FLOWER AND INFLORESCENCE DEVELOPMENT IN THE GARDEN PEA

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"Be Bold, Be Bold; But Not Too Bold,
Lest That Your Hearts Blood Should Run Cold"

'Mr. Fox'
English Fairy Tales
F.A. Steele 1979

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

S. Taylor

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Abstract

This thesis explores the action and nature of several flowering genes in pea (*Pisum sativum* L.), focusing on genes concerned with the transition to the flowering state and specification of the inflorescence and floral meristems. Some of these genes also influence development of vegetative organs, establishing genetic links between vegetative and reproductive development.

Stp and *Pim* are essential for the identification of the floral meristem in pea, and mutations at either gene result in proliferous flowers. Characterisation of two new alleles at *Stp* indicates that this gene is also involved in leaf and inflorescence development. These effects may be mediated through the regulation of cell division. Molecular analyses revealed that *Stp* is likely to be the pea homologue of *Fim* from *Antirrhinum*, and *UFO* from *Arabidopsis*. Two independently isolated alleles at *Stp* possess single base substitutions in the *peafim* sequence. The disruption to the predicted amino acid sequences reflects the severity of the mutant phenotypes. Unlike *Stp*, the effect of mutations in *Pim* are flower specific. Similar (collaborative) studies have revealed that *Pim* is the pea homologue of *Squa / AP1*. *Peasqua* (*Pim*) is expressed early in the developing floral primordia, and later in the outer two whorls, suggesting an underlying conservation of the *Squa / AP1 / Pim* activity.

The *Lf* and *Gi* genes in pea control the transition to flowering, whilst *Det*, *Veg1* and *Veg2* are required to specify the inflorescences and flowers. The genetic interactions between mutations in these five genes have revealed possible mechanisms underlying the transition to flowering, and of flower and inflorescence development in pea. *Lf* appears to suppress *Veg2* activity resulting in delayed flowering. Flowering is only promoted when the level of stimulus produced under the control of *Gi* is sufficient to inhibit *Lf* activity. Thus, flowering may be controlled through a negative regulatory pathway. *Veg1* acts in combination with *Veg2* to identify secondary inflorescences and flowers.

Mutations in *Aero* promote flecking on the leaves. The *aero* mutant phenotype is associated with an acceleration of plant development – plants carrying *aero* attain their adult leaf form, flower and senesce at an earlier node than their wild-type siblings. Similar effects were seen for two independent *aero* alleles, but were not associated with a second gene (*Arg*) which also promoted leaf flecking. Thus, *Aero* identifies an additional gene influencing both flower and leaf development in pea.

Mutations affecting the response to the environment have been well characterised in pea, and the current studies provide an opportunity to link environmental responses with inflorescence and floral meristem identification. The homologies between *Stp*, *Fim* and *UFO*, and *Pim*, *Squa* and *AP1*, provide links between flowering in pea and other model species, and contribute to the general understanding of the control and evolution of flowering.

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Abbreviations

C-*x*	First node bearing *x* or greater than *x* leaflets
cDNA	complementary DNA
cv.	cultivar
DNA	Deoxyribonucleic acid
EDTA	ethylene-diamine-tetra-acetic acid
FLR	Flower / leaf relativity
FT	Flowering time
IAA	Isoamyl alcohol
JIC	John Innes Centre, Norwich, UK
L*x*-*y*	Length between nodes *x* and *y*
LB	Lurio-Bertoni media
LD	Long day photoperiod (18-h daylength unless otherwise noted)
MB	Meldolablue
MOPS	3-(N-morpholino) propanesulphonic acid
MTT	3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NFI	Node of flower initiation
NFD	Node of flower development
PCA	Principal component analysis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SD	Short day photoperiod (8-h daylength unless otherwise noted)
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride, sodium citrate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TN	Total nodes
TL	Total length
VEI	very early initiating

Chapter I. Introduction

The flower is the most derived structure produced on any plant. Not only does it contain the reproductive structures: the stamens bearing pollen and the carpel bearing ovules, but it also often includes two further specialised structures – the calyx and perianth; these act to protect the reproductive structures and attract pollination vectors. It is thought that these four specialised structures have evolved from leaves, and that the entire flower is a highly derived, determinate shoot (Eames 1961, Scagel *et al.* 1966)

The transition to flowering is one of the most fundamental developmental changes an angiosperm can make. It requires a dramatic reorganisation of the plant body to produce inflorescences and flowers, each of which possesses specialised organs, and often a unique phyllotaxy. It also marks the attainment of reproductive maturity, flowers producing the micro- and megaspores necessary for genetic recombination and production of the next generation. The transition to flowering also requires a marked shift in the patterns of gene expression. Those genes specific to vegetative development must be down-regulated, and genes required to organise the specialised organs present in the inflorescence and flower expressed. Furthermore, the expression of these flower-specific genes must be controlled both spatially and temporally.

The conserved, four-whorled structure of the angiosperm flower provides an opportunity to examine the divergence of a developmental pathway. Despite the conservation of the basic flower structure (Endress 1994a), flowers form the most diverse structure produced on flowering plants, differing in size, shape, colour, and positioning of the flowers on the inflorescence. In addition, the typically conserved four-whorled structure is often modified, and flowers containing only three types of floral organs are also found. For example, members of the Amaryllidaceae may have flowers where the sepals and petals are indistinguishable and brightly coloured; alternatively, some monoecious and dioecious species fail to develop carpels or stamens, producing single sex – staminate or pistillate – flowers.

This introduction gives a brief account of the origin of flowers, both in terms of the evolution of the angiosperm group from 'non-flowering' ancestors, and a consideration of the genes involved in the conversion of a leafy shoot into a flower. This reflects an increase in the general interest in the relationship between these two apparently separate areas of study (Chasan 1993, Doyle 1994, Meyerowitz 1994). It

also reflects a requirement to understand where a system has arisen from in order to understand why a system acts as it does – natural selection acts on a phenotype, not on individual genes (Murfet 1977). Thus, an understanding of where the genetic systems controlling floral meristem identification and development may have evolved from will provide insights into why the systems act as they do.

The origin of the flower - evolution of the angiosperms

The first accepted angiosperm fossils are of dispersed organs found in the Early Cretaceous, over 140 million years ago (Mya) (Friis *et al.* 1987, Sanderson and Donoghue 1994). Within 15 million years the angiosperms formed a diverse assemblage with aquatic, herbaceous and shrubby members (Dilcher and Crane 1984, Crane *et al.* 1989, 1995, Taylor and Hickey 1990). By the mid-Cretaceous both wind- and insect-pollinated types, and bi- and unisexual flowers were present, and the monocots, Magnoliid dicots, and the eudicots (a highly diverse group that contains over 70% of extant species) had evolved and begun to diversify (Friis *et al.* 1986, 1994, Crane *et al.* 1986, 1989, 1994, Stewart and Rothwell 1993).

