTSFEGIIVQDLLAK : 38
MaFD4 : D--ISLSTLHQDVPSTP-----VLAFHPAT-----TISSFRAVMLQDFIAD : 50

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CsFD      : D--ISLSSLHSRSDHDFSSAAPTPISLHLHHHH-----SAANLRHIILQDFLST : 53
MdFD2     : D--ISLASLSRNASDNTT-----TSTHH-----PTAAAFRGITFQDFLAS : 44
PpFD2     : E--ISLASLS-HANDHHG-----HCPP-----AAAAAFRGMIFQDFEAT : 56
FvFD2     : D--ISLASLCDHSSPGAAG-----SSATR-----NDPAGVRGFIQDFMAA : 47
ZmDfL1    : NTASSPSASPPQPVAGSVST-----CSAFISTQLSLN-SRLH-LLSS : 50
SbFD1     : NTASSPSASPPQPVAA--AST-----CSAFISTQLSLN-SRLH-LLSS : 53
SiFD1     : KTASSPSASP-TPLTTAVVAP-----CGAFISTQLSLN-SRLQ-LLST : 54
TaFD1     : AVTTSPSASPPPPSSA-----AAISTALSLN-TRLQ-LLAA : 46
HvFD1     : AVTTSPSASPPP-----AISTALSLN-TRLQ-LLAA : 41
BdFD1     : AVTTSPSASPPATAAA-----AAISTALSLN-PRH-LLAS : 45
OsFD1     : NTSS-PSASPPRPR-----GFISTALSLNSTHLQGLLPS : 44
OsFD4     : -----TGSG-----GDDDELVLV : 19
SiFD4     : GLDSSASRSGQHGGRA-----KRGMEPERGDAAELLVW : 45
OsFD2     : YQMAAAAAVWPREPD-----SPQLSIMSGCSSLFLSIS : 42
OsFD5     : YQMAAAAAVWPREPD-----SPQLSIMSGCSSLFLSIS : 42
OsFD6     : YQMAAAAAVWPREPD-----SPQLSIMSGCSSLFLSIS : 42
ZmFD2     : AVHAAAAAAWRREP-----SPQLSFVSGCSSLFLSIS : 43
SbFD2     : AVHAAAAAAWR-EPD-----SPQLSFVSGCSSLFLSIS : 42
SiFD2     : AV--AAAAAAWR-EPD-----SPQLSFVSGCSSLFLSIS : 40
BdFD2     : -GGQYVGTGAWMREPE-----SPQLSLMSGCSSLFLSIS : 36
HvFD2     : GNGHYEMAAAWREPE-----SPQLSLMSGCSSLFLSIS : 42
ZmFD3     : -----MPR-----SPQLS-LSGCSSLFLSIS : 19
SbFD3     : -----MPR-----SPQLS-LSGCSSLFLSIS : 19
SiFD3     : -----MPR-----SPQLS-LSGCSSLFLSIS : 19
OsFD3     : -----MAEQLGGVG-----SPQLS-LSGCSSLFLSIS : 25
TaFDL15   : -----MSWE--EPG-----SPQLS-LSGFSSLPLSIS : 23
HvFD3     : -----MSWE--EPG-----SPQLS-LSGFSSLPLSIS : 23
TaFDL13   : -----MSWE--EPG-----SPQLS-LSGFSSLPLSIS : 23
BdFD3     : -----MSREEAGGS-----PGQLS-LSSFSSLPLSVS : 25

                *          200          *          220          *          240
AtDPBF4   : AGVVTET-----VVP--QENVV-----NIASNGQWVEYH : 143
AtAREB3   : AGVVTET-----IPGSNHDGPVGGGSAGSGAGLQONITQVGPWQYH : 172
OsAREB3   : AGVVTDPNLPGNMDVVGAAAAAAGTSDLNAGAQLQQYHQQALEPQHPSIGAPYMATH : 211
TaFDL2    : -----MAGPFMASH : 9
TaFDL3    : ----- : -
TaFDL6    : AGIVTGQYQKD-----AEELIDLVTGESAHLMTRVQDFPQGTSAIDAYVRQ : 189
GmFDc1    : -----LT : 74
GmFDc2    : PLSTFLT-----LT : 130
PvFDc     : -----LS : 73
GmFDb1    : PFTNFSTIASA-----DPSPPVTTALTLS : 110
GmFDb2    : PFTNFSTIAS-----DPSPPVTTALTLS : 104
PvFDb     : PFN-----PSP-VTALTLS : 46
MtFDb     : -----PSSLHSTINLN : 66
PsFDa     : PLNINPPKNIDHHY-----SSNNSSSSVASDQN---PSFPCPTVSTAPPPLVTALSLN : 93
MtFDa     : PLTLDPKSLD--Y-----SSNNSSSSVASDQNNNNASFYCP-ISTTPPLVTALSLN : 92
PvFDa     : PFTIDPPNATL-----SSQTSSSLYGPPS-----SPAPSLLTALSLN : 72
LjFDa     : HF---PPNTTV-----SSSLASAPQSTSL-----YSPAPPLVTALSLN : 74
GmFDa1    : PFTTIDPPNTTLLS-----SASSETAANSLFSP-----ASPGPPLVTLSLS : 77
GmFDa2    : -LTTIDSPN-IILS-----SASSETG---FS----- : 34
SlSPGB    : PFANE-SSPAA-----AAAAS-----PVS--ATTMLNLN : 107
StFD1     : PFANE-SSP-----AAAAS-----PVS--ATTMLNLN : 97
SlFD2     : PFANDPSTAAV-----TRAYGS-----PVPPPVTMLTLN : 103
StFD2     : PFANNPSTAAA-----YGSPVP-----PLPPPVTMLTLN : 109
AquaFD    : PFSREPPSSLG-----YCTTSSIDQFGHFG-----SAPQPPTYLCLN : 143
AtFD      : SLNQEPAPTSQTTG-----SAPNGDSTTVTVLY-----SSFPPPPATVLSLN : 155
AtFDP     : PLNQPPPPP-----PPPSSTIVTALYG-----SLPLPPPATVLSLN : 119
AcFD      : PAGHNQPPS-----AGY-----GSPASPPAVALSLN : 125
MeFD1     : PFSKDPQTRR-----VSAHAQGDPD--LY-----GSPVPPPATVLSLN : 99
MeFD4     : PFSKDTPTRI-----VSAHVESG--LC-----GSSVPPPATILSLN : 123
PtFD1     : PFNKDPPTRM-----VSIIRDSTP----F-----GSSAPPPATVLSLN : 148
GrFD1     : RSDPPPQQQ-----TNGGGDTNTM--LY-----GSPLPPPATVLSLN : 103
GrFD2     : PFSKDPANR-----VSANGDT-TF--LV-----SPPPPPATLLSLN : 133
VvFD2     : PFNKEPPTS-----ASLDQSTVTEAR-IY-----GSLPPPATVLSLN : 137
PtFD3     : SSNKDTPTRV-----ASKEPSSGGGNNFLK-----NSLGGPPATMLNLN : 82
PtFD2     : PSNKDTSTRA-----ASKEPSSGGGNSFLK-----NSLGGPPATMLSLN : 85
MeFD2     : PFNKEPPT-----EPS-SGRANDFL-----DSLGRPPATMLSLN : 114
MeFD3     : PFNKDSPT-----PPSSAGRATDFP-----NSLAPRTATILTLN : 71
GrFD3     : PINKE-PPTP-----RTS-VVSSNDTSSTE-----GPAPFVPGTIILSLK : 79
GrFD4     : PFNNI-PPTT-----SSG-VLS-NEEPTFL-----HSLSLTPATVLRIN : 82
GrFD5     : PINKEMPPTA-----RSGCGTSLTEETTLF-----GSLPPTPATLFTLN : 78
VvFD1     : PSNMDPLRTG-----TSTADPSSAGAATPF-----ASPLPPPPTLLSLN : 82
MdFD1     : PFAHDS-----AAAAALVSAAASPP-----SPLAPAAPTLLGLN : 135
PpFD1     : PFSHNEPPVSLVS-----TPTEQATLNSPASPP-----LP-PGPPPPVLSLT : 150
FvFD1     : PFANVVSPTPDHH-----HHNNNNHITLNSPPP-----PPPPPPPATVLSLN : 75

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EgFD      : PSHKDP-----TPANITSPSSLP-----PH---HPLTFLSLN : 75
MaFD1     : PLNRPHAVPPDD-----VPRPR-----SSTPLTQLSLN : 77
MaFD5     : PLNRPHAAAKN-----SPLP-----PSTMPPTALSLS : 122
MaFD2     : PLNRPLTISPPAVE-----ELPVPPPPS-----PPAHPQTVLNLN : 82
MaFD3     : HFKATS-----DRSPPPS-----TALGLNSNLGGG : 92
PdFD1     : TFKEPSLASAPFG-----DLCPPPF-----SGLSLNS---GH : 67
MaFD4     : AFKAEN-----RAPPPS-----THSPPAS-LSPT : 73
CsFD      : STSKLDSSSS-----SSSALALP-----PVPAPPPPTLLSLN : 84
MdFD2     : TST-----TSSNI-----DPPPP-----AAASTVLRIN : 67
PpFD2     : CAS---SNKDK-----GRPPQP-----TIAAPATVLSLN : 83
FvFD2     : ASTKLAPSSLAT-----DLGPP-----PPANLLSLN : 73
ZmDLF1    : AAAGGGSSP---V-----RGGAYGADGVRHHH-MALGGG--FRNAAASQGP-FFPYN : 95
SbFD1     : AAAAGGSSPTTRGV-----GAGAYAADGVRHHMSLGVGGG--FRNAVASPASFFPYN : 104
SiFD1     : TAAAGGSSPP-HSV-----GAGIFAADGLRHHVGLDGGGGGFRNAPASPAPFFSAYG : 106
TaFD1     : TGVGGGSPFHPGGV-----GAGSPFHPG-----GGCYRNGG-ASPTFFSSA : 87
HvFD1     : -----AGSPFHPG-----GGCYRNAA-ASPSPFFSSA : 67
BdFD1     : -----SSSPFHPGF-----GGCSRNAASPPCFFSAA : 73
OsFD1     : SFVDAAASP-----CHASGNNG-----GGDGRNAAPMSSIFFASAS : 81
OsFD4     : -----PASFDG-----LPSSRSYP- : 34
SiFD4     : GTT-----GAGNDDG-----AAAACLCP- : 64
OsFD2     : TLRDDDDGG-----GVRLAG-----AALPATPVSLAGTA : 71
OsFD5     : TLRDDDDGG-----GVRLAG-----AALPATPVSLAGTA : 71
OsFD6     : TLRDDDDGG-----GVRLAG-----AALPATPVSLAGTA : 70
ZmFD2     : TLQDDDDDDRA-----AAVVIAAG-----HALPSTPVSLAGFV : 75
SbFD2     : TLQDDDDDDGR-----PAVVIAG-----HAMPSTPVSLAGFA : 73
SiFD2     : TLQDDDDG-----AVVIAG-----HALPSTPVSLAGFA : 68
BdFD2     : VLRDGD-----DLGGV-----RSLPATPVSLAGFV : 62
HvFD2     : GLRDDDTDL-----HLLAGA-----RSLPSTPVSLAGFA : 71
ZmFD3     : STSTSRD-----SAALKAPVPAPPAGA---VPPRRTPLLSL SVGEEE : 58
SbFD3     : STSTSRDND-----SAAALTAPVPAPPAGASLHPLPRLPILSL SVGEE : 66
SiFD3     : STSTSRDND-----SAAAAAPPSS-----LHPLPPR-GPLLSL SVGGG : 58
OsFD3     : SAGTSAADG-----APHLSLGVG--- : 43
TaFDL15   : STAHPPAR-----LPSLSLSIG--- : 40
HvFD3     : STAVRPAR-----LPSLSLSIG--- : 40
TaFDL13   : STAAPPAR-----LPSLSLSIG--- : 40
BdFD3     : SATSTPTPH-----LPPLPSLSLSIGGGG : 49

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          *          260          *          280          *          300
AtDPBF4   : HQP-----QQQQGFMTYPVCEMQDMV---MMGGLSD : 171
AtAREB3   : QLPS-----MPQPQAFMPYPVSDMQAMVSQSSLMGGLSD : 206
OsAREB3   : LAPQP-----LAVATGAVLDPISYDQGIT--SPMLGALSD : 244
TaFDL2    : LGPQP-----LSVATGAIMEPIYPDGQIT--SPMLDALSD : 42
TaFDL3    : TRPQP-----LSVAIPSTMDSIYPDRQMS--ISSLELSD : 33
TaFDL6    : SIAQP-----LSVAIPSTMDSIYPDRQMS--ISSLELSD : 222
GmFDc1    : STSS-----SVFHKHHDHSLSVSNT----SFEASG : 102
GmFDc2    : STSS-----SSSVFHKHHDH--SLLSVSDP----- : 152
PvFDc     : SS-----HNH-HSLLSVSNASFPSN----- : 92
GmFDb1    : TRSE-----FHFDPLTHKDLQLGQPHHKNGSKVEP-- : 140
GmFDb2    : TRSE-----FHFDsATHKDLQLGQPHHKNDKSKVEP-- : 134
PvFDb     : TRSS-----EYLP--LHKDLQL----LHTASKTEP-- : 70
MtFDb     : SLPE-----FHFDPLAHNDLQLEQNHHHTTTLK--- : 95
PsFDa     : TRPDH---FPFDTLIRHNKDN-----SQLLFQQQHQQQRNITVSKVS-HVNVNTP : 140
MtFDa     : SRPD---FLYDPLIRHNKHN-----SQLLLQQQ---QHNIGVSNVSPCFVNASP : 136
PvFDa     : SHPH---LLFDHLT-----HKPSHHH---PPSPNPS----- : 99
LjFDa     : ARPD---FAFDPLRPNKSQL-----PQPLHHH---HPPSKPSSPCFPINPPP : 118
GmFDa1    : -----SLPHHHHFRFEHPSSIPPPS----- : 98
GmFDa2    : -----LPHFHFQ--SQPSSQHPPPS----- : 52
SlSPGB     : SVP-----ELHFFD--NPLRQNSILHQPN----- : 129
StFD1     : SVP-----ELHFFD--NPLRQNSILHQPN----- : 119
SlFD2     : SVP-----EFHFFSNLNPiRQNNQKHISNDALASP-- : 133
StFD2     : SGP-----EFHFFSTLNPiRQTQQKNISNEGLASP-- : 139
AquaFD     : TDPS-----QLPLVNHNSDAASSFVESLNNPFDASFAS- : 175
AtFD      : SGAG---FEFLDNQD-PLVTS-----NSNLHTHHHLSNAHAFNTSFREALVPSSS : 200
AtFDP     : SGVG---FEFLDTTE-NLLAS-----NP-----RSFEESAKFGC : 149
AcFD      : SGPDR---FGPMASDD-PIGTD-----MILQTPP--VSNISSMNVSFREALASAAS : 169
MeFD1     : TGPg---FDFLDNDYPLRPT-----SHLPS--HPVS---SFNSPFEALDSSS- : 139
MeFD4     : SGPV---FDFLDND-PLRPA-----SHLPS--RPVSNFSSFNSSLEALDSSSG : 166
PtFD1     : SGPg---FDFLDNDYPLRPT-----SQLQS--NPISNISSFTSPFEGLDSSPG : 192
GrFD1     : SGPg---FDFLDN-----PRLQS--SPISTLPTFNSPFEALASSTT : 139
GrFD2     : SGPg---FDFLDNDY-PLRSK-----PRLQMNNPISDLNPMNCPFEALASPSG : 178
VvFD2     : SGPE---FHFLSSH-PARPH-----SHLVQH-NPISNVASFAPFDALASSTG : 181
PtFD3     : YG----- : 84
PtFD2     : SGSDHFHYLESSNTVPVRPNPQ-----MHSANGGTISFDSSLDSPFDALGSSS- : 134
MeFD2     : SGSD-FEILESGTASRRPN-PQ-----MNTHARVDTPSFGWLISPLDALGSSS- : 161
MeFD3     : SGPS-PDILESDSVRTRPNNPQ-----LHTHASIG-----ALGSSS- : 106

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GrFD3 : SGFG-SACRCTETGDPMRPNLE-----VNARAGVPAAPSFSSYSSGFRGFGSPE- : 127
GrFD4 : TGS-----DPLRSNPA-----VNNRTAIAPSSLGAHNSTFQGFSSAR : 121
GrFD5 : ARS-----IASVAVKAPAPS----- : 93
VvFD1 : SLPD---FHCIDDTD-PMKPH-----SHLHNN----- : 105
MdFD1 : NPGSE---FQLFYASDS-LRPPSSGFIDHH-QANHNNISSSVSNSFS-GCPFESLAASSF : 189
PpFD1 : NSGSDH--FQLFYVSDPLIRPPSSEFHPHHRQSNPNNISSSVSNSFS-ASPESLAASSS : 207
FvFD1 : HLDSQ--FQLFCIENLNTTRP-----FVNLNHPNTSTVSEKSFNGAYPFEGLGSSQSS : 126
EgFD : NSGPE---FHFLDDSSPLGLST-----TRFQSHSRAGMACFS-TSPFEALAGSAD : 121
MaFD1 : PTLE-----IQPMGSDAHANSSSS--GYNASFISPAF : 107
MaFD5 : PRLE-----IQLMGSDAHGNSNSSSNGCRASFISPAF : 154
MaFD2 : SGLE-----FQHLVVDANSRSNAS--NSNGRYSS-AF : 111
MaFD3 : SNGL-----SSSSASSFFSDGVGD--GVVACCSKK-- : 120
PdFD1 : LVGY-----PHPNPDSNSGASPA--GFFAYCSKKRA : 97
MaFD4 : SDNS-----RQFFGYDLNASASAS--GSNAAQSES-T : 102
CsFD : STRE---LHFDPNNSIATATA-----AAHFRHHDPSLSAFAHSPFDGLGPPP : 130
MdFD2 : CGTRSDLKQHLLTTSATITGPA-----LLKP--NPITRPTSTSVTTTTTRPNS- : 114
PpFD2 : ---SDI-QLLPESESTTSTAPAP-----LLKHPLNPQLLQTSAAATPSFHFLNSS : 129
FvFD2 : SSGSDHFQQYQVHNSSATTSTNTS-----APPNLAKPNPDSTTRPSSSLISFSNK- : 123
ZmDLE1 : LAGA-----GADVEPFDDG--RGVLEDDMSVGAAASGT-- : 126
SbFD1 : LDSA-----AADVAPLDAGPA-RGLLEDEMCLGAGAAAT-- : 137
SiFD1 : LD-A-----GGGVAPIDAGAA-RSALEDEMCVGPGTAWA-- : 138
TaFD1 : AAS-----FPRIAPVDAGPA-RRALEREMCYGHGAAAWP- : 120
HvFD1 : AAS-----FPRIAPLDAGPA-RRALEREMCYGHGAAAWP- : 100
BdFD1 : AASS-----FPHFAAAPLDGA-ARG---MCGLAPVPAWS- : 103
OsFD1 : YHQQQHH-----LPAPAPLDGAILPARRFGLDMCAAAAAAPA-- : 118
OsFD4 : -----SCIGGGS-AAAASASLERELLYRAELHQQQ : 63
SiFD4 : -----AAVAAAPGSVFPRHALEQEMLRRGDLQLQ-- : 93
OsFD2 : GGA-----STPGGDEV- : 82
OsFD5 : GGA-----STPGGDEV- : 82
OsFD6 : GGA-----STPGGDEV- : 81
ZmFD2 : G-----DEV- : 79
SbFD2 : AG-----DEV- : 78
SiFD2 : G-----DEV- : 72
BdFD2 : GA-----GDEV- : 68
HvFD2 : G-----GDEV- : 76
ZmFD3 : EDEG-----EDEEYLLGAGALDLQLTGPG- : 83
SbFD3 : ENE-----DEEYLLGAGALDLQLTGAG- : 89
SiFD3 : DEEQ-----EEEEYLLGG--LDLQLTGAG- : 81
OsFD3 : -----GAEELDLLQVGI- : 56
TaFDL15 : -----NGSA- : 44
HvFD3 : -----TG-- : 42
TaFDL13 : -----TG-- : 42
BdFD3 : N-----DEQP- : 54

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*          320          *          340          *          360
AtDPBF4 : TP-----QAPGR-----KRVA-GEIVEKTVERRQKRMKNRESAAR : 206
AtAREB3 : T-----QTPGR-----KRVASGEVVEKTVERRQKRMKNRESAAR : 241
OsAREB3 : P-----QTPGR-----KRCATGEIADKLVERRQKRMKNRESAAR : 279
TaFDL2 : P-----QTPRR-----KRGASDGVTDKVVVERRQKRMKNRESAAR : 77
TaFDL3 : L-----QSPSH-----KRMSSQDVVYKVADRRQKRMKNRESAAR : 68
TaFDL6 : L-----QSPSR-----KRMSSQDVVYKVADRRQKRMKNRESAAR : 257
GmFDc1 : -----SKKTTLLD-----QRHARIKNRESAVR : 125
GmFDc2 : -----NTLQD-----QRHTRVIKNRESAVR : 172
PvFDc : -----QTTHQD-----QRHVRIMKNRESAVR : 114
GmFDb1 : -----FGKPSG-----IKRIILQSG-----DMRKARIMKNRESAAR : 170
GmFDb2 : -----FGNPFG-----NMRILPSG-----DMRKARIMKNRESAAR : 164
PvFDb : -----FAHPFS-----NERAPPASR-----DMRNARIMKNRESAAR : 101
MtFDb : -----VEALLSNSI-----ERHHRIMKNRESAAR : 120
PsFDa : FDPNVGV-VSNAFTCFG--KRF-----GEPPDVS---PGERRNKRMKNRESAAR : 184
MtFDa : CDQNVGVPASSSFTCFG--KRF-----GEAPDIS---PGERRNKRMKNRESAAR : 181
PvFDa : -----LG-TKRF-----PEP-DCN---LGDRRNKRMKNRESAAR : 129
LjFDa : FDNHLPF--TSPLPCFGSNKRF-----AEPADYG---LGDRRNKRMKNRESAAR : 163
GmFDa1 : -----NKRF-----AQPADHCS--TIGDRRNKRMKNRESAAR : 129
GmFDa2 : -----NKTS-----AQP-----AADRNKRMKNRESAAR : 78
SlSPGB : -----ASGRKRVV-----PET--EDN--STGDRRNQRMKNRESAAR : 162
StFD1 : -----VSGRKRVR-----PET--EDN--STGDRRNQRMKNRESAAR : 152
SlFD2 : -----APGRKRHS-----ESDNNNNN--NSSELKNRMKNRESAAR : 168
StFD2 : -----APGRKRHP-----ES---NNN--NSGDQSKRMKNRESAAR : 171
AqcaFD : -----SSEFSPFGKKRGS-----ESE---D--NTGDTRHKRMKNRESAAR : 211
AtFD : -----FGKKRG-----QDSNEGSG-----NRHHRIMKNRESAAR : 230
AtFDP : -----LGKKRG-----QSDDTRG-----DRRYKRMKNRESAAR : 179
AcFD : L-----PE-----LGKKRF-----PESDGNPG-----NRHHRIMKNRESAAR : 202
MeFD1 : -----FSKKRV-----QESDNSSC-----DRHHRIMKNRESAAR : 169
MeFD4 : -----MPS-----FGKKRV-----QESDNSSG-----DRHHRIMKNRESAAR : 199
PtFD1 : -----LPS-----FCKKRT-----QESDGSSG-----DRHHRIMKNRESAAR : 225

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GrFD1 : -----LAT---FGKKRT-----QSDNSNG-----NRHKRMKNRESAAR : 172
GrFD2 : -----LAC---FDKKRF-----QDPDNNSG-----DRHKRMKNRESAAR : 211
VvFD2 : -----LTS---FGKKRF-----SEDNSNC-----DRHKRMKNRESAAR : 214
PtFD3 : -----KRP-----QENDDVSG---GDRHERMIKNRESAAR : 112
PtFD2 : V-----FLS---ICKKR-----QENGDVSG---GDRHKRMKNRESAAR : 169
MeFD2 : V-----CPS---ICKKRL-----QENHNNSV-----DRHKRMKNRESAAR : 195
MeFD3 : A-----FPS---ICKKRV-----QENDNTS-----DRHKRMKNRESAAR : 140
GrFD3 : A-----FPS---FCTKRA-----PESNENSN-----DRHKRMKNRESAAR : 161
GrFD4 : V-----FPS---FCRKRA-----QGNNENPD-----DLRLKLMKNRESAAQ : 155
GrFD5 : -----FPS---SCRKKG-----QENEENS-----DRHKRMKNRESAAR : 126
VvFD1 : -----ATA---ATPYLP-----RGSDDNSG-----DRHKRMKNRESAAR : 138
MdFD1 : G-----LPS---FGKRSF-----TESDNSN---SGG-DRHKRMKNRESAAR : 225
PpFD1 : G-----LPS---FGKRAF-----PDSHNSN---SGG-DRHKRMKNRESAAR : 243
FvFD1 : PSPGL---VMPSGFGYGNERL-----PEPDDRSNFGGG-DRHKRMKNRESAAR : 172
EgFD : PGNPG---DVA---FGNIKK-----RVSESEG---GSG-DRHKRMKNRESAAR : 162
MaFD1 : SGNMRPPSPIGLFSFCS-KEA-----VSENPSAS---CDRHKRMKNRESAAR : 153
MaFD5 : SDNMVRPPSPIGLFSFCS-KEA-----MSDPAAC---GDLRHKRMKNRESAAR : 200
MaFD2 : S-----PTGLFSCCSNRKM-----MRESLAIG---IDRHKRMKNRESAAR : 150
MaFD3 : -----HMEHELN-----EESGDRSD---VDRHKRMKNRESAAR : 153
PdFD1 : SE-----QQMEAGFG-----NGNGNGNG---ADRHKRMKNRESAAR : 132
MaFD4 : KK-----RSPEKRPN-----RSVDRQSG---VEQRHKRMKNRESAAR : 137
CsFD : -----FAKKRL-----SDSNSG-----DRHKRMKNRESAAR : 159
MdFD2 : ---LNNPSSVF---PSCCKR---ALDDHDQNRDHHNST---NGRHKRMKNRESAAR : 161
PpFD2 : SCSDALDSSSLFHVPSYNNKRKRPIDHHHHQSNQNSR---DRHKRMKNRESAAR : 184
FvFD2 : -----SALEALGSSSYCKR---AVEE---ENGNSR---HVRHKRMKNRESAAR : 165
ZmDLF1 : -----WAG---GGT---DRHKRMKNRESAAR : 148
SbFD1 : -----WAG---GGS---DRHKRMKNRESAAR : 159
SiFD1 : -----GAGVGGS---DRHKRMKNRESAAR : 161
TaFD1 : -----GAPGAGGAAAPVDRHKRMKNRESAAR : 149
HvFD1 : -----GPPGAGAGAPAPVDRHKRMKNRESAAR : 129
BdFD1 : -----GAAPTGTGAG---PVERRHKRMKNRESAAR : 131
OsFD1 : -----GVPAAG---DRHKRMKNRESAAR : 140
OsFD4 : -----LGGGGGVERRKRPMKNRESAAR : 86
SiFD4 : -----GGVG---DRRERKMKNRESAAR : 113
OsFD2 : -----DMEVRQSGSGGDDRTIRMMRNRESAAR : 111
OsFD5 : -----DMEVRQSGSGGDDRTIRMMRNRESAAR : 111
OsFD6 : -----DMEVRQSGSGGDDRTIRMMRNRESAAR : 110
ZmFD2 : -----DMEVQASG---DDRSIRMMRNRESAAR : 105
SbFD2 : -----DMEVQASG---DDRSIRMMRNRESAAR : 104
SiFD2 : -----DMEVQVSGSGGDDRTIRMMRNRESAAR : 101
BdFD2 : -----EMMDHLRQSGDEDRTVRMMRNRESAAR : 97
HvFD2 : -----D-MELPQGGSGGDDRTVIRMMRNRESAAR : 104
ZmFD3 : -----GGSNSGG---YDDERKNIRMMRNRESAAR : 110
SbFD3 : -----GGSNSGGG---CDEERKNIRMMKNRESAAR : 117
SiFD3 : -----G-SNSGCCDGDDEKNIRMMKNRESAAR : 109
OsFD3 : -----GGGGGGGDEEEERKTIRMMKNRESAAR : 85
TaFDL15 : -----DGEDQLGVSSDDGHKSVIRAMKNRESAAR : 73
HvFD3 : -----GEDQLAVSSDDGQKSVIRAMKNRESAAR : 70
TaFDL13 : -----GEDQLGVSSDDGHKSVIRAMKNRESAAR : 70
BdFD3 : -----PLSSSSAVNQEEDEQSVIRMMKNRESAAR : 83