The angiosperms are thought to have originated as weedy species of arid disturbed habitats and many of the specialisations seen in early angiosperms and closely related groups were adaptations for an accelerated lifecycle, rapid regeneration, and seasonally dry conditions (Bakker 1978, Doyle and Donoghue 1986, Crane *et al.* 1995). Many of the differences noted between angiosperms and gymnosperms may be explained by the attainment of adult characteristics in an essentially juvenile plant, suggesting an origin of angiosperms through paedomorphosis, resulting from precocious maturation (progenesis) (Meyen 1984, Doyle and Donoghue 1986). The early colonising lifestyle and rapid reproductive behaviour of the angiosperms may have been promoted by the large scale destruction of established plants by the foraging of large herbivorous dinosaurs (Bakker 1978, Crane *et al.* 1995). In addition, these early flowering plants were probably pollinated by insects (Crane *et al.* 1995), and the current diversity of flowering plants is in part due to plant-pollinator specialisations (Crepet and Friis 1987).

A number of angiosperm characters must be considered when looking for possible ancestors in the fossil record: the reticulate venation pattern of the leaves, the presence of two integuments, the ovules surrounded by a carpel, and the clustering of the micro- and megasporophylls (pollen and ovule bearing 'leaves') together with sterile 'bracts' into a flower. However, the defining feature of the angiosperms may

not be the production of a 'flower', but rather the production of a carpel, and the double integument (Meyen 1984, Stewart and Rothwell 1993, Taylor and Kirchner 1996). The presence of the carpel prevents the pollen from having direct access to the ovule, and reflects the trend of land plants to enclose and hide their female gametophytes. It has been suggested that the closure of the carpel, and subsequent stigmatic germination of the pollen was central to the angiosperms radiation in the early Cretaceous (Doyle and Donoghue 1986). Stigmatic pollen germination allows the development of self-recognition systems (self-incompatibility), which are present in many primitive angiosperms (Meyer 1984, Crepet and Friis 1987) and is suggested to have increased species diversity by encouraging outcrossing and thus genetic recombination.

The original angiosperm

The flower structure of the 'original' angiosperm was once envisioned as somewhat like that of the present-day magnolias; leaf-like sepals, and large showy petals surrounding the many stamens and carpels that were spirally arranged around an elongate receptacle (e.g. Cronquist 1981, Dilcher and Crane 1984, Doyle and Donoghue 1986, Crane *et al.* 1995, Thorne 1996). The flowers terminated the branches produced on woody understorey shrubs, or small trees found in tropical or semitropical forests. This assumption was based primarily on the recognition that members of the magnolia family, particularly members of the Winteraceae (Thorne 1996), have retained many primitive characteristics. Although fossil evidence suggests that magnolia-like species arose early in angiosperm evolution (e.g. Dilcher and Crane 1984), an herbaceous origin of the angiosperms has begun to find favour, and the search for early angiosperms is shifting to primitive herbaceous plants, the paleoherbs (Taylor and Hickey 1990, 1996, Endress 1994a). This hypothesis suggests that the original angiosperm was a rhizomatous or scrambling plant bearing simple leaves, with small, trimerous, bisexual flowers in their axils, much like those of *Cabomba*, *Lactoris*, and the monocots (Taylor and Hickey 1990, 1996, Doyle 1994, Doyle *et al.* 1994, Tucker and Douglas 1996, **Figure I.1**). Thus, the 'original' flower would have had carpels with several anatropous ovules, anthers differentiated into a filament and stamen, and seed containing both perisperm and endosperm (Doyle 1994, Doyle *et al.* 1994). The 'original' flower may have had an undifferentiated perianth (**Figure I.1**) – the distinction between sepals and petals was a later derived specialisation in the angiosperms; the petals may have developed from stamens (or stamenoids) independently in a number of different lineages (Crepet *et al.* 1992, Drinnan *et al.* 1994, Kosuge 1994).

Alternatively, the 'original' flower may have been derived from a reduction of a branched shoot (a pseudanthial origin of floral parts), where the male and female sporangia were borne on indeterminate axes that had been simplified during the development of the flower (Doyle and Donoghue 1986, 1987, Friis *et al.* 1987, Taylor and Hickey 1990, Hickey and Taylor 1996). The simple, perianthless flowers of the Chloranthaceae or the Piperaceae would then be considered to be the most primitive (basal) (Taylor and Hickey 1990, Hickey and Taylor 1996). More complex flowers may then have been formed from a compressed inflorescence of such flowers, with the sepal and petal whorls derived from subtending bracts (Hickey and Taylor 1996). The pseudanthial hypothesis is supported by the inflorescence structure of fossil angiosperm-like plants such as *Sanmiguelia* (Stewart and Rothwell 1993, Taylor and Hickey 1996). Differentiation between the two possible origins of the angiosperm flower may not be possible if based solely on morphological analyses.

Fossil angiosperms from mid-Early Cretaceous (early Barremian-Aptian) deposits support the herbaceous origin of the angiosperms. These fossils consist of small, bisexual or unisexual flowers, with an undifferentiated perianth, and massive stamens (Taylor and Hickey 1990, Crane *et al.* 1994, Friis *et al.* 1994) although even at this early age there is considerable morphological variation. The herbaceous origin of the angiosperms is also supported by the lack of identifiable angiosperm wood in early fossil deposits containing angiosperm derived leaf and pollen deposits (Dilcher and Crane 1984, Taylor and Hickey 1990).

Cladistic analyses of extant seed-bearing plants (angiosperms and gymnosperms) do not resolve the most basal angiosperm groups; various studies using molecular and/or morphological characteristics place an aquatic herb (*Ceratophyllum*), small woody shrubs or vines (Illiciales), or the Nymphaeales as the earliest branching angiosperms – those presumably closest to the ancestral form (Chase *et al.* 1993, Doyle *et al.* 1994, Soltis *et al.* 1997). Although *Ceratophyllum* possesses many primitive characteristics and a long fossil history (Les 1988, Dilcher 1989, Endress 1994b), its placement as an outgroup to the angiosperms based on *rbc*L sequence data is probably an anomaly as it shows variable placement in unrooted trees (those without a gymnosperm outgroup), and in trees from rRNA sequence data (Qiu *et al.* 1993, Soltis *et al.* 1997). Most often, the secondary basal groups belong to the Piperales or Nymphaeales, both paleoherb groups (Chase *et al.* 1993, Doyle *et al.* 1994, Soltis *et al.* 1997), and these two families support alternative flower origins. Although the Chloranthaceae have also been considered to be remnants of the original angiosperm type (Taylor and Hickey 1990, Hickey and Taylor 1996), phylogenetic analyses

indicate that these are more closely related to the woody magnoliids (e.g. see Tucker and Douglas 1996). The differences between phylogenetic analyses do not clarify the most basal groups and tend to produce conflicting results. Thus, the most basal flowering group, and by implication the original flower phenotype, cannot yet be determined from these cladistic analyses. However, all cladistic analyses are consistent in indicating that the angiosperms are monophyletic - they have a single common ancestor, and that their closest living relatives are the Gnetales.

The anthophytes

The Gnetales consists of three loosely related families that share a number of characteristics in common with angiosperms. These include the presence of vessels and a tunica and corpus, reduced megagametophyte, and double fertilisation (Cronquist 1981, Doyle and Donoghue 1986, Friedman 1990). The double fertilisation found in *Ephedra* results in the production of additional diploid embryos, rather than the nutritive triploid endosperm (Friedman 1990, 1992), but suggests that this process arose in the common ancestor of the angiosperms and the Gnetales. Although members of the Gnetales are now either monoecious or dioecious, the presence of an apparently aborted ovule in male flowers of *Welwitschia*, and the occasional bisexual flowers of *Ephedra* (Crane 1985, Doyle and Donoghue 1992), suggest a bisexual ancestor with microsporophylls surrounding the central megasporophylls.

Although characteristics such as double fertilisation, and the presence of a tunica layer are unlikely to be recognised in fossil species (Doyle and Donoghue 1986, 1992), cladistic analyses that include both fossil and extant groups support the close relationship between angiosperms and the Gnetales and have added two further, extinct groups – the Bennettitales (Upper Triassic to Upper Cretaceous), and *Pentoxylon* (Jurassic) (Crane 1985, Doyle and Donoghue 1986, 1992, Albert *et al.* 1994, Doyle *et al.* 1994). These four groups form a clade referred to as the anthophytes (**Figure I.2**), with angiosperms sister to the Bennettitales, *Pentoxylon* and the Gnetales. The anthophyte clade is very stable, and they may therefore represent a natural group (Doyle and Donoghue 1986). All members possess some characteristics that suggest an acceleration of reproductive development: clustering of their micro- and megasporangia into bisexual strobili, production of small seeds, and the possibility of insect pollination (Doyle and Donoghue 1986, Crepet and Friis 1987). Therefore all the anthophytes may have been weedy, early colonising species and may have been adapting to the same environmental conditions (Sanderson and Donoghue 1994, Crane *et al.* 1995). The anthophyte clade also implies that the flower, as a bisexual

reproductive structure, existed before the separation of these lineages, and is of more ancient origin than the angiosperms lineage.

The cladistic analyses of Doyle and Donoghue (1986, 1992), and to a lesser extent Crane (1985), suggest that the angiosperms diverged from the anthophytes early in their evolution (Triassic, 200 Mya) and simplified the microsporophylls to produce the anther, whilst retaining the primitive complex megasporophyll (**Figure I.2**). In contrast, the Bennettitales and Gnetales first reduced their megasporophyll, and the microsporophyll of the Gnetales was secondarily reduced (**Figure I.2**). Alternative scenarios are possible (Crane 1985, Doyle and Donoghue 1986, Doyle *et al.* 1994). For example, the angiosperms may be derived from the Bennettitales, although this requires regaining the complexity of the megasporophyll. A second alternative is that the Gnetales / Bennettitales / *Pentoxylon* and the angiosperm lineages arose separately from the extinct seed-fern group Caytoniales, which is basal to the anthophyte group (Doyle and Donoghue 1986). This would suggest that either the Caytoniales are also members of the anthophytes, or that the 'flower' evolved separately in the two lines. Whichever scenario is correct, these cladistic analyses (Crane 1985, Doyle and Donoghue 1986, 1987, Albert *et al.* 1994, Doyle *et al.* 1994) point to the seed ferns as the likely progenitor of the anthophytes and the angiosperms, and therefore the likely source of the flower.

The Caytoniales

Fossil evidence suggests that the Caytoniales seed fern group became distinct from other seed ferns in the Middle Triassic, and survived to be contemporaries of the angiosperms in the Early Cretaceous (Crane 1985, Stewart and Rothwell 1993). *Caytonia* bore several small ovules with single integuments in fleshy cupules on a bilaterally symmetrical, pinnate megasporangia (Crane 1985). The small size of *Caytonia* seeds (2-4 mm), the reduced megaspore wall, and thick nucellular cuticle are also characteristics of the anthophyte clade (Crane 1985, Doyle 1994), and suggest a trend for progenetic acceleration of the lifecycle had begun before the anthophytes arose (Doyle and Donoghue 1992). *Caytonia* leaves were palmate, bearing four leaflets, with an angiosperm-like epidermis, stomata, and venation (Thomas and Spicer 1987, Trivett and Pigg 1996). The microsporophylls were also bipinnately divided (Crane 1985, Stewart and Rothwell 1993) and, during early stages of development, the microsporophyll possessed bilateral symmetry akin to that seen in the anther (Stewart and Rothwell 1993). Thus, progenesis could also explain the origin of angiosperm stamens from *Caytonia* microsporophylls.

The microsporophylls, megasporophylls and leaves of *Caytonia* have not been found joined, so the basic organisation of the *Caytonia* plant is unknown. However, aggregation of the sporophylls into a 'flower' is considered unlikely (Doyle 1994). The stem of *Caytonia* is represented by small twigs, and it has been suggested that *Caytonia* was a small shrubby plant (Crane 1985).

The Corystospermaceae were Gondwanic woody plants with characteristics that place them in the Caytoniales (Stewart and Rothwell 1993). Although their pinnate leaves suggests that they are more primitive than *Caytonia* and fossil Corytosperms are found earlier (in the middle Triassic) the Coryosperms possessed fewer (to solitary) ovules in each cupule (Crane 1995, Stewart and Rothwell 1993) and bracts subtending, or forming part of, the megasporophyll (Taylor and Archangelsky 1985). These characteristics are prerequisites for the development of the double integumented angiosperm ovule, and a possible source of the carpel wall. The reduction in number of ovules per cupule and pollen wall characteristics place the Corystosperms as the most likely family from which the anthophytes were derived (Crane 1985). Thus, the combined characteristics of the Caytoniales supports the proposed relationship to the anthophytes and angiosperms, and the possibility of a Caytoniales-angiosperm lineage (Trivett and Pigg 1996).

The Glossopteridales

Glossopteris was a diverse group of large gondwanic trees. They bore their ovules in cupules that in some species were fused to their subtending leaf (Crane 1985, Stewart and Rothwell 1993). The bifurcating microsporophylls were also adnate to a leaf. Although falling below the Caytoniales in cladistic analyses of Doyle and Donoghue (1986, 1987) and Crane (1985) (**Figure I.2**), the Glossopterids have also been suggested as possible angiosperm ancestors (Meyen 1984, Stewart and Rothwell 1993), with the ovules per cupule reduced to one, and the subtending leaf rolling over to form the carpel wall. However, *Caytonia* had a reduced megaspore wall and an anatropous cupule, considered characteristic of the anthophyte group, and these characters are absent from the Glossopterids (Crane 1985, Doyle and Donoghue 1986).