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*          380          *          400          *          420
AtDPBF4 : SRARKQAYTHLEIKVSRLEENELRRL---LKEVEKILPS---EPPPD---PKWKLRRTNS : 259
AtAREB3 : SRARKQAYTHLEIKVSRLEENELRRL---KEVEKILPS---VPPPD---PKRQLRRTSS : 294
OsAREB3 : SRARKQAYTNLENKVLRLLEENELRRL---KELDEILNS---APPPE---PKYQLRRTSS : 332
TaFDL2 : SRARKQAYTNLENKVLRLLEENELRRL---KELDMITS---APPPE---PKYQLRRTSS : 130
TaFDL3 : SRARKQAYTNLECKVSCLEENELRRL---EKELDMLKS---APPPE---PKKHHRTS : 121
TaFDL6 : SRARKQAYTNLECKVSCLEENELRRL---EKELDMLKS---APSPE---PKRHLRRTS : 310
GmFDc1 : SRARKQAYRKGLVEVRLTEENSRLRKQ---LKEQLCCLSDDNPPTP---RMAALCRTSS : 181
GmFDc2 : SRARKQAYRKGLVEVRLTEENSRLRKQ---LKEQLRCLCSS---HTP---RMAAPCRTSS : 225
PvFDc : SRARKQAYRRGLEAEVRLTEENSRLRKQ---LKEQLCSLSSSEN---TPD---RISAPCRTSS : 169
GmFDdb1 : SRARKQAYLFELKQKLQLEENARLRQ---QL-LRETAAN---QG---KKGNYLRTYT : 221
GmFDdb2 : SRARKQAYLFELH---LEENARLRQ---QL-VLCETAAN---QG---KRGNYLRTYT : 210
PvFDdb : SRARKQAYLLEIKQKLEQLQLEENARLRQ---QL-LRETATN---QW---KKGNYLRTYT : 152
MtFDdb : SRARKQAYIFELKKVKSLLEENARLRQ---QH-VLCDTASNHKQK---KKGNYLRTST : 173
PsFDa : SRARKQAYTTLEKQVDFLLLEENAKLRQ---QQL-LWEAA-SSAP---KKNSLHRTLT : 235
MtFDa : SRARKQAYTNLEKQVQLQLEENARLRQ---QQL-LWEAE-SGGQK---KKSILYR---T : 232
PvFDa : SRARKQAYMNGLESEVHLKEENARLRQ---QQL-LNEVA-GSEQ-K---KKGSLYR---T : 181
LjFDa : SRARKQAYTNELKHKVDHLLLEENARLRQ---QQL-LWEAA-AGQK-K---KKNLYRSAT : 215
GmFDa1 : SRARKQAYTNLELEVEHLKEENARLRQ---QQL-LYEA-VSEQK---KG-TLCRAST : 181
GmFDa2 : SRARKQAYTNLELEVEHLKEENARLRQ---QQQ-LCEAA-SSEQK---KGTTLYRST : 131
SlSPGB : SRARKQAYMNGLESEVHLVEENARLRQ---QQQ-LRVA---ANQVP---KKNLYRST : 214
StFD1 : SRARKQAYLNELETEVAHLVEENARLRQ---QQQ-LRLAA---AIQVP---KKNSLHRTST : 204
SlFD2 : SRARKQAYTNLEMEVANLMEENARLRQ---QQQ-LCLASSAGAQLP---KKSINRTST : 222
StFD2 : SRARKQAYTNLEMEVANLMEENARLRQ---QQQ-LCLAS-AGALLP---KKSINRTST : 224

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AquaFD : SRARKQAYTTLELENKVDHLSKENERILRQ--QQE-LIKAV-AAAQQA---KKRTLQRTST : 264
AtFD : SRARKQAYTNELELEVAHLQAENARLKRQ--QDQ-LKMAA--AIQPP---KKNTLQRSST : 282
AtFDP : SRARKQAYTNELELEVAHLQATENARLKIQ--QEQ-LKIAE--ATQNQ---VKRTLQRSST : 231
AcFD : SRARKQAYTNELELEVAHLMEENARLRSQ--QQQ-LYLAA--ASQVP---RKKTLLHRTST : 254
MeFD1 : SRARKQAYTNELELEVAHLMEENARLKRQ--QEE-LLAA--STQHP---KKHTLHRTST : 221
MeFD4 : SRARKQAYTTLELEVRHLMENARLKRQ--QGE-LYLAA--AAQLP---KKHTLHRTST : 251
PtFD1 : SRARKQAYTNELENEVEQLKENARLKRQ--QEE-LYLAA--AAQLP---KKHTLQRTST : 277
GrFD1 : SRARKQAYTNELELEVAHLMEENAKLRQ--QEQ-LRVAA--TAQLS---RNRMLQRTST : 224
GrFD2 : SRARKQAYTNELELEVAHLMEENARLKRQ--QEQ-LRVAA--ATPLS---GKRTLQRTST : 263
VvFD2 : SRARKQAYTNELELEVAHLMEENARLKRQ--QEQ-LTSAT--AAQLP---KKNTLHRTST : 266
PtFD3 : SRARKQAYTTLEELKVALLGEENAKLRQ--QER-FLAAA--PAQPP---KKHTLYRTST : 164
PtFD2 : SRARKQAYTVLELREAAHLAQENAKLRQ--QER-FLAAA--PAQLP---KKNTLYRTST : 221
MeFD2 : SRARKQAYTQELELEVANLABENARLRRQ--QEKLFLLA--PAQLP---KKHSLYRTST : 248
MeFD3 : SRARKQAYTQELEQEVANLQKENARLRRQ--QE--KFQAA--PAQLL---KKPSLYRTST : 191
GrFD3 : SRARKQAYTTLEELKVALLQENAKLRQ--QEK--ILAA--RDQVP---KKHTLYRTST : 212
GrFD4 : SRARKQAYKKKLREVAYLKEENAKLRKH--QKK--VMAA--INQLP---KKNRLSRSLT : 206
GrFD5 : SRARKQAYTNELELEVARLLEENVKLRRQ--QDK--LLAS--PRQIP---KKNTLCRTLT : 177
VvFD1 : SRARKQAYTNELELEVAHLIEENARLKR--LQK-FCPEA--SAQLP---KKHTLYRTST : 190
MdFD1 : SRARKQAYTNELELEVAHLMEENARLKRQ--LEQ-L-IAA--ASQPP---KKHTLYRTST : 276
PpFD1 : SRARKQAYTNELELEVAHLMEENTRLKRQ--QEQ-LCFAA--ASQPP---KKHNLHRSST : 295
FvFD1 : SRARKQAYTNDLEIRQLLMEENARLRRQ--NEQ-LSDAA--ASQLP---KKHSLNRSST : 224
EgFD : SRARKQAYTNELELEVAHLLEENARLKRQ--QEQ-LWMAA--ASQIP---KKHSLHRTST : 214
MaFD1 : SRARKQAYMNELELEVAHLLEENARLKR--LEE-LRSAMAAKH--P---KKALQRSST : 205
MaFD5 : SRARKQAYINELELEVARLLEEQRSLQK--LEE-LRLAMAGKH--S---KRNTLRSST : 252
MaFD2 : SRARKQAYTIQLELEVSHLKEENAKLRQ--NEE-LRLAMATQL--P---KRNTLQRSST : 202
MaFD3 : SRARKQAYTTLEQEVHDLVNEENRLKRQ--FEE-LKKAQDHLFVP---AKHTLQRTLT : 207
PdFD1 : SRARKQAYTQELKQAAHLLDENRRLK-Q-LSLA-METPP-----P---TKRTLQRTST : 180
MaFD4 : SRARKQAYRNELELEAARLLNENMLKR--ESEQ-LRMTVAAQNPTA---SKPTLQRTLT : 191
CsFD : SRARKQAYANLELEVSNLKEENAKLRQ--QEE-LQAVA--MAQVP---RKHRLQRTST : 211
MdFD2 : SRARKQAYTNELEIEIQLREENARLKRQLQSLAPSTLAQRLLP---KQTLFRTST : 218
PpFD2 : SRARKQAYTTLELEVAHLQKENARLK----TQAEPLPTTKRHTL-----TLFRSST : 232
FvFD2 : SRARKQAYTSELEKLLELKEENARLKRQ--QQKFCLATPVAPAT-----TLKRAHS : 215
ZmDLE1 : SRARKQAYVRELETKVQLLQENESLEVKYDELRESV---EVAVPM---VRKTLQRMPS : 201
SbFD1 : SRARKQAYVRELERKVMQLQDENESLEVKYDQLRVSV---EVAVPI---VRKTLQRMPS : 212
SiFD1 : SRARKQAYVRELEREVQLLQENESLEVKYELRVSV---EVPVP---VRKTLQRMPS : 213
TaFD1 : SRARKQAHVTQIESEVHQLRENEQLRLKYDQLKASV---EVSVPV---RKTQLQVLS : 201
HvFD1 : SRARKQAHVTQIESEVHQLRENEQLRLKYDQLKASV---EVSVPV---VRKTLQVLS : 183
BdFD1 : SRARKQAHVTQIESEVRELQLENDELRIKYDQLKASV---EAPVPV---KRTLKRVLS : 183
OsFD1 : SRARKQARVNNLETEVEQLKQENKMLVVKYELRKTV---EVPVPV---RRTLQVLS : 192
OsFD4 : SRARKQAYLQELEQEVRLLRANALRHQCHQLKAAAAEAEEAAAA---AAAACKPTSS : 143
SiFD4 : SRARKQAYVNELEKEVSALRAENEELRLKCEELKEA---EAPAKKA---NQLRQ-TSS : 166
OsFD2 : SRARKRAYVELEKEVRRLVDDNLNKKCKELKQEVAAALVMP-----TKSSLRRTSS : 164
OsFD5 : SRARKRAYVELEKEVRRLVDDNLNKKCKELKQEVAAALVMP-----TKSSLRRTSS : 164
OsFD6 : SRARKRAYVELEKEVRRLVDDNLNKKCKELKQEGGC-----TGDAYKELTA : 159
ZmFD2 : SRARKRAYVENLEKEVRRLVDDNLNKKCKELKREVAALVLP-----TKSSLRRTSS : 158
SbFD2 : SRARKRAYVENLEKEVRRLVDENLKLKKCKELKLEVAALVLP-----TKSSLRRTSS : 157
SiFD2 : SRARKRAYVENLEKEVRRLVDENLKLKKCKELKLEVAALVLP-----TKSSLRRTSS : 154
BdFD2 : SRARKRAYVELEKEVRRLVDDNLNKKCKELKQEVAAALVLP-----SKSSLRRTSS : 150
HvFD2 : SRARKRAYVELEKEVRRLVDDNLNKKCKELKREVAALVLP-----TKSSLRRTSS : 157
ZmFD3 : SRARKRAYVQLEKEVRRLVNENLKLKRCQQLKLDMAALVQSS--SSKSSSHIRRTSS : 168
SbFD3 : SRARKRAYVQLEKEVRRLVNENLKLKRCQQLKVDMAALIQSS--SSKSSSHIRRTSS : 175
SiFD3 : SRARKRAYVQLEKEVRRLVNENLKLKRCQQLKVDMAALIQTS--SSKGNSHIRRTSS : 167
OsFD3 : SRARKRAYVQLEKEVRRLVNENLKLKRHCQQLKTEMAALIQPP--TNKQSSHRR--SSS : 141
TaFDL15 : SRARKRAYTQLEKEVRRLVEDNLKLKRCQQLQSEIAALTAQQ--ASNKQSSPHRTSS : 131
HvFD3 : SRARKRAYTQLEKEVRRLVEDNLKLKRCQQLQSEIAALNAQQ--PSNKQSSPHRTSS : 128
TaFDL13 : SRARKRAYTQLEKEVRRLVEDNLKLKRCQQLARLLFLQCH---LSFLLACLGMGN-- : 125
BdFD3 : SRARKRAYVQLEKEVSRLVDHNLKLKRCQQLKTEMAALVQAQQPSKSPQYRRTSS : 143

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AtDPBF4 : ASL-- : 262
AtAREB3 : APF-- : 297
OsAREB3 : APF-- : 335
TaFDL2 : APV-- : 133
TaFDL3 : TSF-- : 124
TaFDL6 : TSF-- : 313
GmFDc1 : SPF-- : 184
GmFDc2 : SPF-- : 228
PvFDc : SPF-- : 172
GmFDdb1 : APF-- : 224
GmFDdb2 : APF-- : 213
PvFDdb : APF-- : 155
MtFDdb : APF-- : 176
PsFDa : APF-- : 238
MtFDa : SSF-- : 235
PvFDa : APF-- : 184

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LjFDa	: APF--	: 218
GmFDa1	: APF--	: 184
GmFDa2	: APF--	: 134
SlSPGB	: APF--	: 217
StFD1	: APF--	: 207
SlFD2	: APF--	: 225
StFD2	: APF--	: 227
AqucaFD	: APF--	: 267
AtFD	: APF--	: 285
AtFDP	: APF--	: 234
AcFD	: APF--	: 257
MeFD1	: APF--	: 224
MeFD4	: APF--	: 254
PtFD1	: APF--	: 280
GrFD1	: APF--	: 227
GrFD2	: APF--	: 266
VvFD2	: APF--	: 269
PtFD3	: APF--	: 167
PtFD2	: APF--	: 224
MeFD2	: APF--	: 251
MeFD3	: APF--	: 194
GrFD3	: APF--	: 215
GrFD4	: TPF--	: 209
GrFD5	: APF--	: 180
VvFD1	: APF--	: 193
MdFD1	: APF--	: 279
PpFD1	: APF--	: 298
FvFD1	: APF--	: 227
EgFD	: APF--	: 217
MaFD1	: APF--	: 208
MaFD5	: APF--	: 255
MaFD2	: PPF--	: 205
MaFD3	: APF--	: 210
PdFD1	: APF--	: 183
MaFD4	: APF--	: 194
CsFD	: APF--	: 214
MdFD2	: APF--	: 221
PpFD2	: APF--	: 235
FvFD2	: M----	: 216
ZmDLF1	: APF--	: 204
SbFD1	: APF--	: 215
SiFD1	: APF--	: 216
TaFD1	: APF--	: 204
HvFD1	: APF--	: 186
BdFD1	: APF--	: 186
OsFD1	: APF--	: 195
OsFD4	: ATF--	: 146
SiFD4	: ATF--	: 169
OsFD2	: TQE--	: 167
OsFD5	: TQE--	: 167
OsFD6	: TNEIN	: 164
ZmFD2	: TQE--	: 161
SbFD2	: TQE--	: 160
SiFD2	: TQE--	: 157
BdFD2	: TQE--	: 153
HvFD2	: TQ---	: 159
ZmFD3	: TQL--	: 171
SbFD3	: TQL--	: 178
SiFD3	: TQL--	: 170
OsFD3	: T----	: 142
TaFDL15	: TQE--	: 134
HvFD3	: TQE--	: 131
TaFDL13	: -----	: -
BdFD3	: THL--	: 146

The following alignment shows predicted protein sequences from online resources and corrected versions (indicated with single asterisks) of sequences that are clearly incorrect (sequence names and incorrect portions highlighted in red) for *LARPIC* homologs. Medtr5g022790, the gene immediately upstream of MtFDa (Medtr5g022780), includes a possible domain for ATP synthase CF0 subunit IV which was not present in expressed sequences of this *Medicago* gene (TC187722 at DFCI TGI) and was not present in syntenic regions of other sequenced legume species. Correcting the annotation of this gene to match an expressed sequence and corresponding genes from other legumes, resulted in the exclusion of this putative domain. The end of Glyma11g08500 was also corrected. Although the annotation for the end of Medtr8g075120 does not match other legumes, it matches an expressed sequence for this gene (TC185276 at DFCI TGI).

Details for sequences are given in parenthesis with full locus names from available genome resources for *Medicago* (Mt3.5), soybean (v1.0) and common bean (v1.0): Medtr5g (Medtr5g022790), Medtr8g (Medtr8g075120), Glyma01g (Glyma01g36801), Glyma02g (Glyma02g05090), Glyma11g (Glyma11g08500), Glyma16g (Glyma16g23170), Phvul.002G (Phvul.002G105500), Phvul.003G (Phvul.003G266200).

341

		*	500	*	520	*	540	
Medtr5g	:	DWEK	WIMSPVQFPN	VTSPEVLNQDMLAEK	MNRNIALETTI	YD	GAGGPVLPD	NSEHTPAE : 491
Medtr5g*	:	DWEK	WIMSPVQFPN	VTSPEVLNQDMLAEK	MNRNIALETTI	YD	GAGGPVLPD	NSEHTPAE : 491
Glyma01g	:	DWR	WIMHPPVQFS	NATTICVLNPD	KLAEQV	QVHNIALETS	NYD	GAGGLDVQPDTSQHRSTF : 477
Glyma11g	:	DWR	WIMHPPVQFS	NATTICVLNPD	MALAEQV	HNIALETS	YD	GAGGPVLPDTSRHRSTF : 474
Glyma11g*	:	DWR	WIMHPPVQFS	NATTICVLNPD	MALAEQV	HNIALETS	YD	GAGGPVLPDTSRHRSTF : 474
Phvul.002G	:	DWR	RWLM-PPVQFS	NATSPGESN	PDILAEQV	HNIALETS	NYD	GGVEVLHDTSQYSSTF : 479
Medtr8g075	:	---	WITLS-SQF	HNVN--GSQ	IAAQLAKN	IONIDLE	NKNGAG	---ELHVSNI--- : 455
Glyma02g	:	DWR	RWIMPG-GV	PNVR--GSQ	TVGQLAE	RQVNTITLER	NNNDAG	---VLDVSQNR-PE : 466
Glyma16g	:	DWR	RWILPA-GL	PNVR--GSQ	TVGQLAEQV	HNIALETTI	NDNAG	---ILDDVSQNR-PE : 452
Phvul.003G	:	DWR	WIMPP-AQL	PNR--GSQ	TIGOLAEQV	HNIALETTI	NDNAG	---VLDVSQNR-PE : 452

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      *           560           *           580           *           600
Medtr5g : GDISSPLQLSTSEITG--EVDIHDPKKLCMLEINFLSRPEEVAWIIDILSLSLSLSLTK : 549
Medtr5g* : GDISSPLQLSTSEITG--EVDIHGSDFS-----TSRR : 521
Glyma01g : GDI---QQLSTS---QVGIQGSDFH-----IPAR : 500
Glyma11g : RDI---QLSTSEGPGPGQVGIQEAQITP-----FQEEIRAV : 507
Glyma11g* : RDI---QLSTSEGPGPGQVGIQGSDDH-----IPGR : 502
Phvul.002G : GDISSPLQLSTSEGAG--QVGIQGSNQS-----IPAR : 509
Medtr8g075 : ----- : -
Glyma02g : GDINSQYLHSTSEGTA--QVGIQVSDHS-----ISAR : 496
Glyma16g : GDSNSQYLISTSEGTA--QVGIQVSDHS-----VSAR : 482
Phvul.003G : GDINGQYLLSTGESTG--QVGIQVSDHS-----ISAR : 486

      *           620           *           640           *           660
Medtr5g : QVLEERSQSAAGGNVVLARRHLMAVIQLFHGGGGLALHGLQGRFRLSRVEEYFTKPLSI : 609
Medtr5g* : N----- : 522
Glyma01g : N----- : 501
Glyma11g : NVT----- : 510
Glyma11g* : N----- : 503
Phvul.002G : N----- : 510
Medtr8g075 : ----- : -
Glyma02g : N----- : 497
Glyma16g : N----- : 483
Phvul.003G : N----- : 487

      *           680           *           700           *           720
Medtr5g : SFRLFGNILADELVVVVLVSLVPLVVPVPMFLGLFTSGIQALIFATLAAAYIGESMEGH : 669
Medtr5g* : ----- : -
Glyma01g : ----- : -
Glyma11g : ----- : -
Glyma11g* : ----- : -
Phvul.002G : ----- : -
Medtr8g075 : ----- : -
Glyma02g : ----- : -
Glyma16g : ----- : -
Phvul.003G : ----- : -