Conclusions

Phylogenetic analyses and hypotheses based on fossil remains provide tantalising glimpses of the possible origins of the anthophytes and the angiosperms (Crane 1985, Doyle and Donoghue 1986, 1987). However, these certainly do not cover all possible origins of the angiosperms and alternatives to the anthophyte concept exist (e.g. Doyle and Donoghue 1992, Doyle 1994, Loconte 1996). Many characters significant in the definition of the angiosperms or anthophytes (such as double fertilisation to produce an endosperm, the production of a pollen tube, the presence of a tunica layer in the apical meristem, and lignin chemistry) are not preserved in fossil material (Doyle 1994). Therefore, their status cannot be determined for many groups significant to the origin and diversification of the anthophytes. If these states, or characteristics such as a flower-like structure in the Caytoniales or Glossopterids, were to be identified in fossils this would strengthen the relationships between the anthophytes (Doyle 1994), but fail to identify the origin of the flower. It appears that the flower is of more ancient derivation than the angiosperms, and it raises the possibility that the genetic controls of floral meristem identification and development identified in higher angiosperms is of similar ancient origin.

The origin of the flower - the genetics of floral meristem identification

The timing of flowering in relation to autonomous and environmental signals provides a significant and diverse area of study, and a number of reviews have been published regarding the physiological and genetic control of flowering time (Lang 1965, Zeevaart 1976, Murfet 1977, Bernier 1988, Bernier *et al.* 1995). Both natural and induced variation in flowering time has been observed in a wide range of plants (for examples see reviews in Halevy 1985, 1986, and 1989) and some of this variation is heritable. However, the genetic variation identified in most species examined results from allelic differences at only a few genes. Two exceptions to this are the mouse-eared cress, *Arabidopsis thaliana* L., a diminutive member of the Brassicaceae; and the garden pea, *Pisum sativum* L. of the Fabaceae. In *Arabidopsis*, over 20 genes have been examined that influence flowering behaviour (e.g. Koornneef *et al.* 1981, Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995, Amasino 1996), whilst the genetic interactions and physiological activities of approximately ten flowering time mutants have been examined in detail in pea (reviewed in Murfet 1985, 1989, Murfet and Reid 1993, Reid *et al.* 1996, Weller *et al.* 1997b). Contrasting models for the control of flowering time based on the characterisations of the mutants in these two model species have been presented (Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995, Reid *et al.*

1996, Weller *et al.* 1997b). The differences between these two models may reflect the evolutionary plasticity of the environmental control of flowering time. Despite this plasticity, similar differences between plants grown under inductive versus non-inductive conditions has been observed in a wide range of species, including *Arabidopsis* and pea (Reid and Murfet 1984, Kelly and Davies 1988, Shannon and Meeks-Wagner 1991, Schultz and Haughn 1993, Wallace *et al.* 1993b, Martínez-Zapater *et al.* 1995). Thus, the possibility remains that the genetic control underlying environmental responses has been conserved.

Few of the genes known to affect flowering time have been cloned (Lee *et al.* 1994, Putterill *et al.* 1995, Bradley *et al.* 1997, Macknight *et al.* 1997), and although evidence exists for their presence in a number of distantly related species (Bradley *et al.* 1996a, Simon and Coupland 1996, Wilson and Dean 1996), the identification of homologies between flowering time genes from different species has yet to be presented. Thus, it is premature to conduct a synthesis of this work with a view to considering their evolutionary implications. However, interest in this area is growing and it will provide a rich area of future research.

In contrast, a detailed picture of floral meristem identification and flower development has emerged from studies of mutants in *Arabidopsis*, and in the distantly related *Antirrhinum majus* L. (Scrophulariaceae) (for reviews see Schwarz-Sommer *et al.* 1990, Coen and Meyerowitz 1991, Weigel and Meyerowitz 1993, Ma 1994, Clark and Meyerowitz 1994, Yanofsky 1995). Identification of homologous genes from other distantly related eudicot and monocot species has indicated that the specification of the flower has been largely conserved during the evolution of flowering plants (e.g. Pnueli *et al.* 1991, van Tunen 1996). These same homologies have also indicated how the systems controlling flower development have diverged.

Identification of the floral meristem

Although flower development has been studied most extensively in *Arabidopsis*, none of the mutations identified in this species completely prevents the transition to flowering. In contrast, two mutants in the garden pea have a completely vegetative phenotype. Mutants *vegetative1* (*veg1*) and *vegetative2-1* (*veg2-1*) are characterised by the continued production of leafy shoots that replace the secondary inflorescences normally produced in this species (Reid and Murfet 1984, Murfet and Reid 1993, Reid *et al.* 1996). Therefore these genes are essential for the transition from the vegetative to the flowering state. That the *Veg1* and *Veg2* genes of pea have

separate functions is exemplified by their distinct interactions with a third mutant *determinate* (*det*), which converts the apical meristem into a terminal stub (Singer *et al.* 1990). Although both the *veg2-1* and *veg2-2* mutant alleles are epistatic to *det*, the addition of *det* to a *veg1* mutant background results in the production of a terminal flower directly from the apical meristem (Singer *et al.* 1994, Murfet *et al.* 1995, Reid *et al.* 1996). The complete prevention of flowering in *veg2-1* mutants in a *det* background suggests that *Veg2* is essential for the identification of floral meristems. The phenotype of plants with the intermediate allele, *veg2-2* (Murfet and Reid 1993, Reid *et al.* 1996), suggests that *Veg2* may also be required for defining the secondary inflorescence of the garden pea.

– *the* Floricaula *homologues*

The *floricaula* (*flo*) mutation of *Antirrhinum* also completely prevents the production of flowers (Carpenter and Coen 1990, Coen *et al.* 1990). However, in contrast to *veg1* and *veg2*, the inflorescence is identified normally, whilst the flowers of *flo* mutants are replaced by reiterating indeterminate inflorescences. The wild-type *Flo* allele is also required to maintain the indeterminacy of the inflorescence meristem, as mutant *flo* inflorescences may eventually produce clusters of sepals and carpelloid structures (Carpenter *et al.* 1995). Mutations in *LEAFY* (*LFY*), the *Arabidopsis* homologue of *Flo*, delay the production of floral characteristics and early flowers may be replaced by coflorescences (Schultz and Haughn 1991, Huala and Sussex 1992, Weigel *et al.* 1992) (**Figure I.3b**). *lfy* mutants also fail to suppress bract development in the inflorescences (**Figure I.3b**). However, even null *lfy* mutants eventually produce abnormal flowers that consist entirely of sepals and carpels (Schultz and Haughn 1991, Huala and Sussex 1992, Weigel *et al.* 1992). Thus, the phenotypes of *lfy* mutants are less severe than those of *flo*. The *Flo* and *LFY* homologues are single copy genes, and are thought to act as transcription factors (Coen *et al.* 1990, Weigel *et al.* 1992). Both *Flo* and *LFY* are expressed early during the development of the floral primordia (Coen *et al.* 1990, Weigel *et al.* 1992), and *Flo* is also expressed in the bract subtending *Antirrhinum* flowers (Coen *et al.* 1990). The expression of *Flo* and *Lfy* occurs before any recognisable floral primordia arises, and may be the first signal identifying the flower (Coen *et al.* 1990, Weigel *et al.* 1992, Bradley *et al.* 1996b). However, recent evidence indicates that *LFY* is expressed in three-day-old seedlings, and this expression gradually increases during plant development (Bradley *et al.* 1997, Blázquez *et al.* 1997). The increase in *LFY* expression occurs at a greater rate under inductive compared with non-inductive conditions (Blázquez *et al.* 1997). However, *lfy* mutations do not affect leaf

development, suggesting that *LFY* does not play a positive role during leaf development in *Arabidopsis* (Blázquez *et al.* 1997).

Expression of *NFL1* and *NFL2*, the *Flo* homologues from tobacco (an allotetraploid), and the *Flo* homologue from *Impatiens* also occurs in both vegetative and floral tissues (Kelly *et al.* 1995, Pouteau *et al.* 1997). Vegetative expression of the *Flo* homologues is also seen in *Eucalyptus* and poplar (Southerton *et al.* 1995, Strauss *et al.* 1995), suggesting that vegetative expression of *Flo* homologues may be common among angiosperms. The identification of *Unifoliata* as the pea homologue of *Flo* (Hofer *et al.* 1997), further supports the more generalised role of the *Flo* homologues in plant development, as *uni* mutations affect both flower and leaf development. The *uni* mutant is primarily characterised by the reduction of the compound pea leaf to a simple leaf, and by the simplification and proliferation of the flower (Marx 1987, Hofer *et al.* 1997). The phenotype of the *uni* flower in pea is similar to that of late-produced flowers on *lfy* mutants, although *uni* flowers contain additional flowers axillary to the sepals (Hofer *et al.* 1997). However, *uni* mutants do not show the complete transformation of flowers to proliferating inflorescences seen in both *lfy* and *flo* mutants, and the secondary inflorescence is correctly identified. It has been suggested that the more general effect of the *uni* mutation on plant development reflects an ancestral role of *Uni* in controlling indeterminacy of primordia (either leaf or flower) (Hofer *et al.* 1997).

It is still open to question whether the ancestral role of the *Flo* homologues was specific to the flower, or more generalised, and included roles in vegetative development. However, the results from tobacco, *Eucalyptus*, aspen, pea, *Impatiens* and *Arabidopsis* seem almost overwhelming. Therefore it appears likely that the lack of vegetative expression in *Antirrhinum* is an evolutionarily derived characteristic, and that *Flo* has lost any role in vegetative development. The expression of *Flo* in the bracts on the *Antirrhinum* inflorescence (Coen *et al.* 1990) may represent remnants of this ancestral activity.

– *the* Squamosa *homologues*

Unlike *Flo* and its homologues, *Squamosa* (*Squa*) from *Antirrhinum* and its *Arabidopsis* homologue, *APETALA1* (*AP1*) are specifically expressed in the floral primordia (Huijser *et al.* 1992, Mandel *et al.* 1992, Gustafson-Brown *et al.* 1994), and may mark floral determination (Hempel *et al.* 1997). The expression of the *Impatiens* and *Eucalyptus* homologues of *Squa* is also floral specific, which contrasts with the expression of the *Flo* homologue in these species (Harcourt *et al.* 1995, Kyozuka *et al.*

1997, Pouteau *et al.* 1997). In addition, the mutant phenotypes of *squa* and *ap1* are also specific to the flower (Irish and Sussex 1990, Schwarz-Sommer *et al.* 1990, Huijser *et al.* 1992, Bowman *et al.* 1993). Although the transition to flowering is severely affected in *squa* mutations (as it is in *flo*), these plants usually produce some aberrant flowers (Huijser *et al.* 1992). Severe *ap1* mutants are also able to undergo the transition from inflorescence to flower production, but the flowers produced retain many inflorescence-like characteristics (Irish and Sussex 1990, Bowman *et al.* 1993, **Figure I.3c**). The addition of a second *Arabidopsis* mutant, *cauliflower* (*cal*), to an *ap1* mutant background completely transforms the aberrant flowers into proliferating inflorescences (producing a phenotype much like that of the common cauliflower) (Bowman *et al.* 1993). However, the *cal* mutation has no effect in a wild-type (*AP1*) background (Bowman *et al.* 1993). The functional redundancy of *ap1* and *cal* mutations reflects their close molecular relationship (Kempin *et al.* 1995, Prugganan *et al.* 1995). The mutant phenotypes of the *squa* (and *flo*) homologues in other species have yet to be confirmed, although a number of mutants from other species have been proposed as potential homologues (e.g. Singer *et al.* 1994, Coen and Nugent 1994, van Tunen 1996, Allen and Sussex 1996).

The combination of the *lfy* and *ap1* mutations in *Arabidopsis* completely prevents the production of flowers, and a proliferating leafy inflorescence – much like that seen in severe *flo* mutants – is produced (Huala and Sussex 1992, Weigel *et al.* 1992, Bowman *et al.* 1993). This phenotype suggests that *LFY* and *AP1* may act in concert to correctly identify the floral meristem. Despite their synergy, the *Flo* and *Squa* homologues probably act in distinct pathways to regulate floral meristem identification (Shannon and Meeks-Wagner 1993, Schultz and Haughn 1993). The *ap1 lfy* double mutant phenotype is also very similar to that of the *veg1* and *veg2-1* single mutants of pea. However, the relationship between *Veg1* and *Veg2*, and *Uni* and the *Squa* homologue in pea has yet to be determined.

– *overexpression of* LFY *and* AP1

Although aspen is only distantly related to *Arabidopsis*, overexpression of *LFY* in this tree species is able to reduce the flowering time from years to months (Weigel and Nilsson 1995). The phenotype of the *LFY*-overexpressing tree is very similar to that produced by overexpression of *LFY* in *Arabidopsis* itself – solitary flowers are produced in the axils of the leaves, and the main apex is consumed in the production of a terminal flower (Weigel and Nilsson 1995). Although the number of rosette leaves produced before the production of the terminal flower in the transgenic *Arabidopsis* is identical to that produced in the wild-type plant when grown under long days, fewer

leaves are produced under short day conditions. The phenotype of the *LFY*-overexpressing plants is highly dependent on the activity of *AP1*, and in *ap1* mutants, the production of axillary flowers is at least partially suppressed (Weigel and Nilsson 1995). Significantly, expressing the *Antirrhinum* gene *Flo* in *Arabidopsis* is unable to complement the *lfy* mutant phenotype (Coen and Nugent 1994), although overexpressing *LFY* in a *lfy* mutant is able to restore a wild-type phenotype (Weigel and Nilsson 1995).