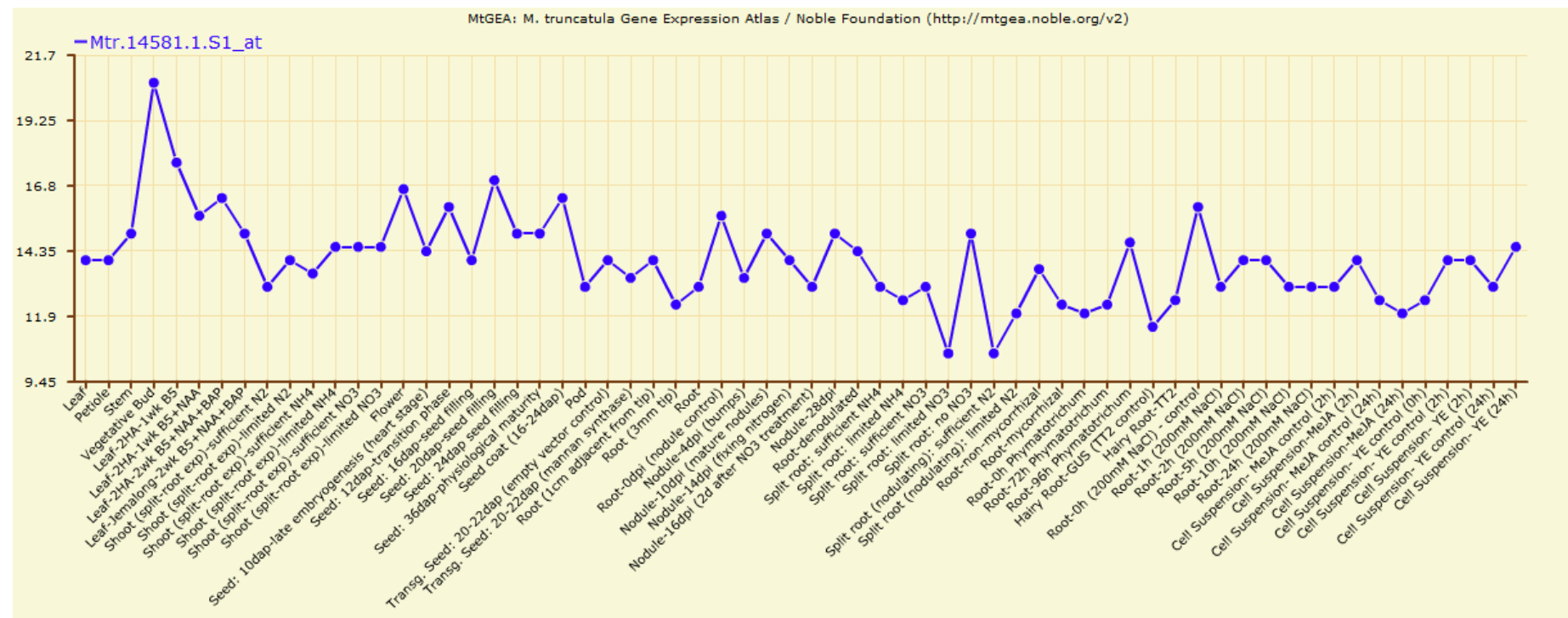
Medtr5g : H : 670
Medtr5g* : - : -
Glyma01g : - : -
Glyma11g : - : -
Glyma11g* : - : -
Phvul.002G : - : -
Medtr8g075 : - : -
Glyma02g : - : -
Glyma16g : - : -
Phvul.003G : - : -

```

### Appendix 3: Expression profiles for legume *FD* genes from gene expression atlases

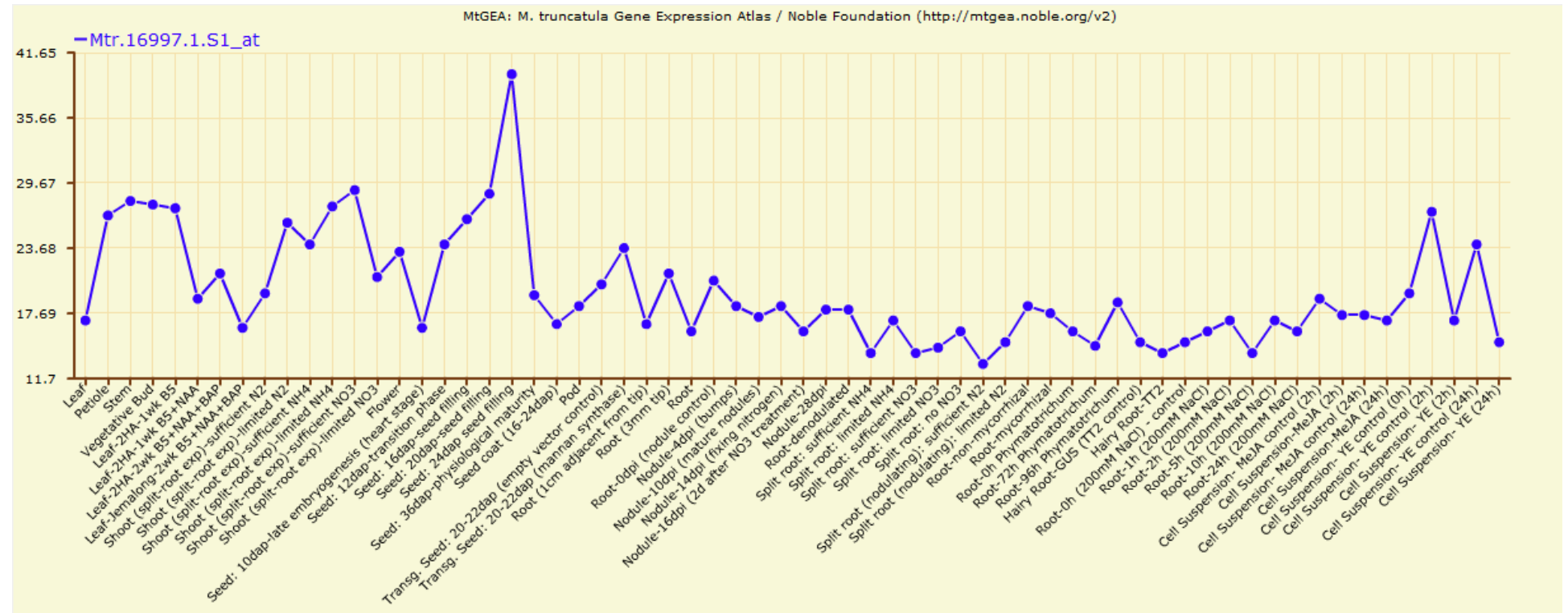
The gene expression profiles found during searches for expression of legume *FD* genes in available gene expression atlases for *Medicago* (<http://mtgea.noble.org/v2/>), soybean (<http://soybase.org/soyseq/>) and *Lotus* (<http://ljgea.noble.org/v2/>), discussed in Chapter 4, are shown below.

***MtFDa* (Mtr.14581.1.S1 at)**





***MtFDb* (Mtr.16997.1.S1\_at)**



***GmFDa1 (Glyma01g36810)***

	young leaf	flower	one cm pod	pod shell 10DAF	pod shell 14DAF	seed 10DAF	seed 14DAF	seed 21DAF	seed 25DAF	seed 28DAF	seed 35DAF	seed 42DAF	root	nodule
Raw	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Normalised*	0	0	0	0	0	0	0	1	0	0	0	0	0	0

***GmFDb1 (Glyma02g05100)***

	young leaf	flower	one cm pod	pod shell 10DAF	pod shell 14DAF	seed 10DAF	seed 14DAF	seed 21DAF	seed 25DAF	seed 28DAF	seed 35DAF	seed 42DAF	root	nodule
Raw	0	3	2	0	0	1	2	3	0	1	0	0	0	33
Normalised*	0	1	1	0	0	0	1	2	0	0	0	0	0	7

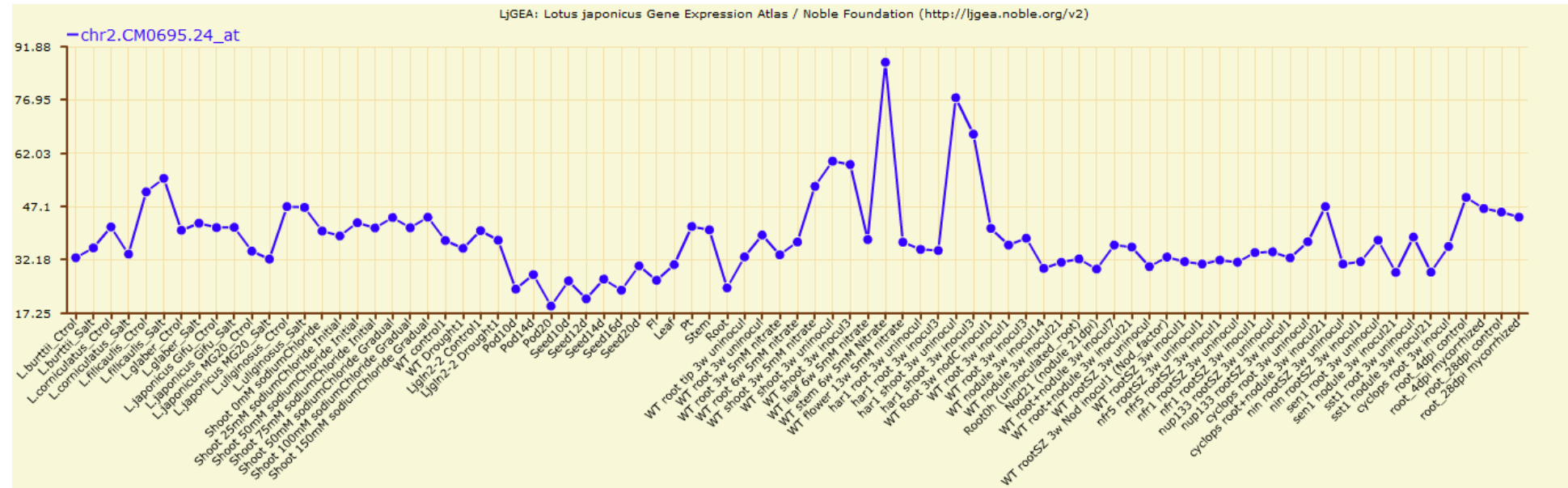
***GmFDc1 (Glyma04g02420)***

	young leaf	flower	one cm pod	pod shell 10DAF	pod shell 14DAF	seed 10DAF	seed 14DAF	seed 21DAF	seed 25DAF	seed 28DAF	seed 35DAF	seed 42DAF	root	nodule
Raw	15	0	0	3	0	3	11	6	17	7	14	2	17	12
Normalised*	3	0	0	1	0	1	3	3	3	1	3	0	3	2

***GmFDc2 (Glyma06g02470)***

	young leaf	flower	one cm pod	pod shell 10DAF	pod shell 14DAF	seed 10DAF	seed 14DAF	seed 21DAF	seed 25DAF	seed 28DAF	seed 35DAF	seed 42DAF	root	nodule
Raw	9	5	0	1	2	4	0	0	0	0	0	2	17	2
Normalised*	2	1	0	0	0	1	0	0	0	0	0	0	3	0

\* Normalised data: Reads/kilobase/million normalization of the raw data (Severin et al., 2010).

***LjFDa* (chr2.CM0695.24\_at)**

## Appendix 4: Sequences and sequence alignments for Chapter 5

**Table A5.1.** Details of sequences used for Chapter 5. Asterisks indicate an altered annotation used (putative intron sequence removed or corrected annotation of exons based on transcript sequence or alignments between species). Source details including websites and relevant references are outlined in Chapter 2 (Table 2.4).

Species	Gene name	Sequence	Source	Reference (s)	
<i>Arabidopsis thaliana</i>	AtFT	NP_176726	GenBank	Kardailsky et al. (1999)	
	AtGF14ε	AAD51785		Rosenquist et al. (2001)	
	AtGF14i	AAK11271			
	AtGF14κ	AAD51783			
	AtGF14λ	AAD51781			
	AtGF14μ	AAD51784			
	AtGF14v	AAD51782			
	AtGF14o	AAG47840			
	AtGF14π	NP_565174			
	AtGF14u	AAB62225			
	AtGF14φ	AAB62224			
	AtGF14χ	AAA96254			
	AtGF14ψ	AAA96252			
	AtGF14ω	AAA96253			
AtTFL1	NP_196004	Bradley et al. (1997)			
AtTSF	NP_193770	Yamaguchi et al. (2005)			
Human	14-3-3θ	NP_006817	GenBank	Fujita et al. (2002)	
<i>Lotus japonicus</i>	LjGF14-1/2	Ljchr1.CM0016.120.r2.m	Lotus genome build 2.5	This study	
	LjGF14-4	Ljchr3.CM0111.260.r2.m			
	LjGF14-5	LjSGA_007953			
	LjGF14-6a	Ljchr2.CM0120.420.r2.m			
	LjGF14-6b	Ljchr4.CM0229.540.r2.m			
	LjGF14-7	Ljchr1.CM0982.540.r2.d			
<i>Medicago</i>	LjGF14-8	Ljchr1.CM0017.330.r2.m	Medicago genome v3.5	This study	
	MtGF14-1	Medtr3g099380			
	MtGF14-2	Medtr5g064580			
	MtGF14-3	Medtr8g086270			
	MtGF14-4	Medtr2g076960	DFCI TGI		
	MtGF14-5	TC193944			
	MtGF14-6a	Medtr5g044160			Medicago genome v3.5
	MtGF14-6b	Medtr4g083060			
Pea	MtGF14-7a	Medtr3g100620	Medicago genome v3.5	This study	
	MtGF14-7b	Medtr3g014060			
	PsDET	AAR03725	GenBank	Foucher et al. (2003)	
	PsFTa1	ADZ05699		Hecht et al. (2011)	
	PsFTa2	ADZ05700			
	PsFTb1	ADZ05701			
	PsFTb2	ADZ05702			
	PsFTc	ADZ05703			
	PsGF14-1	JI896927; CAB42546; P46266		This study; Stanković et al. (1995); May and Soll (2000)	
	PsGF14-2	JI911950; CAB42547		This study; May and Soll (2000)	
	PsGF14-3	JI898567		This study	
	PsGF14-4	JI897751			
	PsGF14-5	JI912187			
	PsGF14-6	JI898267			
PsGF14-7	JI912214				
PsLF	AY343326	Foucher et al. (2003)			
Rice	OsHd3a	NM_001063395	GenBank	Kojima et al. (2002)	

Species	Gene name	Sequence	Source	Reference (s)
Soybean	GmSGF14a	NP_001235653	GenBank	Li and Dhaubhadel (2011)
	GmSGF14b	AK285530		
	GmSGF14c	ADU60530		
	GmSGF14d	AK285774		
	GmSGF14e	ADU60529		
	GmSGF14f	ADU60527		
	GmSGF14g	ACU21064		
	GmSGF14h	ADU60526		
	GmSGF14i	AK286671		
	GmSGF14j	AK285891		
	GmSGF14k	AK286798		
	GmSGF14l	AK286414		
	GmSGF14m	AK286318		
	GmSGF14n	ADU60525		
	GmSGF14o	AK286943		
	GmSGF14p	AAF64040		

### Sequence alignment for Figure 5.3

Alignment for 14-3-3 genes identified in this study in *Lotus* (Lj), *Medicago* (Mt) and pea (Ps) in addition to those known to be expressed in soybean (Gm; Li and Dhaubhadel, 2011) and *Arabidopsis* (At; Lu et al., 1992; Lu et al., 1994; Wu et al., 1997; Rosenquist et al., 2000). Alignment includes full-length predicted sequences for all proteins except for LjGF14-7, for which only partial sequence was available. Shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Accession details for sequences are given above in Table A5.1.

		*	20	*	40	*	60	
PsGF14-6	:	---	MS---	AEKERETQVYMAKLSEQAERYEEMVECMKKVAKLDV---	ELTVEERNLLSVG	:	51	
MtGF14-6a	:	---	MS---	AEKERETQVYMAKLSEQAERYEEMVECMKKVAKLDV---	ELTVEERNLLSVG	:	51	
GmSGF14e	:	---	MS---	AEKERETQVYMAKLSEQAERYEEMVECMKKVAKLDL---	DLTVEERNLLSVG	:	51	
GmSGF14f	:	---	MS---	VEKERETQVYMAKLSEQAERYEEMVECMKKVAKLDL---	DLTVEERNLLSVG	:	51	
LjGF14-6a	:	---	MA---	AEKERETQVYMAKLSEQAERYEEMVECMKNVAKLDL---	ELTVEERNLLSVG	:	51	
LjGF14-6b	:	---	MA---	AEKERETQVYMAKLSEQAERYEEMVECMKAVAKLDL---	ELTVEERNLLSVG	:	51	
MtGF14-6b	:	---	MS---	TEKERETQVYMAKLSEQAERYEEMVECMKTIKLDV---	ELTVEERNLLSVG	:	51	
AtGF14i	:	---	MSSSGSDKERETTFVYMAKLSEQAERYEEMVETMKKVAVRNS---	ELTVEERNLLSVG	:	54		
AtGF14o	:	---	MENERAKQVYMAKLSEQAERYEEMVEAMKKVAKLDV---	ELTVEERNLLSVG	:	49		
PsGF14-3	:	---	MAY-TKERENFVYIAKLSEQAERYEEMVEAMKNVAKLDV---	ELTVEERNLLSVG	:	51		
MtGF14-3	:	---	MAS-TKERENFVYIAKLSEQAERYEEMVEAMKNVAKLDV---	ELTVEERNLLSVG	:	51		
GmSGF14c	:	---	MAS-TKERENFVYIAKLSEQAERYEEMVEAMKNVAKLNV---	ELTVEERNLLSVG	:	51		
GmSGF14l	:	---	MAS-TKERENFVYIAKLSEQAERYEEMVEAMKNVAKLNV---	ELTVEERNLLSVG	:	51		
PsGF14-5	:	---	MAS-TKDERNFVYIAKLSEQAERYEEMVDSMKNVANLDV---	ELTVEERNLLSVG	:	51		
MtGF14-5	:	---	MAS-SKDERNFVYIAKLSEQAERYEEMVDSMKNVANLDV---	ELTVEERNLLSVG	:	51		
LjGF14-5	:	---	MAS-SKDERTFVYIAKLSEQAERYEEMVDSMKKVANLDV---	ELTVEERNLLSVG	:	51		
GmSGF14d	:	---	MTA-SKDERNFVYIAKLSEQAERYEEMVESMKNVANLDV---	ELTVEERNLLSVG	:	51		
GmSGF14o	:	---	MAA-SKDERNFVYIAKLSEQAERYEEMVESMKNVANLDV---	ELTVEERNLLSVG	:	51		
AtGF14μ	:	---	MGS-GKERDTFVYMAKLSEQAERYEEMVESMKSVAKLNV---	DLTVEERNLLSVG	:	51		
PsGF14-4	:	---	MASSTNIRENFVYIAKLSEQAERYEEMVDAMKKLAKMDV---	ELTVEERNLLSVG	:	52		
MtGF14-4	:	---	MASSTNVRENFVYIAKLSEQAERYEEMVDAMKKLAKMDV---	ELTVEERNLLSVG	:	52		
GmSGF14n	:	MTQ	PAMATFSKERENFVYIAKLSEQAERYEEMVDAMKKVAKLDV---	ELTVEERNLLSVG	:	57		
GmSGF14p	:	---	MATFSKERENFVYIAKLSEQAERYEEMVDAMKKVAKLDV---	ELTVEERNLLSVG	:	52		
LjGF14-4	:	---	MAFSSNDRNFVYIAKLSEQAERYEEMVDAMKKVAKMDV---	ELTVEERNLLSVG	:	52		
AtGF14ε	:	---	MENEREKQVYMAKLSEQAERYEEMVEAMKKVAKLDV---	ELTVEERNLLSVG	:	49		
AtGF14n	:	---	MENEREKLIYIAKLGGQAGRYDDVVKSMRKVCELDI---	ELTVEERNLLSVG	:	49		
PsGF14-2	:	---	MATAPTPREEFVYMAKLSEQAERYEEMVEFMEKVTAAVE-SEELTVEERNLLSV	A	:	54		
MtGF14-2	:	---	MATAPTPREEFVYMAKLSEQAERYEEMVEFMEKVTAAVE-SEELTVEERNLLSV	A	:	54		
GmSGF14g	:	---	MAASAPTPREEFVYMAKLSEQAERYEEMVEFMEKVSASAE-SEELTVEERNLLSV	A	:	55		
GmSGF14k	:	---	MASAPTPREEFVYMAKLSEQAERYEEMVEFMEKVSASAE-SEELTVEERNLLSV	A	:	54		
PsGF14-1	:	---	MAAHTPREENVYMAKLSEQAERYEEMVEFMEKVSANAD-SEELTVEERNLLSV	A	:	54		
MtGF14-1	:	---	MAAHTPREENVYMAKLSEQAERYEEMVEFMEKVSANAD-NEELTVEERNLLSV	A	:	54		
GmSGF14h	:	---	MAAAPSPREENVYMAKLSEQAERYEEMVEFMEKVSAAAD-NEELTVEERNLLSV	A	:	54		
GmSGF14i	:	---	MAAAPSPREENVYMAKLSEQAERYEEMVEFMEKVSAAAD-NEELTVEERNLLSV	A	:	54		
LjGF14-1/2	:	---	MAAAPSPREEFVYIAKLSEQAERYEEMVEFMEKLSAAVD-GEELTVEERNLLSV	A	:	54		
AtGF14φ	:	--MA	APPASSAREEFVYIAKLSEQAERYEEMVEFMEKVAEAVD-KDELTVEERNLLSV	A	:	57		
AtGF14ω	:	---	MASGREELVYMAKLSEQAERYEEMVEFMEKVSAAVD-GDELTVEERNLLSV	A	:	51		
AtGF14χ	:	---	MATPGASSARDEFVYMAKLSEQAERYEEMVEFMEKVAKAVD-KDELTVEERNLLSV	A	:	56		
GmSGF14a	:	---	MSDSS--REENVYMAKLSEQAERYEEMVEFMEKVAKTVE-VEELTVEERNLLSV	A	:	52		
GmSGF14m	:	---	MADSS--REENVYMAKLSEQAERYEEMVEFMEKVAKTVE-VEELTVEERNLLSV	A	:	52		
LjGF14-8	:	---	MAAADSSSPREENVYIAKLSEQAERYEEMVEFMEKVAKSVD-AEELTVEERNLLSV	A	:	56		
AtGF14v	:	---	MSS--SREENVYIAKLSEQAERYEEMVEFMEKVAKTVD-TDELTVEERNLLSV	A	:	51		
AtGF14u	:	---	MSSDS--SREENVYIAKLSEQAERYEEMVEFMEKVAKTVE-TEELTVEERNLLSV	A	:	53		
AtGF14ψ	:	---	MST--REENVYMAKLSEQAERYEEMVEFMEKVAKTVD-VEELTVEERNLLSV	A	:	50		
PsGF14-7	:	--MD	VTPENLTREQYVYIAKLSEQAERYEEMVSFMQKLVVVSTPSSSELSVEERNLLSV	A	:	58		
MtGF14-7a	:	--MG	GAIPENLNREQYVYIAKLSEQAERYEEMVSFMQKLVVGSTPSSSELSVEERNLLSV	A	:	58		
LjGF14-7	:	MA	VAGGV PENLSREQYVYIAKLSEQAERYEEMVSFMQKLVVGSTPAAELSV	A	:	60		
GmSGF14b	:	---	MAA--AEGLNREQYVYIAKLSEQAERYEEMVSFMQKLVVGSTPASBLTVEERNLLSV	A	:	56		
GmSGF14j	:	--MA	G--AEGLNREQYVYIAKLSEQAERYEEMVSFMQKLVVGWTPASBLTVEERNLLSV	A	:	56		
MtGF14-7b	:	--MG	FTVAERLRREYVYIAKLSEQAERYEEMVSFMQKLVVGYTPASBLSEEMNLLSV	A	:	58		
AtGF14λ	:	---	MAATLG RDQYVYMAKLSEQAERYEEMVQFMEQLVTGATPAEELTVEERNLLSV	A	:	54		
AtGF14κ	:	---	MATTLSDQYVYMAKLSEQAERYEEMVQFMEQLVSGATPAGELTVEERNLLSV	A	:	54		
Human	:	---	MEKTEL IQAKLAQAERYDDMATCMKAVTEQGA--ELTVEERNLLSV	A	:	47		

		*	80	*	100	*	120	
PsGF14-6	:	YKNVIGARRASWRIMSSIEQKEESKGN	-EHNVKLIKNYCQKVEEELS	KICGDIIITIIDQH	:	110		
MtGF14-6a	:	YKNVIGARRASWRIMSSIEQKEESKGN	-EHNVKMIKSYCQKVEEELS	KICGDIIITIIDQH	:	110		
GmSGF14e	:	YKNVIGARRASWRIMSSIEQKEESKGN	-EHNVKLIKSYCQKVEEELS	KICGDIIITIIDQH	:	110		
GmSGF14f	:	YKNVIGARRASWRIMSSIEQKEESKGN	-EHNVKLIKSYCQKVEEELS	KICGDIIITIIDQH	:	110		
LjGF14-6a	:	YKNVIGARRASWRIMSSIEQKEETKGN	-EHNVKQIKNYRQKVEEELS	KICGDIIITIIDQH	:	110		
LjGF14-6b	:	YKNVIGARRASWRIMSSIEQKEESKGN	-ESNVKLIKGYCHKVEEELS	KICIDIIITIIDQH	:	110		
MtGF14-6b	:	YKNVIGARRASWRIMSSIEQKEEAKGN	-ENNVKLIKSYCQKVEEELS	KICSDIIEIIDKH	:	110		
AtGF14i	:	YKNVIGARRASWRIMSSIEQKEESKGN	-ESNVKQIKGYRQKVEEELS	ANICQDIIITIIDQH	:	113		
AtGF14o	:	YKNVIGARRASWRILSSIEQKEESKGN	-EQNAKRIKDYRTKVEEELS	KICYDILAVIDKH	:	108		
PsGF14-3	:	YKNVVGARRASWRILSSIEHREDTKGN	-DVSVKRIREYRNKVEEELS	NICSDIITIIDDH	:	110		
MtGF14-3	:	YKNVVGARRASWRILSSIEHKEESKGY	-DVNVKRIKEYRHKVEEELS	NICSDIMSIIDDH	:	110		
GmSGF14c	:	YKNVVGARRASWRILSSIEQKEEAKGN	-DVSVKRIKEYRLKVEEELS	NICSDIMTVIDEY	:	110		
GmSGF14l	:	YKNVVGARRASWRILSSIEQKEEAKGN	-DVSVKRIKEYRQKVEEELS	NICSDIMTVIDEH	:	110		
PsGF14-5	:	YKNVIGARRASWRILSSIEQKEESKGN	-DVNAKRIKEYRHKVEEELS	NICSDIMTVIDEH	:	110		
MtGF14-5	:	YKNVIGARRASWRILSSIEQKEETKGN	-DVNAKRIKEYRNKVEEELS	NTICNDVMRVIDEH	:	110		
LjGF14-5	:	YKNVIGARRASWRILSSIEQKEETKGN	-DVNAKRIKEYRQKVEEELS	ADICNDVMRVIDEH	:	110		
GmSGF14d	:	YKNVIGARRASWRILSSIEQKEETKGN	-ELNAKRIKEYRQKVEEELS	NICNDVMRVIDEH	:	110		
AtGF14o	:	YKNVIGARRASWRILSSIEQKEETKGN	-ELNAKRIKEYRQKVEEELS	NICNDVMRVIDEH	:	110		
AtGF14μ	:	YKNVIGARRASWRIFSSIEQKEEAVKGN	-DVNVKRIKEYMEKVEEELS	NICIDIMSVLDEH	:	110		
PsGF14-4	:	YKNVVGARRASWRILSSIEQKEESKGN	-ELNVKRIKGYRQKVEEELS	ICNDIMTIIDEH	:	111		
MtGF14-4	:	YKNVVGARRASWRILSSIEQKEESKGN	-ELNVKRIKEYRQKVEEELS	ICNDIMTIIDEH	:	111		
GmSGF14n	:	YKNVVGARRASWRILSSIEQKEESKGN	-ELHVKRIIRDYRNKVEEELS	NICSDIMTVLDEH	:	116		
GmSGF14p	:	YKNVVGARRASWRILSSIEQKEESKGN	-ELHVKRIIRDYRNKVEEELS	NICSDIMTIIDEH	:	111		
LjGF14-4	:	YKNVVGARRASWRILSSIEQKEESKGN	-ELSVKRIIRDYRNKVEEELS	NICSDIMTIIDEH	:	111		
AtGF14ε	:	YKNVIGARRASWRILSSIEQKEESKGN	-DENVKRLKNYRKRVEEELS	AKVNDIISVIDKH	:	108		
AtGF14π	:	YKNVMEARRVSLRVISSIEKMEESKGN	-DQNVKLIKQOQEMVKYEFFNVCNDIISLIDSH	:	108			
PsGF14-2	:	YKNVIGARRASWRIISSIEQKEESRGN	-DEHVTVIRDYRSKIEEELS	NICNGIIEKLLDSR	:	113		
MtGF14-2	:	YKNVIGARRASWRIISSIEQKEESRGN	-DEHVTVIRDYRSKIEEELS	NICNGIIEKLLDSR	:	113		
GmSGF14g	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVAVIRDYRSKIEEELS	NICDGIIEKLLDTR	:	114		
GmSGF14k	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVAVIRDYRSKIEEELS	NICDGIIEKLLDTR	:	113		
PsGF14-1	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVAVIRDYRSKIEEELS	NICDGIIEKLLDTR	:	113		
MtGF14-1	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVSVIDYRSKIEEELS	NICDGIIEKLLDSR	:	113		
GmSGF14h	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVSVIDYRSKIEEELS	NICDGIIEKLLDSR	:	113		
GmSGF14i	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVSVIDYRSKIEEELS	NICDGIIEKLLDSR	:	113		
LjGF14-1/2	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVSVIDYRSKIEEELS	NICDGIIEKLLDSR	:	113		
AtGF14φ	:	YKNVIGARRASWRIISSIEQKEESRGN	-DDHVTIIRDYRSKIEEELS	KICDGIIEKLLDTR	:	116		
AtGF14ω	:	YKNVIGARRASWRIISSIEQKEESRGN	-DDHVTIAREYRSKIEEELS	GICDGIIEKLLDSR	:	110		
AtGF14χ	:	YKNVIGARRASWRIISSIEQKEESRGN	-DDHVSVIDYRSKIEEELS	ICDGIIEKLLDTI	:	115		
GmSGF14a	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVAIKEYRGKIEEELS	KICDGIIEKLLDSN	:	111		
GmSGF14m	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVAIKEYRGKIEEELS	KICDGIIEKLLDSN	:	111		
LjGF14-8	:	YKNVIGARRASWRIISSIEQKEESRGN	-EDHVSVIDYRSKIEEELS	KICDGIIEKLLDSN	:	115		
AtGF14v	:	YKNVIGARRASWRIISSIEQKEESRGN	-DDHVSVIDYRSKIEEELS	KICDGIIEKLLDSH	:	110		
AtGF14u	:	YKNVIGARRASWRIISSIEQKEESRGN	-SDHVSVIDYRSKIEEELS	KICDGIIEKLLDSH	:	112		
AtGF14ψ	:	YKNVIGARRASWRIISSIEQKEESKGN	-EDHVAIKEYRGEIEEELS	KICDGIIEKLLDSH	:	109		
PsGF14-7	:	YKNVIGSIRAAWRIVSSIEQKEEGRKN	-DDHVVIVKDYRSKVEEELS	NVCASIEKLLDSN	:	117		
MtGF14-7a	:	YKNVIGSIRAAWRIVSSIEQKEEGRKN	-EDHVVIVKDYRSKVEEELS	NTNVCASIEKLLDSN	:	117		
LjGF14-7	:	YKNVIGSIRAAWRIVSSIEQKEESRKN	-DDHVVIVKDYRSKVEEELS	NICASIEKLLDSN	:	119		
GmSGF14b	:	YKNVIGSIRAAWRIVSSIEQKEEGRKN	-DDHVSIVKHYSKVENELTQVCASIEKLLDSN	:	115			
GmSGF14j	:	YKNVIGSIRAAWRIVSSIEQKEEGRKN	-DDHVSIVKHYSKVENELTQVCASIEKLLDSN	:	115			
MtGF14-7b	:	YKNATEPIRAALRIIS---KEEGRKNE	DDHVFVHKYRSKVEEELS	ENVCASIEKLLDSK	:	115		
AtGF14λ	:	YKNVIGSIRAAWRIVSSIEQKEESRKN	-DEHVSIVKDYRSKVEEELS	SVCSGIEKLLDSH	:	113		
AtGF14κ	:	YKNVIGSIRAAWRIVSSIEQKEESRKN	-EEHVSIVKDYRSKVEEELS	SVCSGIEKLLDSH	:	113		
Human	:	YKNVVGARRASWRIISSIEQKTDISDK---	KIQLIKDYREKVEEELS	RSICTTVIEKLLDKY	:	104		

		*	140	*	160	*	180	
PsGF14-6	:	LVP	STS-AEANVFYHKMKGDYFRYLAEFKTDQERKEAAEQSLKGYEAA	SATANTDLPST	:	169		
MtGF14-6a	:	LVE	ASTS-AEANVFYHKMKGDYFRYLAEFKTDQERKEAAEQSLKGYEAA	SATANTDLPST	:	169		
GmSGF14e	:	LIP	SSAS-AEASVFYHKMKGDYFRYLAEFKTDQERKEAAEQSLKGYEAA	SATANTDLPST	:	169		
GmSGF14f	:	LIP	SSGS-AEASVFYHKMKGDYFRYLAEFKTDQERKEAAEQSLKGYEAA	SATANTDLPST	:	169		
LjGF14-6a	:	LIP	SSAS-AEASVFYHKMKGDYFRYLAEFKTDQERKEAAEQSLKGYEAA	SATANTDLPST	:	169		
LjGF14-6b	:	LIP	SSAS-GEATVFYHKMKGDYFRYLAEFKTDQERKEAAEQSLKGYEAA	SATANTDLPST	:	169		
MtGF14-6b	:	LIP	STT-GEATVFYHKMKGDYFRYLAEFKNDQDRKEAADQSLKAYEAA	SATASATDLPST	:	169		
AtGF14i	:	LIP	HATS-GEATVFYHKMKGDYFRYLAEFKTEQERKEAAEQSLKGYEAA	TQAASTELPST	:	172		
AtGF14o	:	LVE	FATS-GESTVFYHKMKGDYFRYLAEFKSGADREAAADLSLKAYEAA	TSSASTELST	:	167		
PsGF14-3	:	LIP	SSSA-GESSVFYHKMKGDYFRYLAEFKNGDERKEAADRSMEAYQT	ASTAAEDELAPT	:	169		
MtGF14-3	:	LIP	SSSA-GESSVFYHKMKGDYFRYLAEFKNGDERKEAADHSMAYQT	ASTAAEDELAPT	:	169		
GmSGF14c	:	LIP	SSSS-GEPSVFYHKMKGDYFRYLAEFKSGDERKEAADHSMKAYQL	ASTTAAEELAST	:	169		
GmSGF14l	:	LIP	SSSA-GEPSVFYHKMKGDYFRYLAEFKSGDERKEAADHSMKAYQ	SASTTAAEELAPT	:	169		
PsGF14-5	:	LIP	SSAA-GESTVFYHKMKGDYFRYLAEFKTGNKKKEAGDQSMKAYE	SATTAEEELAPT	:	169		
MtGF14-5	:	LIP	SATA-GESTVFYHKMKGDYFRYLAEFKTGNKKKEAGDQSMKAYE	SATTAEEELAPT	:	169		
LjGF14-5	:	LIP	SATA-GESTVFYHKMKGDYFRYLAEFKSGNKKKEAADQSMKAYE	SATTAEEELAPT	:	169		
GmSGF14d	:	LIP	SSAA-GESTVFYHKMKGDYFRYLAEFKSGNKKKEAADQSMKAYE	SATTAEEELAPT	:	169		
GmSGF14o	:	LIP	SSAA-GESTVFYHKMKGDYFRYLAEFKACNKKKEAADQSMKAYE	SATTAEEELAPT	:	169		
AtGF14μ	:	LIP	ASE-GESTVFYHKMKGDYFRYLAEFKSGNERKEAADQSLKAYE	IAATTAAEAKLPPT	:	169		
PsGF14-4	:	LIP	STNI-AESTVFYHKMKGDYFRYLAEFKSGDEKKKEVADQSLKAYQ	SASATAENELQPT	:	170		
MtGF14-4	:	LIP	STNI-AESTVFYHKMKGDYFRYLAEFKACDEKKKEVADLSLKAYQ	TASATAENELQPT	:	170		
GmSGF14n	:	LIP	STNI-AESTVFYHKMKGDYFRYLAEFKACNKKKEVADQSLKAYE	TASTTAESELQPT	:	175		
GmSGF14p	:	LIP	STNI-AESTVFYHKMKGDYFRYLAEFKACNKKKEVADQSLKAYQ	TASTTAESELQPT	:	170		
LjGF14-4	:	LIP	STNV-AESTVFYHKMKGDYFRYLAEFKACNKKKEVADQSLKAYE	ASATAESELQPT	:	170		
AtGF14ε	:	LIP	SSNA-VESTVFYHKMKGDYFRYLAEFSSCAERKEAADQSLKAYK	AAVAAAENGLAPT	:	167		
AtGF14n	:	LIP	TTTTNVESTVLEFNVRKGDYFRYLAEFGSDAERKENADNSLDAY	KVAMEMAAENSAPT	:	168		
PsGF14-2	:	LIP	SAAAL-GDSKVFFYLKMKGDYFRYLAEFKSCAERKDAAEESTL	TAYKSAQDIANTELPPT	:	172		
MtGF14-2	:	LIP	SAAAS-GDSKVFFYLKMKGDYFRYLAEFKSCAERKDAAEESTL	TAYKSAQDIANSELPT	:	172		
GmSGF14g	:	LVP	SAAAS-GDSKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	SAYKAAQDIANTELPPT	:	173		
GmSGF14k	:	LVP	SAAAS-GDSKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	SAYKAAQDIANTELPPT	:	172		
PsGF14-1	:	LIP	SASS-GDSKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	SAYKAAQDIANAELPPT	:	172		
MtGF14-1	:	LIP	SAAAS-GDSKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	SAYKAAQDIANAELPPT	:	172		
GmSGF14h	:	LIP	SASS-GDSKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	SAYKAAQDIANAELPPT	:	172		
GmSGF14i	:	LIP	SASS-GDSKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	SAYKAAQDIANAELPPT	:	172		
LjGF14-1/2	:	LIP	AAAAS-GDSKVFFYLKMKGDYFRYLAEFKTGTERKDAAESTL	LAAYKSAQDIANSELPT	:	172		
AtGF14φ	:	LVE	ASAN-GDSKVFFYLKMKGDYFRYLAEFKTGQERKDAAEHTLT	AYKAAQDIANAELAPT	:	175		
AtGF14ω	:	LIP	AAAAS-GDSKVFFYLKMKGDYFRYLAEFKTGQERKDAAEHTLT	AYKSAQDIANAELAPT	:	169		
AtGF14χ	:	LVE	AAAAS-GDSKVFFYLKMKGDYFRYLAEFKSGQERKDAAEHTLT	AYKAAQDIANSELAPT	:	174		
GmSGF14a	:	LIP	SAAAS-PESKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	LAYKSAQDIALADLAPT	:	170		
GmSGF14m	:	LIP	SAAAS-PESKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	LAYKSAQDIALADLAPT	:	170		
LjGF14-8	:	LIP	SSAA-PESKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	LAYKSAQDIALAEAPT	:	174		
AtGF14v	:	LVP	TASL-AESKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	VAYKSAQDIALADLAPT	:	169		
AtGF14u	:	LIP	ASAL-AESKVFFYLKMKGDYFRYLAEFKTCADRKEAAESTL	VAYKSAQDIALADLAPT	:	171		
AtGF14ψ	:	LIP	ASP-AESKVFFYLKMKGDYFRYLAEFKACADRKEAAESTL	VAYKASADIATAELAPT	:	168		
PsGF14-7	:	LIP	SGSS-SESKVFYHKMKGDYFRYLAEFKIGDERKSAEDTMLS	YKAAQDIAAADLPST	:	176		
MtGF14-7a	:	LIP	SASS-SESKVFYHKMKGDYFRYLAEFKIGDERKSAEDTMLS	YKAAQDIAAADLPST	:	176		
LjGF14-7	:	LIP	SASS-SESKVFYHKMKGDYFRYLAEFKFRVGDQRKSAEDTMLS	YKAAQDIAATDLPPT	:	178		
GmSGF14b	:	LVP	SASA-SESKVFYLKMKGDYFRYLAEFKVGDQRKTAAEDTMLS	YKAAQDIASADLPPT	:	174		
GmSGF14j	:	LVP	SVFA-SESKVFYLKMKGDYFRYLAEFKVGDQRKTAAEDTMLS	YKAAQDIASGDLPT	:	174		
MtGF14-7b	:	LIP	SASS-SEIRVYYYQMKGDYQRYMAEFKIGDDKKSAAVEDIIL	SYKAAQDIAAADLRSS	:	174		
AtGF14λ	:	LIP	SAGA-SESKVFYLKMKGDYFRYLAEFKSGDERKTAEDTML	AYKAAQDIAAADMAPT	:	172		
AtGF14κ	:	LIP	SATA-SESKVFYLKMKGDYFRYLAEFKSGDERKTAEDTML	AYKAAQDVAVADLAPT	:	172		
Human	:	LIP	ANATN-PESKVFFYLKMKGDYFRYLAEFVACGDDRKQTIDNSQ	GAYQEAFFDISKEMQPT	:	163		



		*	200	*	220	*	240	
Psgf14-6	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
MtGF14-6a	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
GmSGF14e	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
GmSGF14f	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
LjGF14-6a	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
LjGF14-6b	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
MtGF14-6b	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
AtGF14i	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 232
AtGF14o	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDSIN	NE	ESYKDSTLIMQLLRD	: 227
Psgf14-3	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
MtGF14-3	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDTL	NE	ESYKDSTLIMQLLRD	: 229
GmSGF14c	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
GmSGF14l	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDTL	SE	ESYKDSTLIMQLLRD	: 229
Psgf14-5	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDTL	NE	ESYKDSTLIMQLLRD	: 229
MtGF14-5	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDTL	NE	ESYKDSTLIMQLLRD	: 229
LjGF14-5	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDTL	NE	ESYKDSTLIMQLLRD	: 229
GmSGF14d	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDTL	NE	ESYKDSTLIMQLLRD	: 229
GmSGF14o	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDEAIAELDTL	NE	ESYKDSTLIMQLLRD	: 229
AtGF14μ	:	HP	IRLGLALNFSVFYYEIMNAP	ER	ACHLAKQAFDEAIAELDTL	NE	ESYKDSTLIMQLLRD	: 229
Psgf14-4	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDDGVSELDSTIN	ED	SYKDSTLIMQLLRD	: 230
MtGF14-4	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDDGVSELDSTIN	ED	SYKDSTLIMQLLRD	: 230
GmSGF14n	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDDAVSELDSTIN	ED	SYKDSTLIMQLLRD	: 235
GmSGF14p	:	HP	IRLGLALNFSVFYYEILNSP	ER	ACHLAKQAFDDAVSELDSTIN	ED	SYKDSTLIMQLLRD	: 230
LjGF14-4	:	HP	IRLGLALNFSVFYYEIMNSP	ER	ACHLAKQAFDDAVSELDSTIN	ED	SYKDSTLIMQLLRD	: 230
AtGF14ε	:	HP	IRLGLALNFSVFYYEILNSP	ES	ACQLAKQAFDDATAELDSIN	NE	ESYKDSTLIMQLLRD	: 227
AtGF14π	:	NM	VRGLALNFSVFYYEILNSP	IE	SACKLVKKAYDEAIAELDTL	GD	KNICEESMYTIEMLKY	: 228
Psgf14-2	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACGLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
MtGF14-2	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACGLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
GmSGF14g	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACSLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 233
GmSGF14k	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACSLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
Psgf14-1	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
MtGF14-1	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
GmSGF14h	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
GmSGF14i	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
LjGF14-1/2	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACSLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
AtGF14φ	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 235
AtGF14ω	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 229
AtGF14χ	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 234
GmSGF14a	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 230
GmSGF14m	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 230
LjGF14-8	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACNLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 234
AtGF14v	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACSLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 229
AtGF14u	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACSLAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 231
AtGF14ψ	:	HP	IRLGLALNFSVFYYEILNSP	DR	ACSLAKQAFDDAIAELDTL	GE	ESYKDSTLIMQLLRD	: 228
Psgf14-7	:	HP	IRLGLALNFSVFYYEILNSD	KAC	AMAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 236
MtGF14-7a	:	HP	IRLGLALNFSVFYYEILNSD	KAC	DMAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 236
LjGF14-7	:	HP	IRLGLALNFSVFYYEILNSD	KAC	AMAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 209
GmSGF14b	:	HP	IRLGLALNFSVFYYEILNSD	KAC	AMAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 234
GmSGF14j	:	HP	IRLGLALNFSVFYYEILNSD	KAC	AMAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 234
MtGF14-7b	:	HP	IRLGLALNFSVFYYEILNRFDEGLDMARQAL	DE	ARNELK-LGDEYYKSTVIMQLLRN			: 233
AtGF14λ	:	HP	IRLGLALNFSVFYYEILNSD	KAC	NMAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
AtGF14κ	:	HP	IRLGLALNFSVFYYEILNSSE	KAC	MAKQAFDEAIAELDTL	GE	ESYKDSTLIMQLLRD	: 232
Human	:	HP	IRLGLALNFSVFYYEILNNE	EL	ACTLAKTAFDEAIAELDTL	NE	ESYKDSTLIMQLLRD	: 223

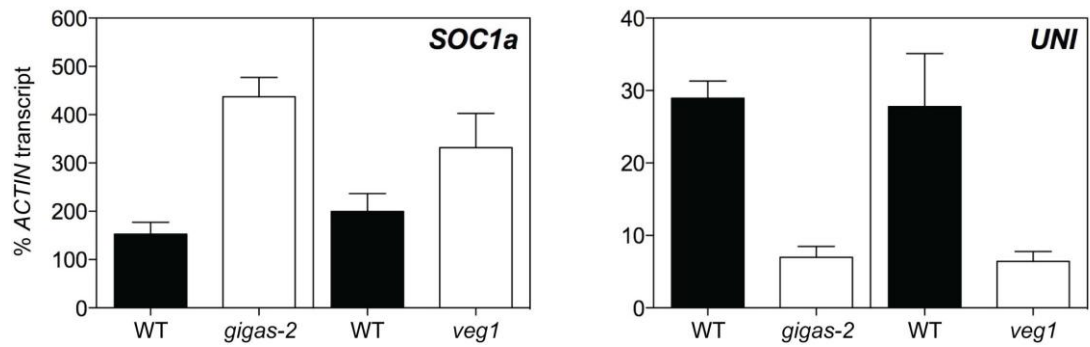
		*	260	*	280	*	300	
PsGF14-6	:	NLTLWTS	DI	PEDGG	EDSIKADEVKPT	EPH-----		: 259
MtGF14-6a	:	NLTLWTS	DI	PEDGGE	ETIKAEEAKPAE	AEH-----		: 259
GmSGF14e	:	NLTLWTS	DI	PEDGG	EDSIKAEEETKP	SEPEH-----		: 259
GmSGF14f	:	NLTLWTS	DI	PEDGG	EDNIKAEEAKP	SEPEH-----		: 259
LjGF14-6a	:	NLTLWTS	DI	PEDGGE	ENIKAEEAKPTE	PEVCG-----		: 261
LjGF14-6b	:	NLTLWTS	DI	PEDGG	DE-IKTEETKPAE	QEVCVW-----		: 261
MtGF14-6b	:	NLTLWTS	DI	PEDGG	EE-LKSEEVKPAE	PEVWQQSRADIF	LVKPNQVGRISMYLS-----	: 282
AtGF14i	:	NLTLWTS	DI	PEDGG	EDNIKTEESKQEQ	AKPADATEN-----		: 268
AtGF14o	:	NLTLWTS	DI	EE-GGK-----				: 241
PsGF14-3	:	NLTLWTS	DI	PEEG-VEE	QKVE-SSRDG-G	DEDA-----		: 259
MtGF14-3	:	NLTLWTS	DI	PEDG-DEE	HKVE-SSGAV-G	GEN-----		: 258
GmSGF14c	:	NLTLWTS	DI	PEDG-AEE	QKVD-SARAA-G	GDDA-----		: 259
GmSGF14l	:	NLTLWTS	DI	PEEG-AEE	QKVD-SARAA-G	GDNA-----		: 259
PsGF14-5	:	NLTLWTS	DI	PEDG-EDS	QKANGTAKFG-G	GDDAE-----		: 261
MtGF14-5	:	NLTLWTS	DI	PEDG-EEN	QKANGTAKLG-G	GDDAE-----		: 261
LjGF14-5	:	NLTLWTS	DI	PEEG-EDS	LKANDTAKVG-G	GEDAEVSS-----		: 264
GmSGF14d	:	NLTLWTS	DI	PEDG-EDA	QKVNGTAKLG-G	GEDAE-----		: 261
GmSGF14o	:	NLTLWTS	DI	PEDG-EDA	QKVNGTAKLG-G	GEEAE-----		: 261
AtGF14μ	:	NLTLWTS	DI	SEEGG	DDAHKTNGSAK	PGAGGDDAE-----		: 263
PsGF14-4	:	NLTLWTS	DI	PEDS--ED	QKMESATKSGQ	EEELGR-----		: 263
MtGF14-4	:	NLTLWTS	DI	PEDG--ED	QKMESATKSGQ	DEDELGR-----		: 263
GmSGF14n	:	NLTLWTS	DI	PEEG--ED	QKMESTTR--G	EDELGR-----		: 265
GmSGF14p	:	NLTLWTS	DI	PEEG--ED	LKMSAARVDQ	GEDELGR-----		: 263
LjGF14-4	:	NLTLWTS	DI	PEDG--DD	QKMSAAMGGEG	EENELGR-----		: 263
AtGF14ε	:	NLTLWTS	DI	NEEGD	ERTKGAD	EPQDEN-----		: 254
AtGF14n	:	NLTLWTS	DI	GDGNG	NKTDG-----			: 245
PsGF14-2	:	NLTLWTS	DI	DDGA-DEI	KEAA---PKGN	DEPQ-----		: 261
MtGF14-2	:	NLTLWTS	DI	DDGA-DEI	KEAA---PKG	ADEQ-----		: 260
GmSGF14g	:	NLTLWTS	DI	DDGA-DEI	KEAA---PKG	DGEQN-----		: 262
GmSGF14k	:	NLTLWTS	DI	DDGA-DEI	KEAA---PKG	DGEQNTYHTLCL	LVLPLFCNVFKGRRC---	: 284
PsGF14-1	:	NLTLWTS	DI	DDGA-DEI	KEAA---PKA	DEQQ-----		: 260
MtGF14-1	:	NLTLWTS	DI	DDGA-DEI	KEAA---PKP	DEQRFLLLISL	GAELGIHVICYEFWMSWAVL	: 287
GmSGF14h	:	NLTLWTS	DI	DDGA-DEI	KEAA---PKQ	DDQ-----		: 259
GmSGF14i	:	NLTLWTS	DI	DDGA-DEI	KEAA---PKP	DDQ-----		: 259
LjGF14-1/2	:	NLTLWTS	DI	DEGA-DEI	KEAA---PKP	DEQ-----		: 259
AtGF14φ	:	NLTLWTS	DI	DESP-EEI	KEAA---APK	PAEEQKEI-----		: 267
AtGF14ω	:	NLTLWTS	DI	DDAA-DEI	KEAA---APK	PTTEEQ-----		: 259
AtGF14χ	:	NLTLWTS	DI	DDVA-DDI	KEAAPAAAKPA	DEQQS-----		: 267
GmSGF14a	:	NLTLWTS	DI	DDAG-DEI	KETSKQQPGE-----			: 257
GmSGF14m	:	NLTLWTS	DI	DDAG-DEI	KETSKQQPGE-----			: 257
LjGF14-8	:	NLTLWTS	DI	DDAG-DEI	KETSKPQSGD	GEQ-----		: 264
AtGF14v	:	NLTLWTS	DI	DEAGG	DEIKEASKHEPE	GKPAETGQ-----		: 265
AtGF14u	:	NLTLWTS	DI	DEAG-DDI	KEAPKEVQKV	DEQAQPPPSQ-----		: 268
AtGF14ψ	:	NLTLWTS	DI	DEAG-DEI	KEASKP-----	DGAE-----		: 255
PsGF14-7	:	NLTLWTS	DI	DQLD----	EP-----			: 252
MtGF14-7a	:	NLTLWTS	DI	DQLD----	EP-----			: 252
LjGF14-7	:	NLTLWTS	DI	DQLD----	EP-----			: -
GmSGF14b	:	NLTLWTS	DI	DQLD----	EP-----			: 250
GmSGF14j	:	NLTLWTS	DI	DQLD----	EP-----			: 250
MtGF14-7b	:	NLTLWTS	DI	EDM-DQLD	-----EH-----			: 249
AtGF14λ	:	NLTLWTS	DI	EQMD----	EA-----			: 248
AtGF14κ	:	NLTLWTS	DI	EQMD----	EA-----			: 248
Human	:	NLTLWTS	DI	SAGEEC	DAAEGAEN-----			: 245

		*	
PsGF14-6	:	-----	-
MtGF14-6a	:	-----	-
GmSGF14e	:	-----	-
GmSGF14f	:	-----	-
LjGF14-6a	:	-----	-
LjGF14-6b	:	-----	-
MtGF14-6b	:	-----	-
AtGF14i	:	-----	-
AtGF14o	:	-----	-
PsGF14-3	:	-----	-
MtGF14-3	:	-----	-
GmSGF14c	:	-----	-
GmSGF14l	:	-----	-
PsGF14-5	:	-----	-
MtGF14-5	:	-----	-
LjGF14-5	:	-----	-
GmSGF14d	:	-----	-
GmSGF14o	:	-----	-
AtGF14μ	:	-----	-
PsGF14-4	:	-----	-
MtGF14-4	:	-----	-
GmSGF14n	:	-----	-
GmSGF14p	:	-----	-
LjGF14-4	:	-----	-
AtGF14ε	:	-----	-
AtGF14π	:	-----	-
PsGF14-2	:	-----	-
MtGF14-2	:	-----	-
GmSGF14g	:	-----	-
GmSGF14k	:	-----	-
PsGF14-1	:	-----	-
MtGF14-1	:	VCPSIFKCFTNYCLFS	303
GmSGF14h	:	-----	-
GmSGF14i	:	-----	-
LjGF14-1/2	:	-----	-
AtGF14φ	:	-----	-
AtGF14ω	:	-----	-
AtGF14χ	:	-----	-
GmSGF14a	:	-----	-
GmSGF14m	:	-----	-
LjGF14-8	:	-----	-
AtGF14v	:	-----	-
AtGF14v	:	-----	-
AtGF14ψ	:	-----	-
PsGF14-7	:	-----	-
MtGF14-7a	:	-----	-
LjGF14-7	:	-----	-
GmSGF14b	:	-----	-
GmSGF14j	:	-----	-
MtGF14-7b	:	-----	-
AtGF14λ	:	-----	-
AtGF14κ	:	-----	-
Human	:	-----	-

Alignment for FT/TFL1 homologs from rice (Os), *Arabidopsis* (At) and pea (Ps), showing putative 14-3-3 binding sites (orange text, marked by asterisks) identified in Hd3a (Taoka et al., 2011). Full-length predicted protein sequences were aligned with ClustalX (Thompson et al., 1997) and manually adjusted with GeneDoc (Nicholas and Nicholas, 1997). Shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Accession details for sequences are given above in Table A5.1.

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## Appendix 5: qRT-PCR results for *UNI* and *SOC1a* in *gigas* and *veg1*



**Figure A5.1.** Apical expression of *SOC1a* and *UNI* in the *gigas* and *veg1* mutants.

Gene expression in dissected shoot apices of *gigas* and *veg1* mutants and corresponding wild-type plants, at a single time-point, 45 days after sowing, after the first appearance of floral buds in wild-type apices grown under LD conditions. Values have been normalised to the transcript level of *ACTIN* and represent mean  $\pm$  standard error for 2-3 biological replicates. Wild-type lines are NGB5839 (left) and wild-type siblings of *veg1* (right). Full details for this experiment are given in Chapter 3.

# Appendix 6: Sequences and sequence alignments for

## Chapter 7

### Sequences for SVP-like genes and other MADS-box transcription factors included in alignments and phylogenetic analyses

**Table A6.1.** Details of sequences used for phylogenetic analyses in Chapter 7. Asterisks indicate a corrected annotation was used for phylogenetic analysis. Source details including websites and relevant references are outlined in Chapter 2 (Table 2.4).

Species	Gene name	Sequence	Source	Reference (s)
<i>Antirrhinum majus</i>	AmINCO	AJ699174	GenBank	Masiero et al. (2004)
Apple ( <i>Malus domestica</i> )	MdJNT	DQ402055	GenBank	Bielenberg et al. (2008)
<i>Arabidopsis thaliana</i>	AtAG	NM_118013	GenBank	Parenicova et al. (2003); Smaczniak et al. (2012)
	AtAGL6	NM_130127		
	AtAGL10	NM_102395		
	AtAGL13	NM_115976		
	AtAGL14	NM_117258		
	AtAGL15	NM_121382		
	AtAGL16	NM_115583		
	AtAGL17	NM_127828		
	AtAGL18	NM_115599		
	AtAGL19	NM_118424		
	AtAGL21	NM_119955		
	AtAGL24	NM_118587		
	AtAGL66	NM_106447		
	AtAGL71	NM_203195		
	AtAGL72	NM_124565		
	AtAGL79	NM_113925		
	AtANR1	NM_126990		
	AtAP1	NM_105581		
	AtAP3	NM_115294		
	AtFLC	NM_121052		
	AtFUL	NM_125484		
	AtFYF	NM_125610		
	AtGOA	NM_001198191		
	AtMAF1	NM_106358		
	AtMAF2	NM_001085329		
	AtMAF3	NM_125905		
	AtMAF4	NM_125906		
	AtMAF5	NM_125907		
	AtPI	NM_122031		
	AtSEP1	NM_121585		
	AtSEP2	NM_111098		
	AtSEP3	NM_102272		
	AtSEP4	NM_126418		
	AtSHP1	NM_115740		
	AtSHP2	NM_129844		
	AtSOC1	NM_130128		
	AtSTK	NM_117064		
	AtSVP	NM_127820		
	AtTT16	NM_203094		
	AtXAL1	NM_105825		
Barley ( <i>Hordeum vulgare</i> )	HvBM1	AJ249142	GenBank	Trevaskis et al. (2007)
	HvBM10	EF043040		
	HvVRT2	DQ201168		
Capsicum ( <i>Capsicum annuum</i> )	CaJ	JQ698661	GenBank	Cohen et al. (2012)
<i>Coffea arabica</i>	CaC10	GU332283	GenBank	de Oliveira et al. (2010)
	CaS21	GU332292		
	CaS22	GU332282		

Species	Gene name	Sequence	Source	Reference (s)
Chinese cabbage ( <i>Brassica rapa</i> )	BrSVP	DQ922945	GenBank	Lee et al. (2007a)
Common bean ( <i>Phaseolus vulgaris</i> )	PvSVPa	Phvul.002G147600	<i>Phaseolus vulgaris</i> genome v1.0	This study
	PvSVPb1	Phvul.006G202300		
	PvSVPb2	Phvul.002G212400*		
	PvSVPb3	Phvul.006G202200		
Cucumber ( <i>Cucumis sativus</i> )	PvSVPc	Phvul.009G037300	<i>Cucumis sativus</i> genome v1	Hu and Liu (2012)
	CsMADS17	Cucsa.104010*		
	CsMADS18	Cucsa.160640		
	CsMADS19	Cucsa.331840*		
	CsMaDS20	Cucsa.190400		
<i>Eucalyptus grandis</i>	EgSVP	AY273873	GenBank	Brill and Watson (2004)
<i>Gnetum gnemon</i>	GGM12	UCGgnemon_isotig04234	ConGenIE	This study
Grape ( <i>Vitis Vinifera</i> )	VvSVP1	GSVIVT01005934001	<i>Vitis vinifera</i> draft genome 2X March 2010 release	Díaz-Riquelme et al. (2009)
	VvSVP2	GSVIVT01009171001*		
	VvSVP3	GSVIVT01011300001		