Overexpression of *AP1* in *Arabidopsis* is also able to convert the apical and vegetative lateral meristems into flowers (Mandel and Yanofsky 1995). Unlike the dependence of *LFY*-overexpression on *AP1*, the addition of a *lfy* mutant allele is unable to suppress the production of lateral flowers in *AP1* overexpressing lines. *AP1*-overexpression also reduces the number of rosette leaves produced under both long and short day conditions (Mandel and Yanofsky 1995), possibly reflecting the more specific role *AP1* plays in identifying the floral meristem. Overexpression of either of the *Eucalyptus* homologues of *AP1*, *EAP1* or *EAP2*, in *Arabidopsis* also results in early flowering, and the conversion of the apical meristem into a flower (Kyozuka *et al.* 1997), thus revealing further conservation of activity underlying the control of flower development. The complete conversion of vegetative shoots into flowers in *LFY*- or *AP1*-overexpressing lines of *Arabidopsis* suggests that either gene alone is sufficient to upregulate all the genes involved in flower development. This suggests the presence of some mechanism for cross communication between the two apparently independent pathways represented by *LFY* and *AP1*.

Inflorescence development – the Centroradialis *homologues*

The *Centroradialis* (*Cen*) mutant from *Antirrhinum* is characterised by the conversion of the normally indeterminate inflorescence meristem into a terminal flower (Stubbe 1966, Coen and Nugent 1994, Bradley *et al.* 1996a). Thus, the *Cen* gene and its putative homologues are considered significant in controlling the difference between the indeterminate and the determinate inflorescence types (Coen and Nugent 1990, Bradley *et al.* 1996a, 1997). Consistent with its mutant phenotype, the *Cen* gene is expressed in a group of sub-apical cells of the inflorescence meristem (Bradley *et al.* 1996a), and *Cen* may act non-cell-autonomously to prevent the expression of floral meristem identity genes, such as *Flo*, in the apical meristem (Huijser *et al.* 1990, Coen and Nugent 1994, Bradley *et al.* 1996a). The ectopic expression of *Flo* in the apical meristem of *cen* mutant plants supports this proposition (Bradley *et al.* 1996a). The *Arabidopsis* homologue of *Cen*, *TERMINAL FLOWER* (*TFL*, **Figure I.3d**), also

actively inhibits the expression of *LFY* and *AP1* (and possibly other flower specific genes) in the apical meristem, maintaining the distinction between the *Arabidopsis* inflorescence and floral meristems (Shannon and Meeks-Wagner 1991, 1993, Alvarez *et al.* 1992, Okamuro *et al.* 1993, Gustafson-Brown *et al.* 1994, Bradley *et al.* 1997). The similar mutant phenotype and activities of the *Cen* and *TFL* homologues further emphasises the conservation of the genetic controls underlying flower and inflorescence development. A significant difference between the *cen* and *tfl* mutants is the earlier flowering behaviour of *tfl* mutants (Shannon and Meeks-Wagner 1991, Zagotta *et al.* 1993, Bradley *et al.* 1997). Unlike *Cen*, *TFL* is also expressed during early plant development, and thus may play an additional role in delaying the commitment to flower production (Bradley *et al.* 1996a, 1997).

The expression of floral (axillary) characteristics in the normally indeterminate (apical) inflorescence meristem in *cen* and *tfl* mutants is similar to the effect of the *det* mutant of pea, which expresses secondary (axillary) inflorescence characteristics in the primary (apical) inflorescence. This results in the conversion of the apical meristem to a hairy, terminal stub (Singer *et al.* 1990). If the *Det* mutant of pea should prove to be homologous to *Cen* and *TFL*, then the requirement for a functional *Veg2* allele to allow expression of the *det* mutant phenotype (Murfet *et al.* 1995, Reid *et al.* 1996) would imply that the *Arabidopsis* and *Antirrhinum* homologue of *Veg2* may also be essential for expression of the *cen* and *tfl* mutant phenotypes.

Floral development – the ABC model

Examination of three classes of floral homeotic mutants from *Arabidopsis* and *Antirrhinum* – each affecting two adjacent whorls – has led to the ABC model of flower development (Schwarz-Sommer *et al.* 1990, Bowman *et al.* 1991, Coen 1991; reviewed in Coen and Meyerowitz 1991, Coen and Carpenter 1992, Clark and Meyerowitz 1994, Weigel and Meyerowitz 1994, Yanofsky 1995, Haughn *et al.* 1995; **Figure I.4a**). In this model, combinations of three classes of gene activity specify the four whorls of the flower: A-class activity alone produces sepals, A activity in combination with B-class activity produces petals, B activity with C-class activity produces stamens, and C activity alone produces the central carpel, and suppresses the indeterminate growth of the floral meristem. Activity of A and C are mutually antagonistic.

Loss of the A, B or C functions results in homeotic changes within the flower. Loss of A-class activity allows ectopic expression of C-class activity resulting in a

flower that consists of carpels in the outer whorl, two whorls of stamens, and a central carpel (**Figure I.4b**). Mutations in *APETALA2* (*AP2*), *AP1* and *LEUNIG* (*LUG*) from *Arabidopsis* produce variations on this expected phenotype (Kunst *et al.* 1991, Bowman *et al.* 1991, 1993, Jofuku *et al.* 1994, Liu and Meyerowitz 1995). These three genes act together to provide A function. While *AP2* acts both to repress C-class activity, and specify organ identity, *AP1* and *LUG* carry out a single function each – organ identity and negative regulation of C-class genes, respectively (Drews *et al.* 1991, Bowman *et al.* 1993, Gustafson-Brown *et al.* 1994, Liu and Meyerowitz 1995).

The overlapping expression of *Deficiens* (*Def*) and *Globosa* (*Glo*) from *Antirrhinum* and their *Arabidopsis* homologues *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in the second and third whorls provides all of the B-class activity (Bowman *et al.* 1989, 1991, Hill and Lord 1989, Schwarz-Sommer *et al.* 1990, Sommer *et al.* 1990, Jack *et al.* 1992, Tröbner *et al.* 1992, Krizek and Meyerowitz 1996). Mutations in any of these genes produces a flower with first and second whorl sepals, and third and fourth whorl carpels (**Figure I.4**). The wild-type *Def* gene from *Antirrhinum* was able to rescue the production of petals and stamens in *ap3* mutants from *Arabidopsis*, suggesting at least some of the activity of these genes has been conserved (Samach *et al.* 1997). Class B activity is restricted to the second and third whorls by *SUPERMAN* a cadastral gene that prevents expression of *AP3* in the fourth whorl (Schultz *et al.* 1991, Bowman *et al.* 1992).