	VvSVP4	GSVIVT01015641001		
	VvSVP5	GSVIVT01001701001		
Japanese apricot ( <i>Prunus mume</i> )	PmDAM1	AB576350	GenBank	Sasaki et al. (2011)
	PmDAM2	AB576351		
	PmDAM3	AB576352		
	PmDAM4	AB576353		
	PmDAM5	AB576349		
	PmDAM6	AB437345		
Kiwifruit ( <i>Actinidia chinensis</i> )	AcSVP1	JF838216	GenBank	Wu et al. (2012)
	AcSVP2	JF838217		
	AcSVP3	JF838218		
	AcSVP4	JF838219		
Leafy spurge ( <i>Euphorbia esula</i> )	EeDAM1	EU334633	GenBank	Horvath et al. (2008); Horvath et al. (2010)
	EeDAM2	EU339320		
	EeDAM3	JX966351		
<i>Lotus japonicus</i>	LjSVPa	TC60537	DFCI TGI	This study
	LjSVPb	TC81268		
	LjSVPc	TC62447		
<i>Medicago truncatula</i>	MtSVPa1	Medtr5g032520	<i>Medicago</i> genome v3.5	Hecht et al. (2005); Jaudal (2011); This study
	MtSVPa2	Medtr5g032150		
	MtSVPb	Medtr4g093970*		
	MtSVPc	TC183050	DFCI TGI	
Maize ( <i>Zea mays</i> )	ZmM19	AJ430633	GenBank	Münster et al. (2002)
	ZmM26	AJ430693		
Peach ( <i>Prunus persica</i> )	PpDAM1	DQ863253	GenBank	Bielenberg et al. (2008)
	PpDAM2	DQ863255		
	PpDAM3	DQ863256		
	PpDAM4	DQ863250		
	PpDAM5	DQ863251		
	PpDAM6	DQ863252		
	PpSVP	ppa011063m	Peach genome v1.0	This study
Perennial ryegrass ( <i>Lolium perenne</i> )	LpMADS10	DQ110009	GenBank	Petersen et al. (2006)
<i>Petunia hybrida</i>	PhFBP13	AF335237	GenBank	Immink et al. (2003)
	PhFBP25	AF335243		
Poplar ( <i>Populus trichocarpa</i> )	PtMADS7	Potri.002G105600	<i>Populus</i> genome v3.0	Leseberg et al. (2006); This study
	PtMADS21a	Potri.005G155300		
	PtMADS21b	Potri.005G155700		
	PtMADS26	Potri.007G010800		
	PtMADS27	Potri.007G115200		
	PtMADS28	Potri.007G115100		
	PtMADS29	Potri.007G115000		
	PtMADS47	Potri.017G044200		
Potato ( <i>Solanum tuberosum</i> )	PtMADS48	Potri.017G044500	GenBank	Carmona et al. (1998); García-Maroto et al. (2000)
	StMADS11	AF008652		
	StMADS16	AY643736		

Species	Gene name	Sequence	Source	Reference (s)
Rice ( <i>Oryza sativa</i> )	OsMADS22	AB107957	GenBank	Sentoku et al.(2005)
	OsMADS47	AY345221		
	OsMADS55	AY345223		
Sapphire dragon tree ( <i>Paulownia kawakamii</i> )	PkMADS1	AF060880	GenBank	Prakash and Kumar (2002)
Soybean ( <i>Glycine max</i> )	GmSVPa1	Glyma01g02880	Soybean genome v1.1	Jung et al. (2012); Shu et al. (2013); This study
	GmSVPa2	Glyma02g04710		
	GmSVPb1	Glyma07g30040		
	GmSVPb2	Glyma08g07260		
	GmSVPb3	Glyma13g33051		
	GmSVPb4	Glyma15g06302		
	GmSVPb5	Glyma13g33031*		
	GmSVPb6	Glyma15g06314		
	GmSVPc1	Glyma06g10020		
Sweet potato ( <i>Ipomoea batatas</i> )	GmSVPc2	Glyma04g10015*		
	IbMADS3	AF345246	GenBank	Kim et al. (2002)
	IbMADS4	AF346303		
Tall fescue ( <i>Festuca arundinacea</i> )	FaVRT2	HM439237	GenBank	Wang et al. (2012)
Tomato ( <i>Solanum lycopersicum</i> )	SIJ	XM_004250097	GenBank	Mao et al. (2000); Hileman et al. (2006)
	SIMBP24	XM_004237945		
Trifoliolate orange ( <i>Poncirus trifoliata</i> , syn. <i>Citrus trifoliata</i> )	CtSVP	FJ373211	GenBank	Li et al. (2010)
Wheat ( <i>Triticum aestivum</i> )	TaVRT2	DQ022679	GenBank	Kane et al. (2005)



**Sequence alignment for Figure 7.1**

Alignment for the phylogenetic neighbour-joining tree of the legume *StMADS11* subfamily and *Arabidopsis* MIKC MADS-box transcription factors shown in Figure 7.1. Full-length predicted protein sequences were aligned with ClustalX (Thompson et al., 1997) and manually adjusted with GeneDoc (Nicholas and Nicholas, 1997). Shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Full sequence details are given in Table A6.1, above.

			20	*	40	*	60	
AtPI	:	-----	MGRGKLEIKRIENANNRVVTF	SKRRNGLVKKAKEITVLCDAKV	:	43		
AtTT16	:	-----	MGRGKLEIKKIENQTAROVTF	SKRRRTGLIKKTRELSILCDAHI	:	43		
MtSVPa1	:	-----	MAREKIQIKKIENSTAROVTF	SKRRRGGLIKKAEELSVLCDADV	:	43		
MtSVPa2	:	-----	MAREKIQIKKIENSTAROVTF	SKRRRGGLIKKAEELSVLCDADV	:	43		
PsSVPa	:	-----	MAREKIQIKKIENATAROVTF	SKRRRGGLFKKAEELSVLCDADV	:	43		
GmSVPa2	:	-----	MVREKIQIKKIDNATAROVTF	SKRRRGGLFKKAEELSVLCDADV	:	43		
GmSVPa1	:	-----	MAREKIQIKKIDNATAROVTF	SKRRRGGLFKKAEELSVLCDADV	:	43		
PvSVPa	:	-----	MAREKIQIRKIDNATAROVTF	SKRRRGGLFKKAEELSVLCDADV	:	43		
LjSVPa	:	-----	MAREKIQIKKIDNATAROVTF	SKRRRGGLFKKAEELSVLCDADV	:	43		
AtSVP	:	-----	MAREKIQIRKIDNATAROVTF	SKRRRGGLFKKAEELSVLCDADV	:	43		
AtAGL24	:	-----	MAREKIRIKKIDNITAROVTF	SKRRRGIFKKAEELSVLCDADV	:	43		
PsSVPc	:	-----	MARQKIKIKKIDNATAROVTF	SKRRRGIFKKAEELSVLCDAEV	:	43		
MtSVPc	:	-----	MARQKIKIKKIDNATAROVTF	SKRRRGIFKKAEELSVLCDAEV	:	43		
LjSVPc	:	-----	MARKKTIKIKKIDNITAROVTF	SKRRRGIFKKAEELSVLCDAEV	:	43		
GmSVPc1	:	-----	MTRTKTIKIKKIDNITAROVTF	SKRRRGGLFKKAEELSVLCDAEV	:	43		
GmSVPc2	:	-----	MTRAKTIKIKKIDNITAROVTF	SKRRRGGLFKKAEELSVLCDAEV	:	43		
PvSVPc	:	-----	MTRAKTIKIKKIDNITAROVTF	SKRRRGGLFKKAEELSVLCDADV	:	43		
PsSVPb	:	-----	MTRKKTIKIKKIDNISSROVTF	SKRRKGLFKKAEELSTLCDAV	:	43		
MtSVPb	:	-----	MTRKKTIKIKKIDNISSROVTF	SKRRKGLFKKAEELSTLCDAI	:	43		
GmSVPb1	:	-----	MTRKRTIKIKKIDNISSROVTF	SKRRKGLFKKAEELSTLCDAI	:	43		
GmSVPb2	:	-----	MTRKRTIKIKKIDNISSROVTF	SKRRKGLFKKAEELSTLCDAI	:	43		
PvSVPb2	:	-----	MTRKRTIKIKKIDNISSROVTF	SKRRKGLFKKAEELSTLCDAI	:	43		
LjSVPb	:	-----	MTRKRTIKIKKIDNISSROVTF	SKRRKGLFKKAEELSTLCDAI	:	43		
GmSVPb3	:	-----	MARKKTIKIKKIDNINAROVTF	SKRRKGLFKKAEELSTLCDAEI	:	43		
GmSVPb4	:	-----	MTRKKTIKIKKIDNINAROVTF	SKRRKGLFKKAEELSTLCDAEI	:	43		
PvSVPb1	:	-----	MARKKTIAMKKIENINAROVTF	SKRRKGLFKKAEELSTLCDAEI	:	43		
GmSVPb5	:	-----	MVRKKTIPIKKIDNVITAROVTF	SKRRSGLFKKARELSILCDAEI	:	43		
GmSVPb6	:	-----	MVRKKTIPIKKIDNVITAROVTF	SKRRSGLFKKARELSILCDAEI	:	43		
PvSVPb3	:	-----	MVRKKTIPIKKIVNVITAROVTF	SKRRSGLFKKARELSILCDAEI	:	43		
AtAGL15	:	-----	MGRGKLEIKRIENANSROVTF	SKRRSGLFKKARELSVLCDAEV	:	43		
AtMAF2	:	-----	MGRKKVEIKRIENKSSROVTF	SKRRNGLIEKARQLSILCESSI	:	43		
AtMAF3	:	-----	MGRKKVEIKRIENKSSROVTF	SKRRKGLIEKARQLSILCESSI	:	43		
AtMAF1	:	-----	MGRKKLEIKRIENKSSROVTF	SKRRNGLIDKARQLSILCESSV	:	43		
AtMAF5	:	-----	MGRRRVEIKRIENKSSROVTF	CKRRNGLMEKARQLSILGSSV	:	43		
AtMAF4	:	-----	MGRKKVEIKRIENKSSROVTF	CKRRNGLMEKARQLSILCESSV	:	43		
AtFLC	:	-----	MGRKKLEIKRIENKSSROVTF	SKRRNGLIEKARQLSVLCDAEV	:	43		
AtAGL18	:	-----	MGRGRIEIKKIENINSROVTF	SKRRNGLIKKAKELSVLCDAEV	:	43		
AtAGL17	:	-----	MGRGKIVIQKIDDSSTROVTF	SKRRKGLIKKAKELSVLCDAEV	:	43		
AtAGL21	:	-----	MGRGKIVIQKIDDSSTROVTF	SKRRKGLIKKAKELSVLCDAEV	:	43		
AtAGL16	:	-----	MGRGKIAIKRINNSTROVTF	SKRRNGLIKKAKELSVLCDAEV	:	43		
AtANR1	:	-----	MGRGKIVIRRIDNSTROVTF	SKRRSGLFKKAKELSVLCDAEV	:	43		
AtAGL14	:	-----	MVRGKTEMKRIENATSROVTF	SKRRNGLLKAFELSVLCDAEV	:	43		
AtAGL19	:	-----	MVRGKTEMKRIENATSROVTF	SKRRNGLLKAFELSVLCDAEV	:	43		
AtSOC1	:	-----	MVRGKTEMKRIENATSROVTF	SKRRNGLLKAFELSVLCDAEV	:	43		
AtFYF	:	-----	MVRGKTEMKRIENATSROVTF	SKRRNGLLKAYELSVLCDAEV	:	43		
AtAGL71	:	-----	MVRGKIEIKKIENVTROVTF	SKRRSGLFKKAEHLSVLCDAEV	:	43		
AtAGL72	:	-----	MVRGKIEIKKIENVTROVTF	SKRRSGLFKKAEHLSVLCDAEV	:	43		
AtSHP1	:	MEEGGSSHDAESS--KK	LGRGKLEIKRIENTTNROVTF	CKRRNGLLKAYELSVLCDAEV	:	58		
AtSHP2	:	MEGGASNEVAESS--KK	LGRGKLEIKRIENTTNROVTF	CKRRNGLLKAYELSVLCDAEV	:	58		
AtAG	:	-MAYQSELGGDSSPLRKSG	RKLEIKRIENTTNROVTF	CKRRNGLLKAYELSVLCDAEV	:	59		
AtSTK	:	-----	MGRGKLEIKRIENTTNROVTF	CKRRNGLLKAYELSVLCDAEV	:	43		
AtXAL1	:	-----	MARGKIQLKRIENPVHROVTF	CKRRRTGLIKKAKELSVLCDAEI	:	43		
AtSEP1	:	-----	MGRGRVELKRIENKINROVTF	AKRRNGLLKAYELSVLCDAEV	:	43		
AtSEP2	:	-----	MGRGRVELKRIENKINROVTF	AKRRNGLLKAYELSVLCDAEV	:	43		
AtSEP3	:	-----	MGRGRVELKRIENKINROVTF	AKRRNGLLKAYELSVLCDAEV	:	43		
AtSEP4	:	-----	MGRGKVELKRIENKINROVTF	AKRRNGLLKAYELSVLCDAEI	:	43		
AtAGL6	:	-----	MGRGRVEMKRIENKINROVTF	SKRRNGLLKAYELSVLCDAEV	:	43		
AtAGL13	:	-----	MGRGKVEVKRIENKITROVTF	SKRRSGLLKAYELSVLCDAEV	:	43		
AtAP1	:	-----	MGRGRVOLKRIENKINROVTF	SKRRAGLLKAEHLSVLCDAEV	:	43		
AtCAL	:	-----	MGRGRVELKRIENKINROVTF	SKRRRTGLLKAEHLSVLCDAEV	:	43		
AtFUL	:	-----	MGRGRVOLKRIENKINROVTF	SKRRSGLLKAEHLSVLCDAEV	:	43		
AtAGL79	:	-----	MGRGRVOLKRIENKINROVTF	SKRRRTGLVKKAEHLSVLCDAEV	:	43		
AtAP3	:	-----	MARGKIQIKRIENQTNROVTF	SKRRNGLFKKAEHLSVLCDAEV	:	43		
AtAGL63	:	-----	MRRGKRVIKKIEEKIKROVTF	AKRRKSLIKKAYELSVLCDAEV	:	43		
AtAGL66	:	-----	MGRVKLEIKRIENTTNROVTF	SKRRNGLIKKAYELSVLCDAEI	:	43		

		*	80	*	100	*	120	
AtPI	:	ALIIIFASNGK	MIDYCCPSMD	GAMLDQYQKLSGK	-----	-----	-----	: 77
AtTT16	:	GLIVFSATGKLSEFC	SEQNR	PQLIDRYLHTNGL	-----	-----	-----	: 77
MtSVPa1	:	ALIIIFSSTGKLF	EYSN--LSMREIL	ERHHLHSK	-----	-----	-----	: 74
MtSVPa2	:	ALIIIFSSTGKLF	EYSN--LSMREIL	ERHHLHSK	-----	-----	-----	: 74
PvSVPa	:	ALIIIFSSTGKLF	EYSN--LSMREIL	ERHHLHSK	-----	-----	-----	: 74
GmSVPa2	:	ALIIIFSSTGKLF	EYSS--SSMKEIL	ERHHLHSK	-----	-----	-----	: 74
GmSVPa1	:	ALIIIFSSTGKLF	EYSS--SSMKEIL	ERHHLHSK	-----	-----	-----	: 74
PvSVPa	:	ALIIIFSSTGKLF	EYSN--SSMKEIL	ERHHLHSK	-----	-----	-----	: 74
LjSVPa	:	ALVVFSSTGKLF	EYSN--LSMKEIL	ERHHLHSK	-----	-----	-----	: 74
AtSVP	:	ALIIIFSSTGKLF	EEFC--SSMKEV	LEHNLQSK	-----	-----	-----	: 74
AtAGL24	:	ALIIIFSATGKL	EEFS--SRMRDIL	GRYSLHAS	-----	-----	-----	: 74
PvSVPc	:	GLIIFSTTGKLF	YEHCS--SSMKDII	TRYNQHSK	-----	-----	-----	: 74
MtSVPc	:	GLVIFSTTGKLF	EYAS--SNMKDII	TRYGQOSH	-----	-----	-----	: 74
LjSVPc	:	GLIVFSATEKLF	EYAS--SSVKSII	TERNQHIQ	-----	-----	-----	: 74
GmSVPc1	:	GLIVFSSTGKLF	EDYSS--SSMNDIV	TYSTHSH	-----	-----	-----	: 74
GmSVPc2	:	GLIVFSSTGKLF	EDYSN--ASMNDII	TYINTHSH	-----	-----	-----	: 74
PvSVPc	:	GLIVFSSTGKLF	EYSS--SSMNDII	TYINTHSP	-----	-----	-----	: 74
PvSVPb	:	ALMVFSATNKL	EYAS--SSMQQVI	ERNGCSE	-----	-----	-----	: 74
MtSVPb	:	ALMVFSATSKL	EYAS--SSMQQVI	ERNGYSA	-----	-----	-----	: 74
GmSVPb1	:	ALIVFSATSKL	EYAS--SSMHQVI	ERDRYSA	-----	-----	-----	: 74
GmSVPb2	:	ALIVFSATSKL	EYAS--SSMHQVI	ERDRSHA	-----	-----	-----	: 74
PvSVPb2	:	ALIVFSATSKL	EDYAS--SSMQQVI	ERDRHSA	-----	-----	-----	: 74
LjSVPb	:	ALIVFSATNKL	EYAS--SSMQKV	IEREQCSG	-----	-----	-----	: 74
GmSVPb3	:	ALIVFSSTGKL	EYAS--SSMQQVI	ERDRHSG	-----	-----	-----	: 74
GmSVPb4	:	ALIVFSATGKL	EYAS--SSMQQVI	ERNQHSG	-----	-----	-----	: 74
PvSVPb1	:	ALIVFSATSKL	EYAT--SSMQQVI	ERNRHSG	-----	-----	-----	: 74
GmSVPb5	:	ALIVFSPGKLF	EDYGS--SSMQKV	IERHILRSEL	-----	-----	-----	: 75
GmSVPb6	:	ALIVFSPGKLF	EDYAS--SSMQKV	IERHILWSEL	-----	-----	-----	: 75
PvSVPb3	:	ALMVFSPGKLF	EDYAS--SSMQK	ITERHILRSEF	-----	-----	-----	: 75
AtAGL15	:	AVIVFSKSGKL	EYSS--TGMKQTI	SRYGNGHQS	-----	-----	-----	: 75
AtMAF2	:	AVLVVSGSGKL	YKSASG--DNMSKII	DRYEIHHAD	-----	-----	-----	: 76
AtMAF3	:	AVVAVSGSGKL	YDSASG--DNMSKII	DRYEIHHAD	-----	-----	-----	: 76
AtMAF1	:	AVVVVSASGKL	YDSSSG--DDMSKII	DRYEQHAD	-----	-----	-----	: 76
AtMAF5	:	ALFIVSSTGKL	YNSSSG--DSMAKII	SERFIQAD	-----	-----	-----	: 76
AtMAF4	:	ALIIISATGR	LYSFSSG--DSMAKII	SERYEQAD	-----	-----	-----	: 76
AtFLC	:	ALLVVSASGKL	YSFSSG--DNLVKII	DRYQKQHAD	-----	-----	-----	: 76
AtAGL18	:	ALIIIFSSTGKI	YDFESS--VCEQI	LSRYGYTTAS	-----	-----	-----	: 75
AtAGL17	:	CLIIIFSTGKL	YDFAS--SSVKSTI	ERFNTAKME	-----	-----	-----	: 75
AtAGL21	:	GLIIFSTGKL	YDFAS--SSMKSVI	DRYNKSIE	-----	-----	-----	: 75
AtAGL16	:	GVIIIFSSTGR	LYDFESS--SSMKSVI	ERYSDAKGE	-----	-----	-----	: 75
AtANR1	:	GVIIIFSSTGKL	YDYASN--SSVKTI	TERYNRVKEE	-----	-----	-----	: 76
AtAGL14	:	ALIIIFSPRGKL	YEFSSS--SSPKT	VERYQKRIQD	-----	-----	-----	: 76
AtAGL19	:	ALVIFSPRSKL	YEFSSS--S	LAATIER	YQORRIKE	-----	-----	: 75
AtSOC1	:	SLIIIFSPKGL	YEFASS--N	QDTH	DRYLRHTKD	-----	-----	: 75
AtFYF	:	SLIIIFSQRGR	LYEFSSS--D	MQKTI	ERYRKYTKD	-----	-----	: 75
AtAGL71	:	AAIVFSQSGRL	HEYSSS--Q	MEKII	DRYGFKSNA	-----	-----	: 75
AtAGL72	:	AAMIFSQKGR	LYEFASS--D	LRNTI	KRYAEYKRE	-----	-----	: 75
AtSHP1	:	ALVIFSTRGR	LYEYANN--SVRG	TIER	YKKACSD	-----	-----	: 90
AtSHP2	:	ALVIFSTRGR	LYEYANN--SVRG	TIER	YKKACSD	-----	-----	: 90
AtAG	:	ALIVFSSRGR	LYEYSNN--SVKG	TIER	YKKAISD	-----	-----	: 91
AtSTK	:	ALIVFSTRGR	LYEYANN--N	IRSTI	ERYKKACSD	-----	-----	: 75
AtXAL1	:	GVVIFSPQKLF	ELATKG--TMEGM	IDRYMKCTGG	-----	-----	-----	: 76
AtSEP1	:	ALIIIFSNRGKL	YEFCS--SNMLKT	IDRYQKCSYG	-----	-----	-----	: 76
AtSEP2	:	SLIVFSNRGKL	YEFCS--SNMLKT	IDRYQKCSYG	-----	-----	-----	: 76
AtSEP3	:	ALIIIFSNRGKL	YEFCS--SSMLRT	IDRYQKCNIG	-----	-----	-----	: 76
AtSEP4	:	ALLIFSNRGKL	YEFCS--SPSG	MARTV	DRYRKHSYA	-----	-----	: 77
AtAGL6	:	ALIIIFSRGKL	YEFGSV--G	HESTI	ERYNRCYNC	-----	-----	: 75
AtAGL13	:	SLIIIFSTGGKL	YEFNSV--GVGR	TIER	YRCKDN	-----	-----	: 75
AtAP1	:	ALVVFSHKGKL	EYSTD--SCMEK	ILERYERYSYA	-----	-----	-----	: 76
AtCAL	:	SLIVFSHKGKL	EYSSE--SCMEK	VLERYERYSYA	-----	-----	-----	: 76
AtFUL	:	ALIVFSSKGKL	EYSTD--SCMER	ILERYDRYLYS	-----	-----	-----	: 76
AtAGL79	:	ALIVFSPKGL	EYSAG--SSMER	ILDRYERSAYA	-----	-----	-----	: 76
AtAP3	:	SIIMFSSSNKL	HEYISPNTTTKE	IVDLYQ	TISDV	-----	-----	: 77
AtAGL63	:	GLIIFSHSNRL	YDFCSNSTS	ENLIMRYQKEKEG	-----	-----	-----	: 77
AtAGL66	:	ALLMFSPSDRL	SLFSGK--TR	EDVFS	EYINLS	DQERENALVFPDQSRPDFQSK	EYLLRT	: 102

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      *           140           *           160           *           180
AtPI      : -----KLWDAK--HENLSNEIDRIK-----KENDSLQLELRHKGEDTQSINLKN : 120
AtTT16    : -----RLPDHDDQQLHHEMELLR-----RETCLNELRLRPFHGHDIASIPNE : 122
MtSVPa1   : -----NLAKLEEPSLE--LQLVENSNCSSL--SKEVAQKSHQLRQVRGEDIQGLSLEE : 123
MtSVPa2   : -----NLAKLEEPSLE--LQLVENSNCSSL--SMEVSKKSHQLRQVRGEDIQGLNVEE : 123
PsSVPa    : -----NLAKMEEPSLE--LQLVENSNCSSL--NKEIAEKSHQLRQVRGEDIQGMNVEQ : 123
GmSVPa2   : -----NLARMEQPSLE--LQLVENSNCSSL--SKEVAEKSHQLRQVRGEDIQGLNIEE : 123
GmSVPa1   : -----NLARMEQPSLE--LQLVENSNCSSL--SKEVAEKSHQLRQVRGEDIQGLNIEE : 123
PvSVPa    : -----NLAKMDQPSLE--LQLVENSNCSSL--SKEVAEKSHLLRQVRGEDIQGLNIEE : 123
LjSVPa    : -----NLAKLEQPSLE--LQLVENSNCSSL--NKEVAEQSRLRLQVRGEDIQGLNIEE : 123
AtSVP     : -----NLEKLDQPSLE--LQLVENSNCSSL--SKEIADKSHRLRQVRGEDIQGLDIEE : 123
AtAGL24   : -----NINKLMDPPST--HLRLENCNLSRL--SKEVEDKTKQLRKVRGEDIQGLNLEE : 123
PsSVPc    : -----QINKLDKT-LP--LQVVK-SMSAEL--QKEFADKTQQLRGLKGEFEGNLDG : 121
MtSVPc    : -----HITKLDKP-LQ--VQVEK-NMPAEL--NKEVADRTQQLRGMKSEDFEGNLEG : 121
LjSVPc    : -----GIRGMDRF-LF--PQGEDYSNLAEL--HKEVANRTEQLRRMTGEGEDFDD : 122
GmSVPc1   : -----GINKLDKPSLE--LQLEA-SNSAKL--SKEIADRTQELSWKGGDDIQGLGNE : 122
GmSVPc2   : -----GVNKLKGPSLE--LQLEA-SNSAKF--SKEIVDRTQELCWLKDDDIQGLGNE : 122
PvSVPc    : -----GINKLDRPSLE--LQLEA-SNSAKL--SKEIADRTQELSWKGGDDIQGLGNE : 122
PsSVPb    : -----NHRLLMDRPSST--DQFQVESESS-DTL--HKKLEDKSHRLRQVRGEDIQGLTVQE : 123
MtSVPb    : -----NHRLLDYPSTDDQLQVESDSNRDTL--RKKLEDKSHRLRQVRGEDIQGLTVQE : 125
GmSVPb1   : -----IHRL-DRPSIE--LQIESDSN-NIL--RKKVEDKTRELRLQVRGEDIQGLTLQE : 121
GmSVPb2   : -----MNRL-DRPSIE--LQIENDSN-EIL--RKKVEDKNRELRLQVRGEDIQGLTLQE : 121
PvSVPb2   : -----MHRSDRPSIE--LQIENDSN-DIL--RKKVEDKSHRLRQVRGEDIQGLTLQE : 121
LjSVPb    : -----IHRL-EHLPIEQFMQFESDSN-DTP--RKKVEEKTHELRLQVRGEDIQGLTLHQ : 123
GmSVPb3   : -----IQGL-VNPSIG--QQLGSDSL-GIL--RKEIEHKTNEMSQVRGEDIQGLTIKE : 121
GmSVPb4   : -----IQGL-DNPSIG--QQLGSDSF-GMLPL--RKEIEDKTNELSQVRGEDIQGLTIKE : 123
PvSVPb1   : -----VQGS-DISSTA--QQLGSESF-DML--HKEIVDKTHELRLQVRGEDIQGLTIQE : 121
GmSVPb5   : -----NLEKLDQSCPT--EQVRCNYADL--NKEFADRTREMRLQVRGEDIQGLTLRE : 122
GmSVPb6   : -----NLEKLDQSCPT--EQLRCNYADL--NKEFGDRIREMRLQVRGEDIQGLALRE : 122
PvSVPb3   : -----NQDKLDQLPPT--EQIRSSHAYL--KKELEDKSHRLRQVRGEDIQGLSFKE : 122
AtAGL15   : -----SASKAEEDCAE-----VDIL--KDQLSKLQEKHLQVRGEDIQGLTFKE : 116
AtMAF2    : -----ELEALDLAEKTRNYLPLK-----ELLEIVQSKLEESNVDNASVDT : 116
AtMAF3    : -----ELKALDLAEKIRNYLPHK-----ELLEIVQSKLEESNVDNVSVDS : 116
AtMAF1    : -----ELRALDLAEKIQNYLPHK-----ELLEIVQSKLEESNVDNVSVDS : 116
AtMAF5    : -----DPETLDLEDKTDYLSHK-----ELLEIVQSKLEESNVDNVSVDS : 116
AtMAF4    : -----DLKTLDLAEKTLNYLSHK-----ELLEIVQSKLEESNVDNVSVDS : 116
AtFLC     : -----DLKALDHQSKALNYGSHY-----ELLEIVQSKLEESNVDNVSVDS : 116
AtAGL18   : -----TEHKQOREHQLLICASHGNEAVLRNDDSMKGELERLQLAIERTKKGELEGMSFPD : 130
AtAGL17   : -----EQELMNPASEVKFWQREAEATLRQEL-----HSLQENYRQRTGEBINGLSVKE : 122
AtAGL21   : -----QQQLLNPASEVKFWQREAAVLRQEL-----HALQENHRRQMGELINGLSVNE : 122
AtAGL16   : -----TSENDPASEIQFWQKEAAAILKRQL-----HNLQENHRRQMGELINGLSVNE : 122
AtANR1    : -----QHQLLNHASEIKFWQREAVASLQQQL-----QYLQECHRKLVGELINGLSVNE : 123
AtAGL14   : -----LG--SNHNRNDNSQSKDETYGLAR-----KIEHLEISTRKMMGELINGLSVNE : 123