Two of the characteristics used to define the flower – determinancy, and the production of fertile sporophylls – depend on the activity of the C-class genes. Mutation in the C-class gene from *Arabidopsis*, *AGAMOUS* (*AG*), or the *Antirrhinum* homologue, *Plena*, results in an indeterminate flower that reiterates three whorls – sepals, petals and petals (**Figure I.4d**) (Bowman *et al.* 1989, 1991, Yanofsky *et al.* 1990, Bradley *et al.* 1993, Mizukami and Ma 1997). This double-flower phenotype results from the ectopic expression of class A activity, and double flowers such as those produced by mutations in *AG* and *Plena* were the earliest recognised floral mutation (Meyerowitz *et al.* 1989).

The emphasis given to these three classes of floral homeotic genes in genetic studies in other species is shown by the large number of homologous, or potentially homologous genes that have been cloned. These come from a wide range of species including other members of the eudicots: tomato (Pnueli *et al.* 1991, 1994a), tobacco (Hansen *et al.* 1993), petunia (van der Krol *et al.* 1992, van der Krol and Chua 1993, Angenent *et al.* 1992, 1993, 1995, Tsuchimoto *et al.* 1993), pea (Beltrán *et al.* 1996), white campion (Hardenack *et al.* 1994), *Eucalyptus* (Harcourt *et al.* 1995), and sorrel

(Ainsworth *et al.* 1995); and the monocots: maize (Schmidt *et al.* 1993, Theissen *et al.* 1995) and rice (Chung *et al.* 1994). These results have suggested that the ABC model may be widely applicable to flowering plants, despite the enormous variation seen in flower morphology.

The conserved MADS-box motif is present in many of the floral homeotic genes identified (Sommer *et al.* 1990, Yanofsky *et al.* 1990, Schwarz-Sommer *et al.* 1990, 1992, Prugganan *et al.* 1995; for a review on the MADS-box family see Shore and Sharrocks 1995). Pursuit of genes containing the MADS-box motif has revealed further genes with both floral and/or vegetative expression patterns from a variety of species (Ma *et al.* 1991, Pnueli *et al.* 1991, Kush *et al.* 1993, Lu *et al.* 1993, van der Krol and Chua 1993, Angenent *et al.* 1994, Hardenack *et al.* 1994, Mandel *et al.* 1994, Colombo *et al.* 1995, Mena *et al.* 1995, Rounsley *et al.* 1995, Beltrán *et al.* 1996, Kang and Hannapel 1996). Although many of these genes have no known mutant phenotype, co-suppression in petunia and tomato has revealed potentially unique functions for some of the MADS-box genes (Tsuchimoto *et al.* 1993, Angenent *et al.* 1994, Pnueli *et al.* 1994b). The many MADS-box genes identified, and the many induced and naturally occurring mutations affecting the development and identification of the flower (e.g. Meyer 1966, Stubbe 1966, Blixt 1972, Komaki *et al.* 1988), indicate that a rich array of genes are involved in flower development. Further analysis of these genes, and their homologues from a wider range of plant species (particularly from primitive angiosperm groups) will provide further progress in understanding the control of flower organisation and development.

A mediator between floral meristem identification and floral organ development

Part of the gap between floral meristem identification and floral organ identification was filled by the characterisation of *Fimbriata* (*Fim*) from *Antirrhinum*, and its *Arabidopsis* counterpart *UNUSUAL FLORAL ORGANS* (*UFO*) (Ingram *et al.* 1995, 1997, Levin and Meyerowitz 1995, Simon *et al.* 1994, Wilkinson and Haughn 1995, **Figure I.3e**). These genes act with or after *Flo/LFY* to regulate the expression pattern of the B-class genes *Def/AP3* and *Glo/PI*. *Fim* may also regulate *Plena* expression (Simon *et al.* 1994, Ingram *et al.* 1997). This role is supported by their interactions with floral meristem and floral organ identity genes, their expression patterns, and the inability of *UFO*-overexpression to rectify the *pi* or *ap3* mutant phenotype (Ingram *et al.* 1995, Levin and Meyerowitz 1995, Simon *et al.* 1994, Wilkinson and Haughn 1995, Lee *et al.* 1997). The *Fim* homologue of *Impatiens*, like its *Flo* homologue, has a vegetative expression pattern, although this expression

pattern is more restricted than that of *Impatiens-Flo* (Pouteau *et al.* 1995). These, and results examining the effect of *UFO* overexpression in *Arabidopsis*, have suggested that *UFO* (and possibly all *Fim* homologues) is expressed independently of, but acts in co-operation with, *LFY* (*Flo* homologues) to control floral identification and also other aspects of plant development (Lee *et al.* 1997).

Conclusions

The identification of homologues of floral meristem identity, floral homeotic, and *Fim* genes from distantly related species points to the conservation of the genetic control of flower identification. This seems at odds with the enormous variety of flowers seen in the over 250, 000 species of extant angiosperms. This variation may reflect the divergence of the systems controlling flower development. A notable example of this is the homology between *green petals* from petunia that only affects the development of the whorl 2 petals, and *deficiens* from *Antirrhinum*, which affects whorls 2 and 3 (van der Krol *et al.* 1992, van der Krol and Chua 1993). In addition, the inability of *Antirrhinum Flo* to complement the *Arabidopsis lfy* phenotype (Coen and Nugent 1994), also suggests that fundamental differences in gene activity have evolved. Differences in inflorescence architecture, particularly the separation between determinate and indeterminate inflorescence types seem directly attributable to the *Centroradialis* homologues, but like *LFY* and *Flo*, the functions of *Cen* and *TFL* have also diverged (Bradley *et al.* 1996a, 1997). Until *Cen* homologues from other species, and their expression patterns are identified, it will remain unclear if the early flowering of *tfl* mutants reflects an ancestral function, or is a later elaboration on a simpler activity.

Evolution and the floral meristem identity genes

The identification of genes required for the correct identification of the flower leads to questions as to their origin; what role do these genes play in non-flowering plants and how have these roles been modified during the evolution of the angiosperms? Homologues of the floral meristem and floral homeotic genes have not yet been isolated from the Gnetales – the closest living relatives of angiosperms. Analyses of such homologues may produce a significant increase in our understanding of the control of floral meristem identification, the relationship between the reproductive structures of the Gnetales and the angiosperms, and the more recent origins of the flower. However, the close evolutionary relationship between the

Gnetales and angiosperms suggests that such studies will not resolve the ancestral role of these genes in reproductive development. The identification of homologues from greater numbers of diverse angiosperms, and also from more distantly related seed, and non-seed, plants are beginning to resolve these questions.