AtAGL19   : -----IG--NNHNRNDNSQSKDETYGLAR-----KIEHLEISTRKMMGELINGLSVNE : 123
AtSOC1    : -----RVS-TKPVSEENMQHLKYEAANMMK-----KIEQLEASKRKLVGELINGLSVNE : 123
AtFYF     : -----HET-SNHDSQIHLQQLKQEAASHMIT-----KIELLEFHKRKLVGELINGLSVNE : 123
AtAGL71   : -----FYVAERPQVERYLQELKMEIDRMVK-----KIDLELVHHRKLVGELINGLSVNE : 124
AtAGL72   : -----YFVAETHPIEQYVQGLKKEMVTMVK-----KIEVLEVHNRKMMGELINGLSVNE : 124
AtSHP1    : -----AVNPPSVTEANTQYYQ-----QEASKLRQIRDIQNSNRHIVGESLGSINFE : 138
AtSHP2    : -----AVNPPSVTEANTQYYQ-----QEASKLRQIRDIQNSNRHIVGESLGSINFE : 138
AtAG      : -----NSNTGSVAEINAQYYQ-----QESAKLRQIISIQNSNRHIVGESLGSINFE : 139
AtSTK     : -----STNTSTVQEIINAQYYQ-----QESAKLRQIISIQNSNRHIVGESLGSINFE : 123
AtXAL1    : -----GRGSSATFTAQEQLOPPNLDPKDEINVLRQELIEMLQKGISYFVGDDGAMNLEE : 131
AtSEP1    : -----SIEVNN--KPAKELN-----SYREYLKLGKRYENLQRRQNLGEDIQGLINSKE : 124
AtSEP2    : -----SIEVNN--KPAKELN-----SYREYLKLGKRYENLQRRQNLGEDIQGLINSKE : 124
AtSEP3    : -----APEPNVPSREALAVELS--SQEYLKLGKRYDALQRTQNRNLGEDIQGLINSKE : 127
AtSEP4    : -----TMDPNQ--SAKDLQD--KYQDYLLKLSRVEILQHSQRHLGEEISEMDVNE : 124
AtAGL6    : -----SLSNNK--PEETQS--WCQEVTKLKSKEYESLVRTNRNLGEDIQGLINSKE : 122
AtAGL13   : -----LLDNDT--LEDT-QG--LRQEVTKLKSKEYESLRLTHRNIVGEDIQGLINSKE : 121
AtAP1     : -----ERQLIAP-ESDVN--TN--WSMEYNRLKAKIELLERNQRHYLGEDIQGLINSKE : 124
AtCAL     : -----ERQLIAP-DSHVNAQTN--WSMEYSRLKAKIELLERNQRHYLGEDIQGLINSKE : 126
AtFUL     : -----DKQLVG--RDVSQSEN--WVLEHAKLKARVEVLEKNKRNFMGEDIQGLINSKE : 124
AtAGL79   : -----GQDIPTP--NLDSQGE--CSTECSKLLRMIDVLQSRSLRHGEEDVDGLSIRD : 124
AtAP3     : -----DVWATQYERMQETKRKLLETN-----RNLRTQIKQLRGLGEDIQGLINSKE : 120
AtAGL63   : -----QTTAEHSFHSQCSDCVKTKESMMR-----ELENLKLNLQLYDCHGILNTYDE : 126
AtAGL66   : LQQLKAENDIALQLTNPTAINSVDVELEHEVYKLLQQLLMAEEELRKYEPDTRFTTME : 162

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      *           200           *           220           *           240
AtPI      : LMAVEHAIEHGIDKVRDHQMEIILISK---RRNEKMAEEQRQLTFQLQQ----- : 166
AtTT16    : LDGLERQLEHSVLKVRERKNELMQQOLENLSRKRMRMEEDNNMYRWLHE----- : 172
MtSVPa1   : LQQLKSLEIGLGRVIETKGEKIMMETNELQTKGRQIMEENNRKRVHSG----- : 173
MtSVPa2   : LQQLERSLEIGLGRVIENKGEKIMMETNDLQRKGRQIMEENDRIKHHVAG----- : 173
PsSVPa    : LQHLERSLEIGLGRVIENKGEKIMMETQHLQRKGRQIMEENDRIKRVHTG----- : 173
GmSVPa2   : LQQLERSLETGLGRVIEKKGEKIMSETDILQRKGMLIMEENERIKRVHAG----- : 173
GmSVPa1   : LQQLMSLETGLGRVIEKKGEKIMSETADLQRKGMLIMEENERIKRVHAG----- : 173
PvSVPa    : LQQLERSLETGLSRVIEKKGEKIMNETDILQRKGMVIMEENERIKHHVAG----- : 173
LjSVPa    : LQQLERSLETGLGRVIEKKGEKIMNETINGLQIKGKQIMEENERIKRVHAG----- : 173
AtSVP     : LQQLERKALETGLTRVIEKSDKIMSETSELQKKGMLMDENKRIRQQGTQLTEENERLGM : 183
AtAGL24   : LQRLKLLSEGLSRVSEKKGEVMSQIFSLEKRGSEIVDENKRIRDKLET----- : 173
PsSVPc    : LQQLERTLETGLKSVIEMKEKIMNETGALQIKSIEQEEENNHKQKMA----- : 170
MtSVPc    : LQQLKSLESGLKRVIEMKEKILNETKALRMKEIMLEENKHLKQKMA----- : 170
LjSVPc    : LLELEKTLQSGIKRVIELKEKIMDETAVQKKEVA----- : 158
GmSVPc1   : LQQLKTLIEIGLDRVTDIKENQIMSQISELQKKGILIEENKHLTKKLAEKEKE----- : 176
GmSVPc2   : LKQFETLTIEIGLDRVIEIKKQIMSQISELQKKGILIEENKHLTKKLIVETEME----- : 176
PvSVPc    : LQQLKTLSEGLDRVIEIKKQIMSQISELQKKGILIEENKHLTKKLIVETEME----- : 176
PsSVPb    : LQKLEELLRRGTSNVSKKDEKFIQENTLKRKEVEIIRENQRIKHHVVP----- : 172
MtSVPb    : LQKLEVLKRSISNVSKIKDEMFRDIDTLKRKEVEIMEENNRKHHVVP----- : 174
GmSVPb1   : LQKLEELLKRSITNVSKVKDAKFMQETSTFKRKGVETMEENQRKKQ-VP----- : 169
GmSVPb2   : LHKLEELLKRGITNVSKVKDEKFMQETSTFKRKGVETMEENQRKKQVVP----- : 170
PvSVPb2   : LQKLEELLKRGTSNVSKLDEKFMQETSAIKRKGVETMEENQRKK-VP----- : 169
LjSVPb    : LQKLEVLKRSIASVSRVKDEKFMQETSTFKRKGVETIEENQRKKQ-VP----- : 171
GmSVPb3   : LQKVEELLQRWTTISKIKDEKFIQENTLKRKEVEIMEENQRKKQ----- : 167
GmSVPb4   : LQKLEDILQRWTTISKTDEKFIQENTLKRKEVEIMEENQRKKQ----- : 169
PvSVPb4   : LHKLEELLRRWTTISKIKDEKFIQENTLKRKEVEIMEENQRKKQ----- : 167
GmSVPb5   : LQKLEERLDSSINRVYKARVENFIKEIGILKEKGKKIMEDNMLIKQMTKL----- : 172
GmSVPb6   : LQKLEERLVSSINRVYKAKVENFTREIDILKQKGNKIMEDNRLMKQRIK----- : 171
PvSVPb3   : LQKLEGRLESSINCVYKARVQNFIRDINILTKQKQNKIMEDNRLKQRTIS----- : 171
AtAGL15   : LQSLQQLYHAFITVREKREKRLTNQIEESRLKEQRAELENETIRROVQELR----- : 168
AtMAF2    : LISLBEQLETAISVTRARKTELMGGEVKSLOKTENLREENQTLAS----- : 162
AtMAF3    : LISMEQLETAISVIRAKKTELMMEDVKSLEFEKLEIEENQTLAS----- : 162
AtMAF1    : LISLBEQLETAISVSRARKAELMMEYIESLKEKEKLREENQVLAS----- : 162
AtMAF5    : LISMEQKLSAISVIRAKTELMELVKNLQDKLEKLKEKNKVLAS----- : 162
AtMAF4    : LKSLBEQKLTASVTRARKTELMMLVKTHQKEKLREENQSLTNQLI----- : 165
AtFLC     : LVQLEHLETAISVTRAKKTELMKLVENKEKEKMLEENQVLAS----- : 162
AtAGL18   : LISLBNQLNESLHSVKDQKTQILNLQIERSRQIEKKALEENQILRQVE----- : 179
AtAGL17   : LQNIQSLESTGRIRMKREQLITNETKEITRKNLWHHENLELSRKVQRIHQEN---VE : 179
AtAGL21   : LNSLENQIEISIRGRMRKEQLITQETQELSQKRNLIHQENLDLSRKVQRIHQEN---VE : 179
AtAGL16   : LQNLBNQLLESLRGVRMKKQDMLIEETQVILNREGNLIHQENLDLHKKNVNLMHQQN---ME : 179
AtANR1    : LQNLBNQLVTSIKGVRLLKQDQMLTNETREINRKQGIIQKENHELQNIQVDIRKEN---IK : 180
AtAGL14   : LQQLBNQLDRSITMKIRAKKYQLRETEKEKKAERNVKENKDLKEKWLG----- : 173
AtAGL19   : LQQLBNQLDRSISIRAKKYQLRETEKEKKAERNVKENKDLKEKWLG----- : 172
AtSOC1    : LQQIEQQLKSQKCIARAKTQVFKEQTEQLKQKEKALAAENEKLEKWSG----- : 173
AtFYF     : LQEIDSQLQRSIGKVRERKALFKEQLEKLKAKEKQLEENVKLHQKN----- : 171
AtAGL71   : LQEIDTQIEKSRIVRSRK-----VQTLGHK-----YIYNI----- : 155
AtAGL72   : LSEIATQIEKSIHMYRLRKAKLYEDELQKIKAKEREIKDERVRLSLKTI----- : 174
AtSHP1    : LKNLEGRLEKGISRVRSKKNELLVAETIYMQRKEMEQHNNMYLRAKIAEGA----- : 190
AtSHP2    : LKNLESRLKGISRVRSKKNELLVAETIYMQRKIEIQNDNMYLRSKITERT----- : 190
AtAG      : LRNLEGRLESTIRIRSKKNELLFSEIDYMQKREVDLHNDNQLIRAKIAENE----- : 191
AtSTK     : LKQVENRLEKASIRRSKKNELLVETENAKREIEIDNENIYLRTKVAEVE----- : 175
AtXAL1    : LLLLEKHLEYWISQIRSAKMDVMLQETQSLRNREGVAKNTNKYLEKLEEN----- : 182
AtSEP1    : LEQLERQLDGSIKQVRSIKTQYMLDQISDLQNKQEMLETNRALAMKLD----- : 173
AtSEP2    : LEQLERQLDGSIKQVRCIKTQYMLDQISDLQCKEHIILLDANRALSMKLE----- : 173
AtSEP3    : LESLERQLDSSIKQIRALRTQFMLDQINDIQSKERMLETNKLRLRLA----- : 176
AtSEP4    : LEHLERQVDASIRQLRSTKARSMLDQISDLKTKKEEMLETNRDLRRKLE----- : 173
AtAGL6    : LQALERQLEAATATRQRKTQVMMEEMDLRKKERQLGDIKQLKIKFETEG----- : 174
AtAGL13   : LQTLERQLEGALSATRKQKTQVMMEQMEELRRKEREGDINN--KLKLETED----- : 171
AtAP1     : LQNLQQLDTAIKHITRKNQLMYESINELQKKEKALQEQNSMLSKQIK----- : 173
AtCAL     : LQNLQQLETAIKHISRKKNQLMNESINHLQRKEKEIQEENSMLTKQIK----- : 175
AtFUL     : LQSLHQLDAATKISRSRKQAMFESTSALQKQKDALQDHNSLLKKIK----- : 173
AtAGL79   : LQGVEMQLDTAKKTRSRKNQIMVESIAQLQKKEKEKELKKQLTKKAG----- : 173
AtAP3     : LRRLEDEMENTFVKLRKFKSLGNQLETTKKKNKSQQDIQKNLIEHELRAEDP----- : 175
AtAGL63   : LLSFLEHLESSIQHARARKSEFMHQQQQQQTDQKLGKGEKQGSSWEQL----- : 175
AtAGL66   : YETCEQQLMDTLTRVNRREHILSQDQLSSYEASALQQQSMGGPFNDVVG----- : 214

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	*	260	*	280	*	300	
AtPI	:	-----QEMAIASNARGMMMR-----		-----DHDGQFG-----			: 188
AtTT16	:	-----HRAAMEFQQAGIDTKPGEYQQFIEQLQCYKPGYQQF-----					: 209
MtSVPa1	:	-MFN-GKMFGGVESEN-MVTEEGQSSESVT-NVYNS-TGPPQDYESSDTSCLKG-----					: 222
MtSVPa2	:	-IIN-DRMVGGESEENENVVNEGQSSESVT-NVYNS-IGPPQDYESSDTSCLKG-----					: 223
PvSVPa	:	-MMNNGKIVGGVESEN-VVIEEGQSSESIT-NVYNS-IGPPQDYESSDTSCLKG-----					: 223
GmSVPa2	:	-IIN-QQRHGGAENSEN-FVMDEGQSSESVT-YVCNS-TGPPQDFESSDTSCLKG-----					: 222
GmSVPa1	:	-IIN-QQRHGGAENSEN-FVMDEGQSSESVT-YVCNS-TGLPQDYESSDTSCLKG-----					: 222
PvSVPa	:	-IVN-GERRGGAENEN-FVVDEGQSSESVT-YVCNS-TGPPQDYESSDTSCLKG-----					: 222
LjSVPa	:	-MISTGLMHGDTESL-LVMEEGHSSSESVT-NVCNSTTGPPLEDSSDTSCLKG-----					: 224
AtSVP	:	QICNNVHAHGAENSENAVYEEGQSSESIT-NAGNS-TGAPVDESSEDTSCLKG-----					: 235
AtAGL24	:	-----LERAKLTTLKEALETESVTNVSSYDSGTPLD-DDSDTSCLKG-----					: 215
PvSVPc	:	-MLFKGKCPPLGD-----LDVSCESMN-NVSCNSGSPLEDDSSDISCLKG-----					: 214
MtSVPc	:	-MLSMGKSPIFGDSIT--MQENVSAESMN-NVSSCNSGSPLEDDSSDTSCLKG-----					: 220
LjSVPc	:	-----SSDSMN-NVSCNSGSPLEDDSSSVTSLKG-----					: 187
GmSVPc1	:	AMLCCKAKIPFMDVSDKGIMQEEGVSLDSTN-NISSCISDPPLDGGSSDISLTG-----					: 229
GmSVPc2	:	AMLCPEPIPFMDLDKGIMQEEGVSLDSTN-SISSFINDPPEDDGSSNISLTG-----					: 229
PvSVPc	:	AMLRKAKMPFMMDSNMG-MQEEGVLLSTN-NVSSSISDPHVEDGSSDTSCLKG-----					: 228
PvSVPb	:	-----DLIIGQR-QQSLE-SVIRRSSYFLEDGGSDTSCLKG-----					: 207
MtSVPb	:	-----DLINVRW-QQSLE-TVISGSSFSLEDDG-SDTSLCLKG-----					: 208
GmSVPb1	:	-----SLIHASYRQSSE-SILSNSSNLPEDGG-SNTSLCLKG-----					: 204
GmSVPb2	:	-----SLIHVH--RQSSE-SILSNSSNLPEDGG-SDTSLCLKG-----					: 203
PvSVPb2	:	-----SLIPLH--RQSSE-SILSNSSNLPEDGG-SDTSLCLKG-----					: 202
LjSVPb	:	-----GLTQYQG-QQSLE-STISSSSYLLEEDG-SDTSLCLKG-----					: 205
GmSVPb3	:	-----SFVREQ--RQPYE-SFTCSSEFFPDGCGNSDTSCLKG-----					: 201
GmSVPb4	:	-----SFLQEQ--RQSYE-SFTCSSEFFPDGCGNSDTSCLKG-----					: 203
PvSVPb1	:	-----KLVPEQ--RQCYE-SITSSSDFPDNGPSDTSCLKG-----					: 201
GmSVPb5	:	-----PRNEICSVQRHEHEQGQLFDTSLTLG-----					: 198
GmSVPb6	:	-----PRNEICSVQRHEHEQGRSFDTSLTG-----					: 197
PvSVPb3	:	-----SRNEICSVQRHEHEQEQSFDTSLTG-----					: 197
AtAGL15	:	-----SFLPSFTHYVPSYIKCFALDPKNALINHDSKCSLQNTDSDTTLQLG-----					: 214
AtMAF2	:	-----QVTKTS-----LEANSS-VDTQ-----					: 178
AtMAF3	:	-----QVGKTF-LVIEGDRG-MSRENGSGNKVPE-----					: 190
AtMAF1	:	-----QMGKNTL-LATDDERG-MFPGSSSGNKIPE-----					: 190
AtMAF5	:	-----EVGKLKILETGDERAVMSPENSSGHSPPE-----					: 192
AtMAF4	:	-----KMGKMKKSVEAEDARA-MSPESSSDNKPE-----					: 194
AtFLC	:	-----QMENNHH-VGAEEAEME-MSAGQISDNLPV-----					: 190
AtAGL18	:	-----MLGRGSGPKVLNERPDQSSPEADPESSSSSEEDENDNEE					: 217
AtAGL17	:	LYKKAYG-----TSNTNGLGHHELVDVYESHQAQVRLQLSQPEQSHYKTSSNS-----					: 227
AtAGL21	:	LYKKAY-----MANTNGFTHREAVADDESHTQIRLQLSQPEHSDYDTPPRA-----					: 226
AtAGL16	:	LHEKVSEVEGVKIANKNLLTNGLDMR-DTSEHVLHQLSQPQH-DHETHSKA-----					: 230
AtANR1	:	LQKKVHG-----RTNAIEGNSSVDPIISNGTTTYAPPQLQLIQPAPREKSIKRLG-----					: 230
AtAGL14	:	-----QGRGIIIRISSSSSTSELDIDDNEMEVVTDLFIG-----					: 207
AtAGL19	:	-----MGTATIASSQSTLSSSEVNIDN-MEVETGLFIG-----					: 205
AtSOC1	:	-----HESEVWSNKNQESTGRGDEESSPSSEVETQLFIG-----					: 207
AtFYF	:	-----VINPWRGSSDQQQEKYKVIDLNLEVETDLFIG-----					: 204
AtAGL71	:	-----YTHVNDVIMSSL-VDLGA-----					: 172
AtAGL72	:	-----YTHLCQVGERPMGMPGSGSK---EKEDVETDLFIG-----					: 205
AtSHP1	:	-----RLNPDQQESSVIQGTTVYESGVSS-HDQSQHYN-RNYIP					: 227
AtSHP2	:	-----GLQ--QQESSVIHQGTTVYESGVTS-SHQSGQYN-RNYIA					: 225
AtAG	:	-----RNN---PSISLMPGGSNYEQLMPPPTQSQPFDSRNYFQ					: 227
AtSTK	:	-----RYQ--QHHHQMVGSEINAEALA---SRNYFAHSIMT					: 208
AtXAL1	:	-----NNSILDANFAVMETNYSYPLTMP-----					: 206
AtSEP1	:	-----DMIGVRSHHMGGGGGWEGGEQ-NVYAHHQAS					: 205
AtSEP2	:	-----DMIGVRHHHIGGG--WEGGDQQNIAYGHPQAS					: 204
AtSEP3	:	-----DGYQMPLQLNPNQ---EEVDHYGRHHHQQQHS					: 206
AtSEP4	:	-----DSDAALTQSFWS---SAAEQQQHQQQQGMSSYSQNP					: 209
AtAGL6	:	-----HAFKTFQDLWANSAAVAGDPNNSEFPVPSHPN-----					: 208
AtAGL13	:	-----HDFKGFQDLLLNPLVITAG---CSTDFSLQSTHQNY----					: 203
AtAP1	:	-----EREKILRAQQEQWDQGNQGHNMPPPLPPQQHQIQH-----					: 208
AtCAL	:	-----ERENILRTKQTQCEQLNRSVDDVP--QPQPFQHPH----					: 208
AtFUL	:	-----EREKKTGQEGQLVQCSNSSSVLLP-----					: 198
AtAGL79	:	-----EREDFQTQNLSHDLASLATPPFESPHLRRTISPP----					: 208
AtAP3	:	-----HYGLVDNGGDYDSVLGYQIEGSRAYALRFHQNHHPYPNHG----					: 216
AtAGL63	:	-----MWQAERQMMTCQRQKDPAPANEGGVFFLRWG-----					: 206
AtAGL66	:	-----GWLTEGPNEAHLFDASAHSAMYETLLQGSSSSSNQNNIMGESNVSNHNGDMFQ					: 268

		*	320	*	340	*	360	
AtPI	:	-----	YRVQPIQPN--LQEKIMSLVID-----					: 208
AtTT16	:	-----	LEQQQQQPN SVLQLATLPSEIDPTYNLQLAQP NLQNDPTAQND-----					: 252
MtSVPa1	:	-----	LPYAG-----					: 227
MtSVPa2	:	-----	LPYAG-----					: 228
PsSVPa	:	-----	LPYAG-----					: 228
GmSVPa2	:	-----	LPYSG-----					: 227
GmSVPa1	:	-----	LPYSG-----					: 227
PvSVPa	:	-----	LPYSG-----					: 227
LjSVPa	:	-----	LPY-----					: 227
AtSVP	:	-----	LPYGG-----					: 240
AtAGL24	:	-----	LPSWE-----					: 220
PsSVPc	:	-----	LPFP-----					: 218
MtSVPc	:	-----	LPFPN-----					: 225
LjSVPc	:	-----	LPFPN-----					: 192
GmSVPc1	:	-----	LPFSN-----					: 234
GmSVPc2	:	-----	LPFSN-----					: 234
PvSVPc	:	-----	LPFSK-----					: 233
PsSVPb	:	-----	LPFHN-----					: 212
MtSVPb	:	-----	LPFLK-----					: 213
GmSVPb1	:	-----	LP-----					: 206
GmSVPb2	:	-----	LP-----					: 205
PvSVPb2	:	-----	LP-----					: 204
LjSVPb	:	-----	LPLLHK-----					: 211
GmSVPb3	:	-----	LSLFE-----					: 206
GmSVPb4	:	-----	LSLFE-----					: 208
PvSVPb1	:	-----	LRLEF-----					: 206
GmSVPb5	:	-----	LPFPAGSK-----					: 206
GmSVPb6	:	-----	LSFPAGSKYRVSEQ-----					: 211
PvSVPb3	:	-----	LPFPSDSK-----					: 205
AtAGL15	:	-----	LPGEAHDRRTNEGERESPSSDSVTNTTSSETAERGDQSSLANSPP EAKRQRF					: 266
AtMAF2	:	-----	-----					: -
AtMAF3	:	-----	TL SLLK-----					: 196
AtMAF1	:	-----	TL PLLN-----					: 196
AtMAF5	:	-----	TL PLLK-----					: 198
AtMAF4	:	-----	TL LLLK-----					: 200
AtFLC	:	-----	TL PLLN-----					: 196
AtAGL18	:		HHS DTS LQLGLSSTGYCTKRKKPKIELVCDNSGSQV ASD-----					: 256
AtAGL17	:	-----	-----					: -
AtAGL21	:	-----	NE-----					: 228
AtAGL16	:	-----	IQLNYFSFIA-----					: 240
AtANR1	:	-----	LQLS-----					: 234
AtAGL14	:	-----	PPETRHFKKFPPSN-----					: 221
AtAGL19	:	-----	PPETRQSKKFPPQN-----					: 219
AtSOC1	:	-----	LPCSSRK-----					: 214
AtFYF	:	-----	LPNRNC-----					: 210
AtAGL71	:	-----	-----					: -
AtAGL72	:	-----	FLKNRP-----					: 211
AtSHP1	:	VN-----	-----LLEPNQQFSG-----QDQPPLQLV-----					: 248
AtSHP2	:	VN-----	-----LLEPNQNSSN-----QDQPPLQLV-----					: 246
AtAG	:	VA-----	-----ALQPNNHYS SAGRQDQTALQLV-----					: 252
AtSTK	:	AG-----	-----SGSGNGGSYSD---PDKKILHLG-----					: 230
AtXAL1	:	-----	-----EIFQF-----					: 211
AtSEP1	:	---	QGLYQPLECNPTLQMG--YDN P VCSEQITATTQAQAQQ--GNGYIPGWML-----					: 251
AtSEP2	:	---	QGLYQSLECDPTLQIG--YSH P VCSEQMAVTVQGQSQQ--GNGYIPGWML-----					: 250
AtSEP3	:	---	QAFFQPLECEPILQIG--YQG-----QDGMGAGPS--VNNYMLGWLPYDTNSI					: 251
AtSEP4	:		PIQEAGFFKPLQGNVALQMSSHYNHNPAN---ATNSATT SQN--VNGFFPGWMV-----					: 258
AtAGL6	:	-----	VLD CNTEPFLQIG-FQQHYVQGE GSSVSKSNVAG--ETNFVQGWVL-----					: 252
AtAGL13	:	-----	ISDCNLGYFLQIG-FQQHY-EQGE GSSVTKSNARSDAETNFVQ-----					: 244
AtAP1	:	-----	PYMLSHQSPFLN-----MGGLYQEDDPAMRRNDLELTLEPVYNCNLGCFA					: 255
AtCAL	:	-----	LYMIAHQTS PFLN-----MGGLYQEDQTAMRRNLDLTLEPIYNY-LGCYA					: 254
AtFUL	:	-----	QYCVTSSRDGFVE-----RVGGENG GASSLTEPN---SLLPAWMLRP TTN					: 241
AtAGL79	:	-----	PPPLSSGDS TQRD-----GVG--EVAAGTLIRRTN---ATLPHWMPQLTGE-					: 249
AtAP3	:	-----	LHAPSASDIITFHLLE-----					: 232
AtAGL63	:	-----	TTHRRSSPP-----					: 215
AtAGL66	:		EWAQAYNSTTAHN PSTLFPPMQHQHGLVVDPNIEEIEIPVMKKDAQADHEVSDYDIRMPQ					: 328

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AtPI      : ---- : -
AtTT16    : ---- : -
MtSVPa1   : ---- : -
MtSVPa2   : ---- : -
PsSVPa    : ---- : -
GmSVPa2   : ---- : -
GmSVPa1   : ---- : -
PvSVPa    : ---- : -
LjSVPa    : ---- : -
AtSVP     : ---- : -
AtAGL24   : ---- : -
PsSVPc    : ---- : -
MtSVPc    : ---- : -
LjSVPc    : ---- : -
GmSVPc1   : ---- : -
GmSVPc2   : ---- : -
PvSVPc    : ---- : -
PsSVPb    : ---- : -
MtSVPb    : ---- : -
GmSVPb1   : ---- : -
GmSVPb2   : ---- : -
PvSVPb2   : ---- : -
LjSVPb    : ---- : -
GmSVPb3   : ---- : -
GmSVPb4   : ---- : -
PvSVPb1   : ---- : -
GmSVPb5   : ---- : -
GmSVPb6   : ---- : -
PvSVPb3   : ---- : -
AtAGL15   : SV-- : 268
AtMAF2    : ---- : -
AtMAF3    : ---- : -
AtMAF1    : ---- : -
AtMAF5    : ---- : -
AtMAF4    : ---- : -
AtFLC     : ---- : -
AtAGL18   : ---- : -
AtAGL17   : ---- : -
AtAGL21   : ---- : -
AtAGL16   : ---- : -
AtANR1    : ---- : -
AtAGL14   : ---- : -
AtAGL19   : ---- : -
AtSOC1    : ---- : -
AtFYF     : ---- : -
AtAGL71   : ---- : -
AtAGL72   : ---- : -
AtSHP1    : ---- : -
AtSHP2    : ---- : -
AtAG      : ---- : -
AtSTK     : ---- : -
AtXAL1    : ---- : -
AtSEP1    : ---- : -
AtSEP2    : ---- : -
AtSEP3    : ---- : -
AtSEP4    : ---- : -
AtAGL6    : ---- : -
AtAGL13   : ---- : -
AtAP1     : A--- : 256
AtCAL     : A--- : 255
AtFUL     : E--- : 242
AtAGL79   : ---- : -
AtAP3     : ---- : -
AtAGL63   : ---- : -
AtAGL66   : LSSQ : 332

```

### Sequence alignment for Figure 7.2

Alignment for phylogenetic neighbour-joining tree of predicted protein sequence for *StMADS11* subfamily genes from legumes and other angiosperm species shown in Figure 7.2. Full-length predicted protein sequences were aligned with ClustalX (Thompson et al., 1997) and manually adjusted with GeneDoc (Nicholas and Nicholas, 1997). Shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Full sequence details are given in Table A6.1, above.

		*	20	*	40	*	60	
PsSVPc	:	---	MAR	---	QKIKIKKIDNATARQVTF	SKRRRGIFKKAE	ELSVL	: 38
MtSVPc	:	---	MAR	---	QKIKIKKIDNATARQVTF	SKRRRGIFKKAE	ELSVL	: 38
LjSVPc	:	---	MAR	---	QKIKIKKIDNATARQVTF	SKRRRGIFKKAE	ELSVL	: 38
GmSVPc1	:	---	MTR	---	TRIKIKKIDNITARQVTF	SKRRRGLEFKKA	ELSVL	: 38
GmSVPc2	:	---	MTR	---	AKIKIKKIDNITARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PvSVPc	:	---	MTR	---	AKIKIKKIDNITARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PtSVPb	:	---	MTR	---	KKIQIKKIDNISSRQVTF	SKRRKGLFKKA	QELSTL	: 38
MtSVPb	:	---	MTR	---	KKIQIKKIDNISSRQVTF	SKRRKGLFKKA	QELSTL	: 38
GmSVPb1	:	---	MTR	---	KRIQIKKIDNISSRQVTF	SKRRKGLFKKA	QELSTL	: 38
GmSVPb2	:	---	MTR	---	KRIQIKKIDNISSRQVTF	SKRRKGLFKKA	QELSTL	: 38
PvSVPb2	:	---	MTR	---	KRIQIKKIDNISSRQVTF	SKRRKGLFKKA	QELSTL	: 38
LjSVPb	:	---	MTR	---	KRIQIKKIDNISSRQVTF	SKRRKGLFKKA	QELSTL	: 38
GmSVPb3	:	---	MAR	---	KKIPIKKIDNINARQVTF	SKRRKGLFKKA	QELSTL	: 38
GmSVPb4	:	---	MTR	---	KKIPIKKIDNINARQVTF	SKRRKGLFKKA	QELSTL	: 38
PvSVPb1	:	---	MAR	---	RKIAMKKIENINARQVTF	SKRRKGLFKKA	QELSTL	: 38
GmSVPb5	:	---	MVR	---	KKIPIKKIDNVITARQVTF	SKRRKGLFKKA	QELSTL	: 38
GmSVPb6	:	---	MVR	---	KKIPIKKIDNVITARQVTF	SKRRKGLFKKA	QELSTL	: 38
PvSVPb3	:	---	MVR	---	KKIPIKKIDNVITARQVTF	SKRRKGLFKKA	QELSTL	: 38
GGM12	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
CsMADS19	:	---	MTR	---	QKIQIKKIDNIAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
CsMADS20	:	---	MTR	---	KKIQIKKIDNIAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
VvSVP4	:	---	MAR	---	QKIQIKKIDNTAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
VvSVP5	:	---	MVR	---	QKIQIKKIDNTAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
VvSVP3	:	---	MAR	---	QKIQIKKIDNTAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PtMADS48	:	---	MTR	---	KKIPIKKIDNTTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PtMADS47	:	---	MAR	---	KKIPIKKIDNTTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PtMADS27	:	---	MTR	---	KKIPIKKIDNTAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PtMADS28	:	---	MTR	---	KKIPIKKIDNTAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PtMADS29	:	---	MTR	---	RKIPIKKIDNTTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
StMADS11	:	---	MVR	---	QKIQIKKIDNLTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PhFBP25	:	---	MVR	---	QKIQIKKIDNLTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
CaS21	:	---	MVR	---	QKIQIKKIDNLTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
AcSVP4	:	---	MVR	---	QKIQIKKIDNLTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
EeDAM2	:	---	MTR	---	QKIQIKKIDNITARQVTF	SKRRRGLEFKKA	ELSVL	: 38
EeDAM3	:	---	MTR	---	QKIQIKKIDNITARQVTF	SKRRRGLEFKKA	ELSVL	: 38
EeDAM1	:	---	MTR	---	QKIQIKKIDNITARQVTF	SKRRRGLEFKKA	ELSVL	: 38
MtSVPa1	:	---	MAR	---	EKKIPIKKIDNSTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
MtSVPa2	:	---	MAR	---	EKKIPIKKIDNSTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PsSVPa	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
GmSVPa2	:	---	MVR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
GmSVPa1	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PvSVPa	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
LjSVPa	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
CsMADS17	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PpSVP	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
MdJNT	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
VvSVP1	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PtMADS26	:	---	MAR	---	ERIKIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
SlJ	:	---	MAR	---	EKKIPIKKIDNSTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
CaJ	:	---	MAR	---	EKKIPIKKIDNSTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
IbMADS3	:	---	MAR	---	EKKIPIKKIDNITARQVTF	SKRRRGLEFKKA	ELSVL	: 38
AcSVP1	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
CtSVP	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
AtSVP	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
BrSVP	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
CaS22	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
PkMADS1	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
AmINCO	:	---	MAR	---	EKKIPIKKIDNTTARQVTF	SKRRRGLEFKKA	ELSVL	: 38
EgSVP	:	---	MAR	---	EKKIPIKKIDNATARQVTF	SKRRRGLEFKKA	ELSVL	: 38
HvBM10	:	---	MAR	---	ERREIKRIEAAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
LpMADS10	:	---	MAR	---	ERREIKRIEAAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
ZmM19	:	---	MAR	---	ERREIKRIEAAARQVTF	SKRRRGLEFKKA	ELSVL	: 38
ZmM26	:	---	MAR	---	ERREIKRIEAAARQVTF	SKRRRGLEFKKA	ELSVL	: 38



```

OsMADS22 : ---MAR-----ERREIRRIESAAARQVTFSKRRRGLEKKAEELSVL : 38
HvVRT2 : ---MAR-----ERRAIRRIESAAARQVTFSKRRRGLEKKAEELAVL : 38
TaVRT2 : ---MAR-----ERRAIRRIESAAARQVTFSKRRRGLEKKAEELAVL : 38
FaVRT2 : ---MAR-----ERREIRRIESAAARQVTFSKRRRGLEKKAEELGVL : 38
OsMADS55 : ---MAR-----ERREIRRIESAAARQVTFSKRRRGLEKKAEELAVL : 38
OsMADS47 : ---MAGGGGGGGRGEGEGRAATGKRERIAIRRIDNLAARQVTFSKRRRGLEKKAEELSVL : 57
HvBM1 : ---MAG-----KRERIAIRRIDNLAARQVTFSKRRRGLEKKAEELSVL : 40
PtMADS21b : ---MAR-----EKIKIKKIDNVAARQVTFSKRRRGLEKKAEELSVL : 38
PtMADS21a : ---MAR-----EKIKIKKIDNVAARQVTFSKRRRGLEKKAEELSVL : 38
PtMADS7 : ---MAR-----EKIKIKKIDNVITARQVTFSKRRRGLEKKAEELSVL : 38
VvSVP2 : ---MAR-----EKIKIKKIDNVSARQVTFSKRRRGLEKKAEELSVL : 38
SlMBP24 : ---MAR-----EKIKIKKIDNITARQVTFSKRRRGLEKKAEELSVL : 38
StMADS16 : ---MAR-----EKIKIKKIDNITARQVTFSKRRRGLEKKAEELSVL : 38
PhFBP13 : ---MAR-----EKIKIKKIDNITARQVTFSKRRRGLEKKAEELSVL : 38
IbMADS4 : ---MAR-----EKIKIKKIDNITARQVTFSKRRRGLEKKAEELAVL : 38
CaC10 : ---MAR-----EKIKIKKIDNITARQVTFSKRRRGLEKKAEELAVL : 38
AcSVP2 : ---MAR-----EKIKIKKIDNITARQVTFSKRRRGLEKKAEELAVL : 38
AcSVP3 : ---MAR-----EKIKIKKIDNITARQVTFSKRRRGLEKKAEELAVL : 38
CsMADS18 : ---MAR-----EKIKIKKIDNITARQVTFSKRRRGLEKKAEELSVL : 38
AtAGL24 : ---MAR-----EKIRIKKIDNITARQVTFSKRRRGLEKKAEELSVL : 38
PmDAM1 : ---MKMMR-----EKIKIKKIDNLPARQVTFSKRRRGLEKKAEELSVL : 40
PpDAM1 : ---MKMTR-----EKIKIKKIDNLPARQVTFSKRRRGLEKKAEELSVL : 40
PmDAM2 : ---MVKTMR-----EKIKIKKIDYLPARQVTFSKRRRGLEKKAEELSVL : 41
PpDAM2 : ---MVKTMR-----KKIKIKKIDYLPARQVTFSKRRRGLEKKAEELSVL : 41
PmDAM3 : ---MMR-----KKIKIKKIDCLPARQVTFSKRRRGLEKKAEELSVL : 38
PpDAM3 : ---MVKMTR-----KKIKIKKIDYLPARQVTFSKRRRGLEKKAEELSVL : 41
PmDAM5 : ---MM-----NKIKIKKIDYLPARQVTFSKRRRGLEKKAEELSVL : 37
PpDAM5 : ---MMR-----NKIKIKKIDYLPARQVTFSKRRRGLEKKAEELSVL : 38
PmDAM6 : ---MVKMMR-----EKIKIKKIDYLPARQVTFSKRRRGLEKKAEELSVL : 41
PpDAM6 : ---MMR-----EKIKIKKIDYLPARQVTFSKRRRGLEKKAEELSVL : 38
PmDAM4 : ---MVKMTR-----KKIKIKKIDYLSARQVTFSKRRRGLEKKAEELSVL : 41
PpDAM4 : ---MVKMMR-----EKIKIKKIDYLPARQVTFSKRRRGLEKKAEELSVL : 41

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*      80      *      100      *      120
PsSVPc : CDAEVGLIIFSTTGKLYEHCS--SMKDIITRYN-QHSGQINKLD---KT--LPLOVVK--- : 89
MtSVPc : CDAEVGLVIFSTTGKLYEYASS--NMKDIITRYG-QQSHHITKLD---KP--LQVQVEK--- : 89
LjSVPc : CDAEVGLIIVFSATGKLYEYASS--SMKSIITERN-QHIQGTGMD---RF--LEPGGEDY-- : 90
GmSVPc1 : CDAEVGLIVFSSTGKLFYSSS--SMNDIVTKYS--THSHGINKLD---KPSLELQLEA--- : 90
GmSVPc2 : CDAEVGLIVFSSTGKLFYSSA--SINDIITIYN--THSHGVNKLK---KPSLELQLEA--- : 90
PvSVPc : CDAEVGLIVFSSTGKLFYSSS--SMNDIITRYN--THSPGINKLD---RPSLELQLEA--- : 90
PsSVPb : CDADVALMVFSATGKLFYSSS--SMQQVIERRNGCSENHRLMDRST--DQFQVSESS-- : 94
MtSVPb : CDADIALMVFSATGKLFYSSS--SMQQVIERRNGYSANHRLDYPSTDDQ--LQVESDSNR-- : 96
GmSVPb1 : CDADIALIVFSATGKLFYSSS--SMHQVIERHDSYAIHRL--DRPSIE--LQIESDSN-- : 92
GmSVPb2 : CDADIALIVFSATGKLFYSSS--SMHQVIERRDSHSAHRL--DRPSIE--LQIENDSN-- : 92
PvSVPb2 : CDADIALIVFSATGKLFYSSS--SMQQVIERLDRHSAMHRS--DRSYME--LQIENDSN-- : 92
LjSVPb : CDADIALIVFSATGKLFYSSS--SIQKVIERRSQCSGIHR--EHLPIEQMFQFESDSN-- : 94
GmSVPb3 : CDABIALIVFSSTGKLFYSSS--SMQQLERRDRHSGIQGL--VNPSIG--QQLGSDSL-- : 92
GmSVPb4 : CDABIALIVFSATGKLFYSSS--SMQQTERRNQHSGIQGL--DNPSIG--QQLGSDSF-- : 92
PvSVPb1 : CDACIALIVFSATGKLFYSSS--SMQQTERRNRHSGVQGS--DISSTA--QQLGSESF-- : 92
GmSVPb5 : CDABIALIVFSPGKLFYSSS--SMQKVIERHILRSELNLE--KLDQSCPTQVRCNYA-- : 94
GmSVPb6 : CDABIALIVFSPGKLFYSSS--SMQKVIERHILWSELNLE--KLDQSCPTQVRCNYA-- : 94
PvSVPb3 : CDABIALMVFSATGKLFYSSS--SMQKVIERHILRSEFNQD--KLDQLPPTQVIRSSHA-- : 94
GGM12 : CDABVALIIFSTTGKLYDYSSS--SMKVLIERYENDFREKGT-----ARDQIDNG-- : 87
CsMADS19 : CDADIALIVFSATGKLFYSSS--SMLDLIRRH-----MPELNS--ISQPPSLEKS-- : 88
CsMADS20 : CDABIGLIVFSATGKLFYSSS--SIQEIIRHNSVHSENP--PNLNE--PS-VELQLENS-- : 92
VvSVP4 : CDABIALIVFSATGKLFYSSS--SVSQVIERHN--QHPQTPEKPEP---PSLELQLENS-- : 91
VvSVP5 : CDABIELIVFSATGKLFYSSS--SVNQVIERHS--QHPQTPEKPEP---PSLELQLENR-- : 91
VvSVP3 : CDABIALIVFSATGKLFYSSS--SVSQVIERHN--QHPQTPEKPEP---PSLELQLENS-- : 91
PtMADS48 : CDABIALMVFSATGKLFYSSS--SIGQVIERRN--LHPKNIDKFSQ---PSVELQDSA-- : 91
PtMADS47 : CDABIALMVFSATGKLFYSSS--SIGQVIERRN--LHPKNIDTFSQ---PSVELQDSA-- : 91
PtMADS27 : CDABIALMVFSATGKLFYSSS--SMGQVIERRN--LHPKNIDTLQ---PSLEKLDGG-- : 91
PtMADS28 : CDABIALIVFSATGKLFYSSS--SMGQVIERRN--LHPKNIDTLHQ---PSLEKLDGG-- : 91
PtMADS29 : CDABIALMVFSATGKLFYSSS--SMGQVIERRN--LHPKNIDMFGQ---PSLEKLDGG-- : 91
StMADS11 : CDADIGLIVFSATGKLFYSSS--SMMQLIEKHK--MQSERSDMDNPEQLHSSNLESEK-- : 94
PhFBP25 : CDADIGLIVFSATGKLFYSSS--SMMQLIEKHK--IHSESD--MDNPEQLQSSNLECKK-- : 93
CaS21 : CDABIALIVFSATGKLFYSSS--SMMQVIERHR--LCSEDTGRQDKHPPHILT--QRENH-- : 92
AcSVP4 : CDABIALIVFSATGRLFYSSS--SMNQVIERHN--LQGDNIQQNQ---PSLELQLENS-- : 91
EeDAM2 : CDABIALIVFSATGKLFYSSS--SVTRVIERYH--LHPKNIDSKMDQ---PSLELQLENG-- : 91
EeDAM3 : CDABIALIVFSATGKLFYSSS--SVTRVIERYH--LHPKNIDSKMDQ---PSLELQLENG-- : 91
EeDAM1 : CDABIALIVFSATGKLFYSSS--RRRASMPY----- : 68
MtSVPa1 : CDADVALIIFSTTGKLFYSSS--SMREIERHH--LHKNIDAKLE---EPSLELQ----- : 87
MtSVPa2 : CDADVALIIFSTTGKLFYSSS--SMREIERHH--LHKNIDAKLE---EPSLELQ----- : 87
PsSVPa : CDADVALIIFSTTGKLFYSSS--SMREIERHH--LHKNIDAKME---EPSLELQ----- : 87
GmSVPa2 : CDADVALIIFSTTGKLFYSSS--SMKEIERHH--LHKNIDARME---QPSLELQ----- : 87
GmSVPa1 : CDADVALIIFSTTGKLFYSSS--SMKEIERHH--LHKNIDARME---QPSLELQ----- : 87

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PvSVPa : CDADVALIIFSSSTGKLFEEYNS-SMKEILERHH-LHSKNLAKMD---QPSTLELQ----- : 87
LjSVPa : CDADVALVVFSSSTGKLFEEYNSL-SMKEILERHH-LHSKNLAKLE---QPSTLELQ----- : 87
CsmADS17 : CDADVALIIFSSATGKLFEEYSSS-SMKGILERHN-LHSKNLQKLE---QPSTLELQ----- : 87
PpSVP : CDADIALIIFSSSTGKLFEEYASS-SMKEILERHN-LHAKNLKIE---QPSTLELQ----- : 87
MdJNT : CDADIALIIFSSSTGKLFEEYASS-SMKEILERHN-LHSKNLEKLE---QPSTLELQ----- : 87
VvSVP1 : CDADVALIIFSSSTGKLFEEYSSS-SMKEILERHS-LQSKNLEKLE---QPSTLELQ----- : 87
PtMADS26 : CDADVALIIFSSSTGKLFEEYSSS-SMKEILERHN-LHSKNLEKLE---QPSTLELQ----- : 87
SlJ : CDADVALIIFSSSTGKLFEEYSSS-SMKQILERRD-LHSKNLEKLD---QPSTLELQ----- : 87
CaJ : CDADVALIIFSSSTGKLFEEYSSS-SMKQILERRD-LHSKNLEKLD---QPSTLELQ----- : 87
IbMADS3 : CDADVALIIFSSSTGKLFEEYASS-SMKGILERRN-LHSKNLEKMD---QPSTLELQ----- : 87
AcSVP1 : CDADVALIIFSSSTGKLFEEYSSS-SMKGILERHN-VHSKNLEKLE---QPSTLELQ----- : 87
CtSVP : CDADVALIIFSSATGKLFEEYSSSRSMKEILERHH-VHSKNLERVD---QPSTLELQ----- : 88
AtSVP : CDADVALIIFSSSTGKLFEEYCSS-SMKEILERHN-LQSKNLEKLD---QPSTLELQ----- : 87
BrSVP : CDADVALIIFSSSTGKLFEEYCSS-SMREVLERN-LQSKNLEKLD---QPSTLELQ----- : 87
CaS22 : CDADVALIIFSSSTGKLFEEYSSS-SMKEILERHN-LHSKNLEKG---AAATLELQ----- : 86
PkMADS1 : CDADVGLIIFSSSTGKLFEEYASS-SMKEILERHN-LHSKNLEKLE---QPSTLELQ----- : 87
AmINCO : CDADVALIIFSSSTGKLFEEYASS-SMKEILERHN-LHSKNLEKME---QPSTLELQ----- : 87
EgSVP : CDADVALIIFSSSTGKLFEEYCSS-SMKEILERHH-SHSENLGKLD---QPSTLELQ----- : 87
HvBM10 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYS-THSKNLEKTD---QPTLDLN----- : 87
LpMADS10 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYS-THSKNLEKAD---QPTLDLN----- : 87
ZmM19 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYS-THSKNLEKAE---QPTLDLN----- : 87
ZmM26 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYN-THSKNLEKTE---QPTLDLN----- : 87
OsMADS22 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYN-THSKNLEKAE---QPTLDLN----- : 87
HvVRT2 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYS-THSKNLEKSDQ---QPAIDLN----- : 88
TaVRT2 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYS-THSKNLEKSDQ---QPAIDLN----- : 88
FaVRT2 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYS-THSKNLEKSE---KPAIDLN----- : 88
OsMADS55 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYT-THSKNLEKTDK---QPTLDLN----- : 94
OsMADS47 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYN-SHSKNLEKRAE---PSQIDLG----- : 107
HvBM1 : CDADVALIIFSSSTGKLFEEYASS-SMNEIDKYN-SHSKNLEKVD---PSQIDLG----- : 90
PtMADS21b : CDADVALIIFSSSTGKLFEEYASS-SMKDVLARYN-LHSNNLEKLN---QPSTLELQ----- : 87
PtMADS21a : CDADVALIIFSSSTGKLFEEYASS-SMKDVLARYN-LHSNNLEKLN---QPSTLELQ----- : 87
PtMADS7 : CDADVALIIFSSSTGKLFEEYASS-SMKDVLARYN-LHSNNLEKIN---PPSTLELQ----- : 87
VvSVP2 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEKLE---GPSTLELQ----- : 87
SlMBP24 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEKVD---QPSTLELQ----- : 87
StMADS16 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEKVD---EPSTLELQ----- : 87
PhFBP13 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEKDD---QPSTLELQ----- : 87
IbMADS4 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEQAT---QPSRELELQ----- : 87
CaC10 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEKTE---QPSTLELQ----- : 87
AcSVP2 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEQMD---QPSRELELQ----- : 87
AcSVP3 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEQID---QPSTLELQ----- : 87
CsmADS18 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEKLE---YPSRELELQ----- : 87
AtAGL24 : CDADVALIIFSSSTGKLFEEYASS-SMKDILGRYT-MHSNNLEKMD---PPSTHLR----- : 88
PmDAM1 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKFN---ERSTLELQ----- : 89
PpDAM1 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKFD---EPSTLELQ----- : 89
PmDAM2 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKSD---EPSTLELQ----- : 90
PpDAM2 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKSD---EPSTLELQ----- : 90
PmDAM3 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKSD---EPSTLELQ----- : 87
PmDAM3 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKSD---KPSVELELQ----- : 90
PmDAM5 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKSN---NQETLELQ----- : 86
PpDAM5 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKLN---NQETLELQ----- : 87
PmDAM6 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKSN---KQFLELQ----- : 90
PpDAM6 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKSN---KQFLELQ----- : 87
PmDAM4 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKSD---EQSTLELQ----- : 90
PpDAM4 : CEEBVAIVIFSSATGKLFEEYSSS-SMKDILGRYT-MHSNNLEKSD---EQSTLELQ----- : 90

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*          140          *          160          *          180
PsSVPc : -----SMSAEIQKEFADKTQQLR-GLKGDFEGINLDGLQQLERTL : 129
MtSVPc : -----NMPAELNKEVADRTQQLR-GMKSEDFEGINLEGLQQLKSL : 129
LjSVPc : -----SNLAELHKEVANRTEQLR-RMTGEDFEGLEFDDLLLEKTL : 130
GmSVPc1 : -----SNSAKLSKEIADRTQQLS-WLKGDDLOGIGLNELQQLKTL : 130
GmSVPc2 : -----SNSAKFSKEIADRTQQLS-WLKGDDLOGIGLNELQQLKTL : 130
PvSVPc : -----SNSAKLSKEIADRTQQLS-WLKGDDLOGIGLNELQQLKTL : 130
PsSVPb : -----DTL---HKKLEDKSRRLR-QMNGEDLQELTVOELQKLETL : 131
MtSVPb : -----DTL---RKKLEDKSRRLR-QMNGEDLQELTVOELQKLEVL : 133
GmSVPb1 : -----NIL---RKKVEDKTRRLR-QMNGEDLQGLTVOELQKLEHL : 129
GmSVPb2 : -----EIL---RKKVEDKTRRLR-QMNGEDLQGLTVOELQKLEHL : 129
PvSVPb2 : -----DIL---RKKVEDKSHRLR-QMNGEDLQGLTVOELQKLEHL : 129
LjSVPb : -----DTP---RKKVEEKTHRLR-QMNGEDLQGLTVOELQKLEVL : 131
GmSVPb3 : -----GIL---RKEIEHKTNEFS-QMNGEDLQGLTVOELQKVEEL : 129
GmSVPb4 : -----GMLP---RKEIEDKTNELS-QMNGEDLQGLTVOELQKLEDL : 131
PvSVPb1 : -----DML---HKEIVDKTHELS-RMCKELQGLTVOELQKLEEL : 129
GmSVPb5 : -----DLN---KEFADRTREMR-QMNGEDLQGLTVOELQKLEERL : 130
GmSVPb6 : -----DLN---KEFGDRIEMR-QMNGEDLQGLTVOELQKLEERL : 130
PvSVPb3 : -----YLK---KEIEDRSREMR-QMNGEDLQGLTVOELQKLEGR : 130
GGM12 : -----DVLKAQQQVAELERARR-QMNGEDLQGLTVOELQKLEANL : 126

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CsMADS19 : -----AHAKTTEFAAKTKELR-HMKGEELQELGTEELKQLEKLL : 127
CsMADS20 : -----IRAKINEEVEKKSHQLR-QMKGEELQGLGTEELKKLEKSL : 131
VvSVP4 : -----TCAALSKETAQQTQRLR-QMKGEELQGLKTEELIELEELL : 130
VvSVP5 : -----TCAALSKETAQQTQRLR-QMRGEELQGLKTEELIELEKLL : 130
VvSVP3 : -----TCAALSKETAQQTQRLR-QMKGEELQVLKTEELTELEELL : 130
PtMADS48 : -----VHAMLNKEIAEKTREL-RTRGEDLQGLNVEELEKLEKLI : 130
PtMADS47 : -----VHAMLNKEIAEKTREL-RTRGEDLQGLNVEELEKLEKLI : 130
PtMADS27 : -----VHAMLIKEIAKKNREL-HMRGEDLQGLDTEELQKLEKIM : 130
PtMADS28 : -----VHAMLIKEIAEKNREL-HMRGEDLQGLSTEELKKLEKLI : 130
PtMADS29 : -----VYATLNKEIAEKTRELS-QVRGEDLQGLNTEELHKLEKLI : 130
StMADS11 : -----THAMLSRDFVEKNREL-QTHGEELQGLGDDIMKLEKLV : 133
PhFBP25 : -----TYGMTSKEFLEKNREL-QIKGEELQGLGTEELMKLEKLV : 132
CaS21 : -----THAMIAEETKEETAELR-HLKGEELVGLSVEDIG----- : 125
AcSVP4 : -----TYAMLCNEVEERTREL-QLRGEETHGLGVEELKNLEKSL : 130
EeDAM2 : -----PLSALSNVEADKTQELR-NMRGEELQGLGFEELQILEKLL : 130
EeDAM3 : -----PLSALSNVEADKTQELR-KMRGEELQGLGFEELQILEKLL : 130
EeDAM1 : ----- : -
MtSVPa1 : -----LVENSNCSTRISKEVAQKSHQLR-QMRGEDLQGLSTEELQOLEKSL : 131
MtSVPa2 : -----LVENSNCSTRISMEVSKKSHQLR-QMRGEDLEGINVEELQOLERSL : 131
PsSVPa : -----LVENSNCSTRINKEIAEKSHQLR-QMRGEDLQGMNVEOLQHLESL : 131
GmSVPa2 : -----LVENSNCSTRISKEVAEKSHQLR-QLRGEDLQGLNTEELQOLERSL : 131
GmSVPa1 : -----LVENSNCSTRISKEVAEKSHQLR-QLRGEDLQGLNTEELQOLEMSL : 131
PvSVPa : -----LVENSNCSTRISKEVAEKSHLLR-QLRGEDLQGLNTEELQOLERSL : 131
LjSVPa : -----LVENSNCSTRINKEVAEQSRLR-QLRGEDLQGLNTEELQOLERSL : 131
CsMADS17 : -----LVENSNYTRINKEIAEKTHQLR-QMRGEELQTLNTEELQOLEKSL : 131
PpSVP : -----LVENSNSYASISKETTAQSQQLR-QLRGEEIQGLNTEELQOLEKSL : 131
MdJNT : -----LVENSNYTRISKEIAAKSHQLR-QMRGEELQGLNTEELQOLEKSL : 131
VvSVP1 : -----LVENNNHSRISKEVADKSHKLR-QMRGEELQGLNTEEDLQOLEKSL : 131
PtMADS26 : -----LVEDSTCSRISKEVAEKSHQLR-QMRGEDTRGLDTEELQOLEKSL : 131
SlJ : -----LVENSNSYRISKEISEKSHRLR-QMRGEELQGLNTEELQOLERSL : 131
CaJ : -----LVENSNSYRISKEISEKSHRLR-QMRGEELQGLNTEELQOLEKSL : 131
IbMADS3 : -----LVENANHSRISKETADMTHRLR-QMRGEDLQGMSTEELQOLERSL : 131
AcSVP1 : -----LVENSNYTRISKEVVEKSHQLR-KMRGEELQGLNTEELQOLERSL : 131
CtSVP : -----LLENNYSMIFKEIAEKSHLLR-QMRGEETHGLSTEELQKLESL : 132
AtSVP : -----LVENS DHARMSKEIADKSHRLR-QMRGEELQGLDTEELQOLEKAL : 131
BrSVP : -----LVENS DHALLSKETAEKSHRLR-QMRGEELQGLNTEELQOLEKAL : 131
CaS22 : -----LVENNNCSPTISKEVAEKSHQLR-QMRGEELQGLTVD----- : 121
PkMADS1 : -----LVEDSNYSRISKEVAERSHQLR-RMRGEELQGLSTEKLQHKKSL : 131
AmINCO : -----LVEDSNCTRISKEVAERSQQLR-RMRGEELQGLNVEELQRLERSL : 131
EgSVP : -----LVENG DYSRISKEVAEKGHQLR-QMRGEELQGLNTEDELQOLEKSL : 131
HvBM10 : -----LEHSKYANLNDQIAEASLRLR-QMRGEELGLSVDELQOLEKNL : 130
LpMADS10 : -----LEHSKYANLNDQIAEASLRLR-QMRGEELGLTVDELQOLEKNL : 130
ZmM19 : -----LEHSKYANLNEQIVAEASLRLR-QMRGEELGLSVDELQOLEKNL : 130
ZmM26 : -----LEHSKYANLNEQIAEASLRLR-QMRGEELGLNVEELQOLEKNL : 130
OsMADS22 : -----LEHSKYANLNEQIAEASLRLR-QMRGEELGLSVDELQOLEKNL : 130
HvVRT2 : -----LEHCKYDSINEQIAEASLRLR-HMRGEELDGLSVGELQOMEKNL : 131
TaVRT2 : -----LEHCKYDSINEQIAEASLRLR-HMRGEELDGLSVGELQOMEKNL : 131
FaVRT2 : -----VEHSKYNSINEQIAEASLHLR-HMRGEELAGLSVGELQOMEKDL : 131
OsMADS55 : LRTYTNSYAYIHLQLLEHSKCSSINEQIAEASLQLR-QMRGEELGLSVDELQOMEKNL : 153
OsMADS47 : -----EDSSTCARISKEIAETSRLR-QMRGEELHRLNVEOLQBLEKSL : 150
HvBM1 : -----EDS-NCARIDEIAEASLWLQ-QMRGEELQSLNVQQLQALEKSL : 132
PtMADS21b : -----LENSNHMRIRKEVSEKSHQLR-RMRGEELQGLNTEELQOLEKVL : 130
PtMADS21a : -----LENSNHMRIRKEVSEKSHQLR-RMRGEELQGLNTEELQOLEKVL : 130
PtMADS7 : -----LENSNHMRISKEVSEKSHQLR-RMRGEDLHGLNTEELQOLEKAL : 130
VvSVP2 : -----LENSNHVRISKEIADKSHQLR-QMRGEDIQGLNTEELLKLEKML : 130
SlMBP24 : -----LENSLNMRLSKQVADKTREL-QMRGEELGLSTEELQOTEKRL : 130
StMADS16 : -----LENSLNMRLSKQVADKTREL-QMRGEELGLSTEELQOTEKRL : 130
PhFBP13 : -----LENSLNMRLSKETADKNREL-QMRGEELGLSTEELQKLEKLI : 130
IbMADS4 : -----LENSLHVRISKEVADKTREL-QMKGEELQGLSTEELQKLEKRL : 130
CaC10 : -----LENSCHVRISKEVADRTHQLR-QMKGEDLQGLKTEELQOLEKVL : 130
AcSVP2 : -----LEDNLAKLGKDVSEKTTQLW-QMRGEDLQGLNTEELQKLEKML : 130
AcSVP3 : -----LEDNLVKGKDVSEKTTQLR-QMRGEDLQGLNTEELQKLEKML : 130
CsMADS18 : -----VEDSNHVQINKEVEDMNQQLR-QMRGEDLQGLNTEEDLKOLEKRL : 131
AtAGL24 : -----LENCNLSRISKEVEDKTKQLR-KLRGEDLDGLNTEELQRLKEL : 130
PmDAM1 : -----PEYENHIRISKEIEEKSRQLR-QMKGEDLEELNFDLQKLEQLV : 132
PpDAM1 : -----PEKENHIRISKEIEEKSRQLR-QMKGEDLEELNFDLQKLEQLV : 132
PmDAM2 : -----LEIENQIRINKEIAEKSRQLR-QMRGEDLEKLNTEDELQKLEQLV : 133
PpDAM2 : -----LEIENHIRISKEIEEKSCQLR-QIKGEDLEELNFDLQKLEQLV : 133
PmDAM3 : -----LENNENHIGISKEIEEKSHQLRQOMKAEDLEELNFDLQKLEQLV : 131
PpDAM3 : -----LENNENQIGISKEIEEKSHQLR-QMKAEDLEELNFDLQKLEQLV : 133
PmDAM5 : -----LENNENHIKISKEIEKTSHQLR-QMKGEDLEGLNTEDELLKLEQLV : 129
PpDAM5 : -----LENNENHIKISKEIEEKSRQLR-QMKGEDLEGLNTEDELLKLEQLV : 130
PmDAM6 : -----LENNENHIKISKEIEEKSRQLR-QMKGEDLEGLNTEDELLKLEQLV : 133
PpDAM6 : -----LENNENHIKISKEIEEKSRQLR-QMKGEDLQGLNTEDELLKLEQLV : 130
PmDAM4 : -----LENNENRIKISTEIEEKNRQLR-RMKGEDLEELDTEDELLKLEQLV : 133

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PpDAM4      : -----LENENHTKISTEIEEKNRQLRQMKGEDLEELDDEILKLEQLV : 133

                *           200           *           220           *           240
PsSVPc      : ETGLKSVIEMKEKRIMNEIGALQIKSIEIOEENNH---LKQKMA-----MLFKG : 175
MtSVPc      : ESGIKRVIEMKEKKILNEIKALRMKEIMIEENKH---LKQKMA-----MLSMG : 175
LjSVPc      : QSGIKRVIEMKEKRIMDEITAVQKKEVA-----LTKKLA-----EKEKEAMLCKA : 182
GmSVPc1     : EIGIDRVTDIKENQIMSQISELQKKGILIEENKH---LTKKLA-----EKEKEAMLCKA : 182
GmSVPc2     : EIGIDRVIEIKKEQIMSQISELQKKGNLIEENKH---LTKKLV-----ETEMEAMLCEP : 182
PvSVPc      : ESGIDRVITQKEKQMSQISELQKKGILIEENRH---LTKKLV-----ETEMEAMLRKA : 182
PsSVPb      : RRSTSSVSKKKDEMFIQGNITLKRREVELIRENQR---LKHVV----- : 171
MtSVPb      : KRSTSSVSKIKDEMFMRDITLKRREVELMEENRR---LKHVV----- : 173
GmSVPb1     : KRSTTNVSKVKDAKFMQESTFKRKGVELMEENQR---LKV-V----- : 168
GmSVPb2     : KRGLINVSKVKDEKLMQESTLKRKGVELMEENQR---LKV-V----- : 169
PvSVPb2     : KRGLSNVSKVKDEKVMQESTLKRKGVELMEENQR---LKV-V----- : 168
LjSVPb      : KRSTASVSRVKDEKFMQESTLKRKEVELMEENQK---LKV-V----- : 170
GmSVPb3     : QRRWTTISKIKDEKIQEINHLKTKEAKLMEENQK---LKV----- : 167
GmSVPb4     : QRRWTTISKIKDEKVIQEINHLKTKEAKLMEENQK---LKV----- : 169
PvSVPb1     : LRRWTTISKIKDEKITQEINSLKTKEAELEENQK---LKV----- : 167
GmSVPb5     : DSSINRVYKAKVENEIKETGILKEKKGKLMEDNML---LKV----- : 168
GmSVPb6     : VSSINRVYKAKVNFTRIDILKQKGNKLMEDNRL---MKV----- : 168
PvSVPb3     : ESSINCVYKAKVQNFTRDNIILTQKQNKLMEDNRL---LKV----- : 168
GGM12       : ETAINRVNRKGVQILKDNLDLQKQGEILEENNR---LRQQLR---QRYNNRAPLEN : 178
CsMADS19    : ENGLNRVIEKKEDEKILKEVTVKKEKGLMEENQR---LKNKLM---ETLINR--- : 174
CsMADS20    : QGGISRVAEIMDGKNTDLSDIGRNVDLIEENKR---LN----- : 168
VvSVP4      : EAGLCSVVEEKAERIRTEISDLQKRGDLREENER---LRKE----- : 169
VvSVP5      : EAGLCSVVEEKEERIQTETISDLQKRGDLREGENER---LRKE----- : 169
VvSVP3      : EAGLCNVVEEKEERIRTEISDLQKRGDLREENER---LRKE----- : 169
PtMADS48    : EGSICRVMETKGEKILKEVDALKSEQQLEENQR---LTKR----- : 169
PtMADS47    : EKSLCRVIEKKEKILKEVDALKSEHQLEENQR---LTKR----- : 169
PtMADS27    : EGSILRLVVEEKGKIIINEIDALKTKGEQLEENQR---LTKQ----- : 169
PtMADS28    : EGSILRVVEEKEEKSTKDNALTKKEQLEENQR---LTKQ----- : 169
PtMADS29    : ETSILCRVVEEKGKIIINEINTLKNEGEQLEENRR---LRQQ----- : 169
StMADS11    : EGGISRVIRIKGDKFMKEISSLKKKEAQLEENSQ---LTKQS----- : 173
PhFBP25     : EGGISRVVMKMGDKFMKEISSLKKKEAQLEENSQ---LTKQS----- : 172
CaS21       : ----- : -
AcSVP4      : EGGIGRIIEKTKDERFEKETITALKRRETRIEENLW---LTKQ----- : 168
EeDAM2      : EGGIKKVVEKTKGNALMNEIKELKSKGNQLEENR---LKHQ----- : 169
EeDAM3      : EGGIKKVVEKTKGNALMNEIKELKSKGNQLEENR---LKHQ----- : 169
EeDAM1      : ----- : -
MtSVPa1     : EIGLGRVIEKKEKIMMEINELQTKGRQLEENNR---LKRH-----VSGMFN-GKMF : 180
MtSVPa2     : EIGLGRVIEKKEKIMMEINDLQKRGQLEENDR---LKRH-----VAGIIN-DRMV : 180
PsSVPa      : EIGLGRVIEKKEKIMMETQHLQKRGQLEENDR---LKRH-----VTGMMNNGKIV : 181
GmSVPa2     : ETGLGRVIEKKEKIMSETDLQKRGMLMEENER---LKRH-----VAGIIN-QGRH : 180
GmSVPa1     : ETGLGRIIEKKEKIMSETADLQKRGMLMEENER---LKRH-----VAGIIN-QGRH : 180
PvSVPa      : ETGLSRVIEKKEKIMNETDLQKRGMLMEENER---LKRH-----VAGIVN-GERR : 180
LjSVPa      : ETGLGRVIEKKEKIMNEINGLQIKGKQLEENR---LKRH-----VAGMISTGLMH : 181
CsMADS17    : ESGLSRVMEKKEGERIMKETDLQKRSALMDENKR---LTKQ-----AEKMNG--VRH : 179
PpSVP       : EAGLGRVIEKKEKIMKETSDLESNAMRVEENER---LTKQ-----VLEKHNSQKP- : 180
MdJNT       : ETGLGRVIEKKEKIMKEIGDLQKNGMLMEENER---LTKQ-----VAKSDGRRRL- : 180
VvSVP1      : EAGLSRVIEKKEGERIMKETDLQKSGVQLEENR---LTKQ-----VVEISNRRRQ- : 180
PtMADS26    : EAGLSCVIEKKEKIMNETDLQKRGMLMEENER---LTKQ-----VVEISNGRKH- : 180
SlJ         : ETGLSRVIEKKGDKIMREINQLQKGMHMEENEK---LTKQ-----VMEISSNNNNN : 181
CaJ         : ETGLSRVIEKKGDKIMREINQLQKGMHMEENEK---LTKQ-----VMEISSNNNNN : 181
IbMADS3     : ETGLSRVIEKKEKIMKEINELQKGMNMEEKER---LTKQ-----VMAISNGQRTV : 181
AcSVP1      : EAGLGRVIEKKEKIMNETHLQKRGMLMEENER---LTKQ-----VMEACKARKHS : 181
CtSVP       : EVGLGRVIEKKEKITKEINELQKRGMLMEENER---LTKQ-----VAEVSN----- : 177
AtSVP       : ETGLTRVIEKKSQIMSEISELQKKGMLMDENKR---LTKQ-----GTQLTEENERL : 181
BrSVP       : ESGLTRVIEKKEKIMNETSYLQKKGMLMDENKR---LTKQ-----GTQLTEENERL : 181
CaS22       : ----- : -
PkMADS1     : ESGLSRVIEKKEKIMKGDQSTSRKQKQLEENR---LTKQ-----VADISNDCKNN : 181
AmINCO      : EVGLSRVIEKKEKIMNEINQLQKRGMLMEENR---LTKQ-----VVDISNYQQIT : 181
EgSVP       : EAGLNRVIEKKEKIMKETDLQKKGAKLMEENKR---LTKQ-----VTEISGRKTTA : 181
HvBM10      : ETGLHKVLOTKDQGFLEQINELHRRSSQLEENKK---LRNQ-----VAQVP-TAGKL : 179
LpMADS10    : ETGLHRVLOTKDQGFLEQINELQKSSQLEENMQ---LRNQ-----VSQIP-IAGKP : 179
ZmM19       : ESGLHRVLOTKDQGFLEQISDLQKSTQLEENRQ---LRNQ-----VSHIP-PVGKQ : 179
ZmM26       : ESGLHRVLOTKDSQGFLEQINDLERSTQLEENMQ---LRNQ-----VSQIP-PAGKQ : 179
OsMADS22    : EAGLHRVMTKDDQFMQSESELQKRSQLEENMQ---LRNQ-----VSQIS-PAEQK : 179
HvVRT2      : ETGLQRVICTKDRQFMQSDLQKRGTOLEENMR---LRNQ-----MHEVP-TASMV : 180
TaVRT2      : ETGLQKVICTKDRQFMQSDLQKRGTOLEENMR---LRNQ-----MHEVP-TVSTV : 180
FaVRT2      : ETGLQRVICTKDDQFMQSDLQKRGTOLEENMR---LRNQ-----MPQVP-T---- : 176
OsMADS55    : EAGLQRVICTKDDQFMQSESELQKRGTOLEENMR---LRDQ-----MPQVP-TAG-L : 201
OsMADS47    : ESGLSVIEKTKSKILDEIDGLERKRMQLEENLR---LKEQ-----VSRMSRMEEMQ : 200
HvBM1       : ESGLSSVIEKTKSKQIMDQISELEKTRVQLEENAR---LKEQ-----ASKM---EMQ : 178
PtMADS21b   : EVGLCCVIEKTKGERIMNETSLERKGAQLEENKH---LTKQ-----MTTICKGKRPA : 180
PtMADS21a   : EVGLCCVIEKTKGERIMNETSLERKGAQLEENKH---LTKQ-----MTTICKGKRPA : 180

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PtMADS7 : EVGTSRVLETGGERIMNEISTLERKGVQLLEENKQ---LKQK-----IATIIYKKGKPA : 180
VvSVP2 : EAGTSRVLKSksDRIMTEIATLQTKGAQLMEENK---LRQK-----MEIICKGKRLM : 180
SlMBP24 : EAGFNrvLEIKGKRIMDEITNLQKKGAELEENKQ---LKHK-----MEIMKKGKLPL : 180
StMADS16 : EAGFNrvLEIKGTRIMDEITNLQKKGAELEENKQ---LKHK-----MEIMKKGKFPL : 180
PhFBP13 : EAGTTRVLTQIKGTRIMDEITNLQKKGADLEENKQ---LKQK-----MVIMSEGKLPL : 180
IbMADS4 : ENGTSRVLETGGERVVTETIATLQKKGAELEMKENKQ---LKEK-----MARVNGEKFPV : 180
CaC10 : EAGTTRVLTQTKGERIMNEITNLQKKGAELEENKQ---LKQK-----MAMLYEGKRPV : 180
AcSVP2 : EAGTSRVLETGGERIMNEIATLQKKGAELEENHR---LKQK-----MKAISEGKLGv : 180
AcSVP3 : EAGTSRVLETGGERIMNEIATLQKKGAELEENQR---LKQKLQ-----MNAISEGKWAV : 182
CsMADS18 : EVGTSRVLTHTKEKKIMREIDELKLGARLEENKM---LKQK-----MLRLSNERLMA : 180
AtAGL24 : ESGTSRVSEKKGECVMSQIFSLERKGSLEVDENKR---LRDK----- : 170
PmDAM1 : DASTGRVLETGKERIMSEIMALERKRAELVKANKQ---LRQR-----MLFRGNIGPEL : 182
PpDAM1 : DASTGRVLETGKDELIMSEIMALKRKRAELVEANKQ---LRQRASNYHNHMLSRGNIGPAL : 189
PmDAM2 : DASTGRVLETKEELIMSEIMALERKGAELVEANNQ---LRQRM-----VMLSRGNIGPAL : 185
PpDAM2 : DASTGRVLETKEELIMSEIMALERKGAELVEANNQ---LRQRM-----VMLSRGNIGPAL : 185
PmDAM3 : DTSISRVLETKEELRMSEIMALERKGAELVEANNQ---LKQT-----VMLSGGNTGPTL : 182
PpDAM3 : DASTGRVLETKEELRMSEIMALERKGAELVEANNQ---LRQT-----MMLSGGNTGPTL : 184
PmDAM5 : EASTGRVMETKEELIKSEIMELERKGAELVEANSQ---LRQTM-----VMLSGGNTGPAL : 181
PpDAM5 : EASTGRVMETKEELIKSEIMALERKGTLEVEANNQ---LRQTM-----VMLSGGNTGPAL : 182
PmDAM6 : EASTGRVLETKEELIMSEIMALEKKGAELVETNNQ---LRHRM-----VMLSGGNTGPAF : 185
PpDAM6 : EASTGRVLETKEELIMSEIMALEKKGAELVEANNQ---LRQKM-----AMLSGGNTGPAF : 182
PmDAM4 : EATIVRVLETKEELIMSDIVALEKKGTLEVEANNQMVMLEDRM---VMLSKRSTGPAL : 188
PpDAM4 : EATIVRVLETKEELIMSDIVALEKKGTLEVEANNQMVMLEDRM---VMLSKRNTGPAL : 188

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*          260          *          280          *          300
PsSVPc : KCPL-----LGD-----LDVSCESMNN-----VCSCNSGPS- : 201
MtSVPc : KSPI-----FGDSGIT--MQENVSAESMNN-----VSSCNSGPS- : 207
LjSVPc : -----SSDSMNN-----VCSCNSGPS- : 174
GmSVPc1 : KIPF-----MVDSDKGIMQEEGVSLDSTNN-----ISSCISDPP- : 216
GmSVPc2 : EIPF-----MVDLDKGIMQEEGVSLDSTNS-----ISSFINDPP- : 216
PvSVPc : KMPF-----MMDSNMG-MQEEGVLLSTNN-----VSSSISDPH- : 215
PsSVPb : -----PDLIGQR-QQSLESVIRR-----SSYFL---- : 194
MtSVPb : -----PDLINVRW-QQSLTIVISG-----SSFSL---- : 196
GmSVPb1 : -----PSLIHAHSYRQSSSILSN-----SSNLP---- : 192
GmSVPb2 : -----PSLIHVH--RQSSSILSN-----SSNLP---- : 191
PvSVPb2 : -----PSLIPLH--RQSSSILSN-----SSNLP---- : 190
LjSVPb : -----PGLTQYGO-RQSLSTISS-----SSYLL---- : 193
GmSVPb3 : -----SfVREQ--RQPYESFTCS-----SSEFP---- : 188
GmSVPb4 : -----SFLQEQ--RQYYESFTCS-----SSEFP---- : 190
PvSVPb1 : -----KLVPQEQ--RQCYESITSS-----SSDFP---- : 188
GmSVPb5 : -----MIKL---PRNEICSV-----QRHEH---- : 185
GmSVPb6 : -----RIK---PRNEICSV-----QRHEH---- : 184
PvSVPb3 : -----RIS---SRNEICSV-----QRHEH---- : 184
GGM12 : FEAE-----SLPIGQLLAHEPPQSQSSDSISTS-----FSLKLGNGV : 216
CsMADS19 : -DDQQ-----EEEEAVVLIAGNSVGSKKSNTSN-----SSSS--QNP : 209
CsMADS20 : ---QL-----EVDKLGEQIMQNIQGHSSSISIGNN-----STSS--NNP : 201
VvSVP4 : -----VENISEA-PLLQGHSSSISITN-----ICSL--SD : 197
VvSVP5 : -----MENISEAQPLLQGHSSSISITN-----ICSL--SD : 198
VvSVP3 : -----MENIFEAQPLL----- : 180
PtMADS48 : -----LMNLSKQGHLLEQGQSSDSMVTN-----ISSN--SAY : 200
PtMADS47 : -----LMSLSKQGHLLEQGQSSDSMVNN-----ISSN--SAN : 200
PtMADS27 : -----VMSLLAGQGHLLPQGQSSDSLVTN-----ISSMG-SVD : 201
PtMADS28 : -----VMNLSAAQGHLLPQGQSSDSLVTN-----ISSMS-SAD : 201
PtMADS29 : -----VMNLSAGQRHLLPDKSSDSLVTN-----TRSMS-SVD : 201
StMADS11 : -----QARLNEEGQNVIEQGHSAISITN-----NRSL-VN- : 202
PhFBP25 : -----EARLS---QNATEPFGHSANSITN-----CPSF-VYG : 199
CaS21 : ----- : -
AcSVP4 : -----RLQIVNASTPEQGQSSSISITN-----NGSS-TA- : 195
EeDAM2 : -----ITSSPLELLHFEKGHSPPDSVTTN-----TSSLI-DHS : 200
EeDAM3 : -----ITSSPLELLHFEKGHSPPDSVTTN-----TSSLI-DHS : 200
EeDAM1 : ----- : -
MtSVPa1 : -----GGVESEN-MVTEEGQSSSISVT-----NVYNS--TGPP- : 209
MtSVPa2 : -----GGDESENEENVVNEGQSSSISVT-----NVYNS--IGPP- : 210
PsSVPa : -----GGVECEEN-VVIEEGQSSSISITN-----NVYNS--IGPP- : 210
GmSVPa2 : -----GGAESEN-FVMDEGQSSSISVT-----YVCNS--TGPP- : 209
GmSVPa1 : -----GGAESEN-FVMDEGQSSSISVT-----YVCNS--TGLP- : 209
PvSVPa : -----GGAENEN-FVVDEGQSSSISVT-----YVCNS--TGPP- : 209
LjSVPa : -----GDTESEL-LVMEEGHSSSISVT-----NVCNST-TGPP- : 211
CsMADS17 : -----LGVEPEI-LVVEDGQSSNSVT-----EVCVNSNSNGPP- : 210
PpSVP : -----VRADSENIVM---EEGQSSSISVT-----NLCNSN-SAP- : 210
MdJNT : -----VQVDSNMFT---EEGQSSSISVT-----NPCNSN-NGP- : 209
VvSVP1 : -----VAGDSNMFH---EEGQSSSISVT-----NVNSN-GPP- : 209
PtMADS26 : -----VTADSENVGY---EEGQSSSISVT-----NVCNSN-GPL- : 209
SlJ : NNGYREAGVVIFEPENGFNNNNEDGQSSSISVT-----NPCNSI-DPPP- : 224
CaJ : --GYKNP--IVFEPEIEFN---YEEGQSSSISVT-----NPCNST-GPP- : 216

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IbMADS3      : -----AVINSDNMLN---EEGLSSESIT-----NVCNST-SPPQ- : 211
AcSVP1       : -----ATDSNVIN---EQGQSSSVT-----NICNST-GPM-- : 209
CtSVP        : -----AYG---EEGQSSSVN-----NICNSS-NAPP- : 200
AtSVP        : GMIICNNVHA-HGGAESENAAVYEEGQSSSESIT-----NAGNST-GAP-- : 222
BrSVP        : GQIYNNVHERYGGGSENIAYVEEGHSESSESIT-----NAGNST-GAP-- : 223
CaS22        : ----- : -
PkMADS1      : -----AASDSENI-VYDEGQSSSVN-----ACNSV-GPP-- : 209
AmINCO       : -----ATPDSEIIHVYEEGQSSSVT-----YTCNST-GLP-- : 211
EgSVP        : T-----DSETIIN---EEGLSSESIT-----NVCSSS-SGPP- : 209
HvBM10       : V-----VVDTENVIAEDGQSSSVSM-----TALHSGSSQ--- : 208
LpMADS10     : V-----VADTENVIAEDGQSSSVSM-----TALHSGSSQ--- : 208
ZmM19        : S-----VADTENVIAEDGQSSSVSM-----TALHSGSSQ--- : 208
ZmM26        : A-----VADTENVIAEGQSSSVSM-----TALHSGSSQ--- : 208
OsMADS22     : -----VVDTENFVTE--GQSSSVSM-----TALHSGSSQSQ- : 208
HvVRT2       : A-----VAD--VVPEDVHSSDSVM-----TAVHSASSQ--- : 206
TaVRT2       : A-----VAEAENVVPEDAHSSDSVM-----TAVHSGSSQ--- : 209
FaVRT2       : ----- : -
OsMADS55     : A-----VPDTENVLTEDGQSSSVSM-----TALNSGSSQ--- : 230
OsMADS47     : P-----GPD-SEIVYEEGQSSSVT-----NASYPRPP- : 228
HvBM1        : V-----AADPLVVVYDEGQSSSVT-----NTSYPRPPL--- : 207
PtMADS21b    : LVDL-----DT-----AVQEERMSSESSTN-----VCCSSSGPPV- : 210
PtMADS21a    : LVDL-----DT-----AVQEERMSSESSTN-----VCCSSSGPPV- : 210
PtMADS7      : LVDL-----DT-----AVQEEGMSSESSTN-----VCSSSGPPV- : 210
VvSVP2       : TMES-----DN-----MILEEGQSSSITN-----VYSCSSGPPQ- : 210
SlMBP24      : VTD-----MVMEEGQSSSIIIT-----TNNPDQDDSSNASLKLGGTT- : 217
StMADS16     : LTD-----MVMEEGQSSSIIIT-----TNNPDQDDSSNASLKLGGTT- : 217
PhFBP13      : HSEL-----EC-----MVMEEGQSSSITTHVCSCSSGPPEDDYSNASLKLGCNG : 226
IbMADS4      : IADV-----EAAG--LIPEEGQSSSITT-----NVCSCNSGPPP- : 213
CaC10        : IPDL-----DKDM--LI-EEGQSSSIT-----NVCSCNSGPPP- : 211
AcSVP2       : TG-----GAESDNMVAEEQGQSSSGTN-----VCSCNSAPPP- : 213
AcSVP3       : TGVV-----GAELDNVVAEEQGQSSSVTN-----VCSCNSAPPP- : 217
CsMADS18     : VLVD-----SSDVRVAAEEGLSSESAAAN-----VYSCNSGPP- : 212
AtAGL24      : -----LETLEAKLTTLKEALETSVTTN-----VSSYDSGTP- : 203
PmDAM1       : MKPE-----RLNNNFSGGGGEEGMSSES-----ATSTTCNSAPSL- : 218
PpDAM1       : MEPE-----RLNNNIGGGGEEGMSSES-----ATSTTCNSAPSL- : 225
PmDAM2       : TEPE-----RFINNIGDGG-EEGMSSESATN-----ATISSCSSLGLSL- : 223
PpDAM2       : TEPE-----RFVNNIGGGG-EEGMSSESATN-----ATISSCSSLGSL- : 223
PmDAM3       : MDPE-----RLNDNVGGGGEEGMSSES-----AISTTCNSALSL- : 218
PpDAM3       : MEPE-----RLSNNIGGGGEEGMSSES-----AISTTCNSALSLSP- : 221
PmDAM5       : MDPE-----RLNNNIEGGGEEGMSSES-----AISTTCNSAVSL- : 217
PpDAM5       : MDPE-----RLNNNIEGGGEEGMSSES-----AISTTCNSAVSL- : 218
PmDAM6       : VEPE-----TLITNVGGGGGDDMSSESAAVI-----ATSTSCNSAFSL- : 224
PpDAM6       : VEPE-----TLITNVGGGGGDDMSSESAAII-----ATSTSCNSAHL- : 221
PmDAM4       : MEP-----SDS-----ATSTSCNSALSL- : 207
PpDAM4       : MEP-----SES-----ATSTSCNSALSL- : 207

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*          320          *
PsSVPc       : --LEDDSSDISLKLGLPFP----- : 218
MtSVPc       : --LEDDSSDTSIKLGLPFPN----- : 225
LjSVPc       : --LDDSSVTSIKLGLPFPN----- : 192
GmSVPc1      : --LEDGSSDISLTLGLPFSN----- : 234
GmSVPc2      : --PEDGSSNISLTLGLPFSN----- : 234
PvSVPc       : --VEDGSSDTSIKLGLPFSK----- : 233
PsSVPb       : --EDGSDSISLKLGLPFPN----- : 212
MtSVPb       : --EDDG-SDTSIKLGLPFLK----- : 213
GmSVPb1      : --EDGG-SNTSLKLGLP----- : 206
GmSVPb2      : --EDGG-SDTSIKLGLP----- : 205
PvSVPb2      : --EDGG-SDTSIKLGLP----- : 204
LjSVPb       : --EEDG-SDTSIKLGLPLLHK----- : 211
GmSVPb3      : --PDCGNSDTSIKLGLSLFE----- : 206
GmSVPb4      : --PDNGSSDTSIKLGLSLFE----- : 208
PvSVPb1      : --PDNGPSDTSINLGLRLFE----- : 206
GmSVPb5      : --EQGQLFDTSITLGLPFPAGSK----- : 206
GmSVPb6      : --EQGRSFDTSITLGLSFPAGSKYRVSEQ-- : 211
PvSVPb3      : --EQEQSFDTSITLGLPFPSPDSK----- : 205
GGM12        : VIPDNEVSDTSLHLGLPSHS----- : 236
CsMADS19     : NSQDYD--DISLKLGL----- : 223
CsMADS20     : -SQDYDSSDTSIKLGLV----- : 217
VvSVP4       : PNQGLHNSDTSIKLGLPFSN----- : 217
VvSVP5       : PNQGHNSDTSIKLGLPFSN----- : 218
VvSVP3       : ----- : -
PtMADS48     : PRQDYDNSCSFLTGLPFPD----- : 220
PtMADS47     : PRQDYDNYSSFLTGLPFPD----- : 220
PtMADS27     : PRQDCDSSCAFLKGLPFPD----- : 221
PtMADS28     : PRQDNDSSCAFLTGLPFPD----- : 221

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PtMADS29	: P-----FLTLGLPFRD-----	: 212
StMADS11	: SHQDYNDSDTSLKLCIAFP-----	: 221
PhFBP25	: DHQDYNDSDTSLKGLPFPY-----	: 219
CaS21	: -----	: -
AcSVP4	: PPQDYDSSDTSLKLSLPFPN-----	: 215
EeDAM2	: QDFGGSDLDTFLRLGLPFPN-----	: 220
EeDAM3	: QDFGGSDLDTFLRLGLPFPN-----	: 220
EeDAM1	: -----	: -
MtSVPa1	: --QDYESSDTSLKGLPYAG-----	: 227
MtSVPa2	: --QDYESSDTSLKGLPYAG-----	: 228
PsSVPa	: --QDYESSDTSLKGLPYAG-----	: 228
GmSVPa2	: --QDFESSDTSLKGLPYSG-----	: 227
GmSVPa1	: --QDYESSDTSLKGLPYSG-----	: 227
PvSVPa	: --QDYESSDTSLKGLPYSG-----	: 227
LjSVPa	: --LEDDSSDTSLKGLPY-----	: 227
CsMADS17	: --QDLESSDTSLKGLPYSG-----	: 228
PpSVP	: --QDYESSDTSLKGLCV-----	: 225
MdJNT	: --QDYDSSDTSLKGLCV-----	: 224
VvSVP1	: --QDYESSDTSLKGLPYSG-----	: 227
PtMADS26	: --HDYESSDTSLKGLPFSN-----	: 227
SlJ	: --QDDSSDTSLKGLPYSG-----	: 242
CaJ	: --QDDSSDTSLKGLPYSG-----	: 234
IbMADS3	: --DYDSSDTSLKGLPY-----	: 227
AcSVP1	: --QDYESSDTSLKGLPYSG-----	: 227
CtSVP	: --PESESSDTSLKGLPYAG-----	: 218
AtSVP	: --VDESSDTSLRLGLPYGG-----	: 240
BrSVP	: --VDESSDTSLRLGLPYGG-----	: 241
CaS22	: -----	: -
PkMADS1	: --QDYDSSDTSLKGLPYSG-----	: 227
AmINCO	: --QDYDCSDTYLKLGLPYSG-----	: 229
EgSVP	: --QEDDSSDTSLKGLPYNG-----	: 227
HvBM10	: --DNDDGSDVSLKIALP---WK-----	: 225
LpMADS10	: --DNDDGSDVSLKGLPCSAWK-----	: 228
ZmM19	: --DNDDGSDVSLKGLPCVAWK-----	: 228
ZmM26	: --DNDDGSDVSLKGLPCVAWK-----	: 228
OsMADS22	: --DNDDGSDVSLKGLPCGAWK-----	: 228
HvVRT2	: --DNDDGSDISLKIALP---WK-----	: 223
TaVRT2	: --DNDDGSDISLKIALP---WK-----	: 226
FaVRT2	: -----	: -
OsMADS55	: --DNDDGSDISLKGLP-----	: 245
OsMADS47	: --DNDYSSDTSLKGLHS-----	: 244
HvBM1	: --DTESSDTSLRLGLSLFNSK-----	: 227
PtMADS21b	: ---EDDSSDTSLKGLAILS-----	: 227
PtMADS21a	: ---EDDSSDTSLKGLAILS-----	: 227
PtMADS7	: ---EDDSSDTSLKGLAI-----	: 225
VvSVP2	: ---EDDSSDTSLKGL-----	: 222
SlMBP24	: -AVEDDCSITSLKGLPFS-----	: 235
StMADS16	: -AVEDECSITSLKGLPFS-----	: 235
PhFBP13	: PTVEDDCSDTFLKGLPFPN-----	: 245
IbMADS4	: ---EDDCSDTSLKGLPIN-----	: 229
CaC10	: ---EDDCSDTSLKGLPFPN-----	: 227
AcSVP2	: ---EDDCADTSLKGLSFY-----	: 229
AcSVP3	: ---EDDCSDTSLKGLPF-----	: 232
CsMADS18	: --ADDDSSDTSLKGLPPCPN-----	: 230
AtAGL24	: ---LEDDSDTSLKGLPSWE-----	: 220
PmDAM1	: -LEDDSDVTLSTLKLGLP-----	: 235
PpDAM1	: -LEDDSDVTLSTLKLGLP-----	: 242
PmDAM2	: -LEDDCSDVTLSTLKLGLP-----	: 240
PpDAM2	: -LEDDCSDVTLSTLKLGLP-----	: 240
PmDAM3	: -LGDDSDVTLSTLKLGLP-----	: 235
PpDAM3	: -SLGDDSDVTLSTLKLGLS-----	: 239
PmDAM5	: -LEDDSSDEVTLSTLKLGR-----	: 234
PpDAM5	: -LEDDSSDEVTLSTLKLGR-----	: 235
PmDAM6	: -LEDDCSDVTLSTLKLGLP-----	: 241
PpDAM6	: -LEDDCSDVTLSTLKLGLP-----	: 238
PmDAM4	: -LEDECSDETLSTLKLGRS-----	: 225
PpDAM4	: -LEDDCSDVTLSTLKLGLTVRAGRPMCLKT	: 237

### Sequence alignment for Figure 7.3

Protein alignment of AtSVP, AtAGL24 and SVP-like proteins from pea and *Medicago*, showing exon boundaries. Where introns were not isolated in pea, their location was predicted from *Medicago*. For each protein, the last amino acid coded by an exon is coloured. Locations of the MADS-box, intervening (I), keratin (K), and C-terminal domains are indicated. This alignment was used to determine which exons corresponded between genes, for colour coding of exons in Figure 7.3. Full-length predicted protein sequences were aligned with ClustalX (Thompson et al., 1997) and manually adjusted with GeneDoc (Nicholas and Nicholas, 1997). For residues that are not at exon boundaries, shading indicates degree of conservation: black = 100%, dark grey = 80%, light grey = 60%. Full sequence details are given in Table A6.1.

		*	20	*	40	*	60	
AtSVP	:	MAREKIQIRKIDNATARQVTFSKRRRGIFKKAEELSVLCDADVALIIFSSSTGKLFEFCSS	:	60				
AtAGL24	:	MAREKIQIRKIDNITARQVTFSKRRRGIFKKAEELSVLCDADVALIIFSSATGKLFEFCSS	:	60				
MtSVPa1	:	MAREKIQIKKIENSTARQVTFSKRRRGIFKKAEELSVLCDADVALIIFSSSTGKLFEYSNL	:	60				
MtSVPa2	:	MAREKIQIKKIENSTARQVTFSKRRRGIFKKAEELSVLCDADVALIIFSSSTGKLFEYSNL	:	60				
PsSVPa	:	MAREKIQIKKIENSTARQVTFSKRRRGIFKKAEELSVLCDADVALIIFSSSTGKLFEYSNL	:	60				
MtSVPb	:	MTRKKIQIKKIENISSRQVTFSKRRRGIFKKAEELSTLCDADIALMVFSATSKLFEYASS	:	60				
PsSVPb	:	MTRKKIQIKKIENISSRQVTFSKRRRGIFKKAEELSTLCDADIALMVFSATSKLFEYASS	:	60				
MtSVPc	:	MARQIKIKKIENSTARQVTFSKRRRGIFKKAEELSTLCDAEVGLVIFSTTGKLYEYASS	:	60				
PsSVPc	:	MARQIKIKKIENSTARQVTFSKRRRGIFKKAEELSVLCDAEVGLIIFSTTGKLYEHCSS	:	60				
	<-		MADS-DOMAIN				-	
		*	80	*	100	*	120	
AtSVP	:	SMKEVIERHNLQSKNLEKLDQPSL--ELQLVE--NSDHARMSKETADKSHRLROMRGEDLQ	:	117				
AtAGL24	:	RMRDILGRYSLHASNINKLMDEPS--THLRLE--NCNLSRLSKEVEDKTKQLRKLRGEDLD	:	117				
MtSVPa1	:	SMREILERRHHLSKNIAKLEEPSL--ELQLVE--NSNCSRLSKEVAQKSHOLROMRGEDLQ	:	117				
MtSVPa2	:	SMREILERRHHLSKNIAKLEEPSL--ELQLVE--NSNCSRLSMEVSKKSHOLROMRGEDLE	:	117				
PsSVPa	:	SMREILERRHHLSKNIAKMEEEPSL--ELQLVE--NSNCTRRLNKEIAEKSHOLROMRGEDLQ	:	117				
MtSVPb	:	SMQOVIERRRNGYSANHRLLDYESTDDQLQ--VESDSNRDTLRKKLEDKSRELRQNGEDLQ	:	119				
PsSVPb	:	SMQOVIERRRNGCSENHRLMDREST-DQFC--VESESS--DTLHKLEDKSRELRQNGEDLQ	:	117				
MtSVPc	:	NMKDIITRYGQQSHHTKLDKE---LQVQ--VE--KNMPAELNKEVADRTQQLRGMKSEDFE	:	115				
PsSVPc	:	SMKDIITRYNQHSKQINKLDKT---LPLQ--VV--KMSMAELQKEFADKTQQLRGLKGEDFE	:	115				
	><-		I-DOMAIN		-><-		K-DOMAIN	
		*	140	*	160	*	180	
AtSVP	:	GLDIEELQOLEKALETGLTRVLETKSKIMSEISELQKKGMOLMDENKRLRQGTQLTEE	:	177				
AtAGL24	:	GLNLEELQRLKLLSEGLSRVSEKKGECVMSOIFSLEKRGSELVDENKRLRDKLET----	:	173				
MtSVPa1	:	GLSLEELQOLEKSLTGLGRVLETKGEKIMMEINELQTKGROLMEENNRKLRHVSG----	:	173				
MtSVPa2	:	GLNVEELQOLERSLEIGLGRVIENKGEKIMMEINDLQKGROLMEENDRLKHHVAG----	:	173				
PsSVPa	:	GMNVEQLQHLERSLEIGLGRVIENKGEKIMMEIQHLQKGROLMEENDRLKRVHTG----	:	173				
MtSVPb	:	BLTVQELQKLEVLKRSLSVSKIKDEMFMRDIDTLKRKEVELMEENNRKLRHVVPD----	:	175				
PsSVPb	:	BLTVQELQKLETLRLRSLSVSKKKDEMFIQGINTLKRKEVELIRENQRLKRVVPD----	:	173				
MtSVPc	:	GLNLEGLQOLEKSLSEGLKRVLEMKEKKILNETIKALRMKEIMLEENKHLKQKMMAM----	:	171				
PsSVPc	:	GLNLDGLQOLELTLETGLKSVLEMKEKRIMNETIGALQIKSIELEQEEKNHLKQKMMAM----	:	171				
			K-DOMAIN		-><-		C-DOMAIN	
		*	200	*	220	*	240	
AtSVP	:	NERLGMQICNVVHAHGAESENAAYVEEGOSSESIT--NAGNS--TGAPVDSESSDTSRLKG	:	235				
AtAGL24	:	-----IERAKLT--TLKE-----ALETESVTNVSSYDSGTPLEDDSS--DTSRLKG	:	215				
MtSVPa1	:	-----MFN--CKMFGGVESEN--MVTEEGOSSESVT--NVYNS--TGPPQDYESSDTSRLKG	:	222				
MtSVPa2	:	-----IIN--DRMVGGESEENENVVNEGOSSESVT--NVYNS--IGPPQDYESSDTSRLKG	:	223				
PsSVPa	:	-----MMNNGKIVGVCEEN--VVEEGOSSESIT--NVYNS--IGPPQDYESSDTSRLKG	:	223				
MtSVPb	:	-----LINVRWQ-----QSLETVISGSSFS-----LEDDG--SDTSRLKG	:	208				
PsSVPb	:	-----LIIGQRQ-----QSLESVIRSSYF-----LEDGGSDTSRLKG	:	207				
MtSVPc	:	-----LSMCKSP--IFGSDSITMQENVSAESMN--NVSSCNSGPSLEDDSSDTSRLKG	:	220				
PsSVPc	:	-----LFKCKCP--LLGD-----LDVSCESMN--NVSCNSGPSLEDDSSDTSRLKG	:	214				
			C-DOMAIN					
AtSVP	:	LPYGG	:	240				
AtAGL24	:	LPSWE	:	220				
MtSVPa1	:	LPYAG	:	227				
MtSVPa2	:	LPYAG	:	228				
PsSVPa	:	LPYAG	:	228				
MtSVPb	:	LPFLK	:	213				
PsSVPb	:	LPFHN	:	212				
MtSVPc	:	LPFPN	:	225				
PsSVPc	:	LPFP-	:	218				
		->						