Conifer homologues of genes involved in floral meristem identification in angiosperms (both *Flo* homologues, and MADS-box containing genes) have been isolated, and, like their angiosperm counterparts, also show reproductive (cone) specific expression (Weigel and Meyerowitz 1993, Tandre *et al.* 1995, Mouradov *et al.* 1996, 1997a, 1997b, Liu and Podila 1997). This is consistent with the idea that these genes have played some role in reproductive development in many primitive species. The four conifers examined – *Ginkgo*, *Picea alba* (Norway spruce), *Pinus resinosa* (red pine) and *Pinus radiata* – are all only distantly related to angiosperms and probably arose as distinct lineages from ancestral seed-fern groups (e.g. Crane 1985, Doyle and Donoghue 1986). Despite this, phylogenetic analyses suggest that the functions of the MADS-box group of transcription factors had already diversified in the common ancestor of *Picea alba* and flowering plants (Tandre *et al.* 1995). Thus, their activities may have already diverged in the seed-ferns, and possibly even the non-seed-bearing (spore producing) ancestor(s) of the seed bearing plants. It is possible that the ancestral activities of the B- and C-class genes may be related to the development of heterospory – the specialisation into the male (many small), and female (few large) spores, with B-class activity required to distinguish the development of the microspores from that of the megaspores. Extant heterosporous ferns such as *Marsilea* may provide a means of testing this possibility. Heterospory is evolutionarily significant as it is a prerequisite for the development of pollen and the seed.

The separation of receptacle or A-class activity from B- and C-class activity also predates the separation of conifers from angiosperms (Tandre *et al.* 1995), suggesting that this divergence is similarly ancient in origin. This may reflect an ancient requirement to distinguish between vegetative and reproductive growth (A-class activity) and to produce fertile structures (C-class activity). The basic separation of these roles may even be found in homosporous plants. Consistent with this is the identification of at least two different classes of MADS-box genes in the homosporous fern *Ceratopteris* (Münster *et al.* 1997): one group that forms a sister group to all angiosperm and conifer MADS genes, and one, *Ceratopteris-MADS3*, that lies within the higher plant clade (Münster *et al.* 1997). However, the function of these genes is currently unknown. The duplication and diversification of MADS-box genes in the seed plant lineage may have been pivotal in the evolution of their more complex reproductive development.

The isolation of the *Floricaula* homologue from the moss *Physcomitrella patens* reveals its extremely ancient origin (Leech *et al.* 1995). Transgenic moss plants carrying an antisense moss-*Flo* cDNA have suggested that the *Flo* homologue is required for the transition from the filamentous protonemal to the leafy gametophye stage (M.J. Leech pers. com.). This suggests an ancestral function of the *Flo* homologue in controlled cellular proliferation and organisation of the meristem. This proposed function is reflected in the effect of the *uni* mutation on leaf and flower development in pea, and on the disruption of floral primordia development in *Arabidopsis* and *Antirrhinum*, as both flower and leaf development require controlled meristem development. The interaction between *LFY* and *UFO*, and the proposed role of *UFO* in cellular proliferation, also lends support to this suggestion.

The terminal flower produced by the *cen* and *tfl* mutants was once considered to be characteristic of the ancestral angiosperm (i.e. Magnolia-like). Thus, it has been proposed that *Cen* homologues may have been independently recruited to produce the indeterminate inflorescences seen in many groups of flowering plants (Bradley *et al.* 1997). However, the proposal that the earliest angiosperms either bore solitary axillary flowers (Doyle 1994), or produced indeterminate inflorescences (Taylor and Hickey 1990, Hickey and Taylor 1996) suggests that the ancestral *Cen* homologue was active in the apical meristem of the earliest angiosperms. Members of the Gnetales also produce axillary flowers and the Bennettitales contained species with both terminal and axillary flowers (Crane 1995, Stewart and Rothwell 1993). Thus the *Cen* homologue may also predate the anthophytes. Examination of the role of *Cen* homologues in the Gnetales, the closest living angiosperm relatives, and in more distantly related conifer species may provide further support for an angiosperm ancestor with indeterminate inflorescences.

The floral meristem identity and floral homeotic genes are apparently universal in angiosperms, and essential for the correct identification of the flower. Evidence suggests that homologues of these genes are also present in distantly related gymnosperms and in the more distantly related mosses. Certainly the flower specific genes in angiosperms have been recruited into their current roles from ancestral functions. However, what these functions were, and how the activity of these genes was modified to produce the current complex forms of the ABC model, remains unknown at present. The combined efforts of paleobotanists and of plant developmental biologists may provide insights into flower development and evolution. Although the origin of the angiosperms is unlikely to be completely resolved, continued examination of homologous genes from non-flowering plants may reveal the origin of the flower.

Aims and scope of this thesis

The garden pea has been used as a model species for the investigation of the control of floral induction for over forty years and these studies have led to a detailed understanding of the genetic control of flower initiation in pea (Murfet 1985, Reid *et al.* 1996, Weller *et al.* 1997b). Despite this, the control of inflorescence and flower identification has received little attention. Although mutations affecting these processes are known in pea (e.g. Monti and Devreux 1969, Blixt 1972, Sharma 1972, Muehlbauer and Konzak 1973, Gottschalk 1974, 1978, 1985, Reid and Murfet 1984, Singer *et al.* 1990, 1994, Beltrán *et al.* 1996), other than typically brief mutant characterisations, the role of the genes in flowering have largely been ignored. The identification and molecular characterisation of floral meristem identity and floral homeotic genes from *Antirrhinum* and *Arabidopsis* has led to a resurgence of interest in mutants with similar phenotypes in pea (Singer *et al.* 1994, Beltrán *et al.* 1996, Reid *et al.* 1996). Although the garden pea is not amenable to direct cloning of genes based solely on mutant phenotype, pea homologues may be identified by heterologous probing using clones from unrelated species (e.g. Beltrán *et al.* 1996, Hofer *et al.* 1997). Such analyses have already revealed unique roles of gene homologues in plant development (Hofer *et al.* 1997).

Although floral meristem identity genes have been cloned from a number of species, it is still unknown how the expression of these genes is regulated by inductive processes occurring in the leaves. The well characterised floral induction processes in pea (e.g. Figure 1 in Reid *et al.* 1996, and Figure 4 in Weller *et al.* 1997b) provides a further motivation for characterisation of genes involved in the identification of the inflorescence and flower in pea. Thus, the aims of this thesis are threefold: firstly, to identify possible homologies between flowering genes in pea and those in other species; secondly, to identify the genetic interactions that control the inflorescence architecture in pea; and thirdly, to determine the relationship of these genes to those genes involved in floral inductive processes in the leaves.

Towards these aims, **Chapters III, IV** and **V** describe the morphological and molecular characterisation of both new and previously described mutations affecting floral meristem identity in pea, while **Chapters VI** and **VII** discuss the interactions between genes involved in inflorescence development, the transition to flowering, and the production of the floral stimulus in the leaves. **Chapter VIII** details the previously unidentified heterochronic effects of a mutation affecting leaf flecking. An integration of these results, and a discussion of their implications in inflorescence and floral meristem identification and evolution, is presented in **Chapter IX.**

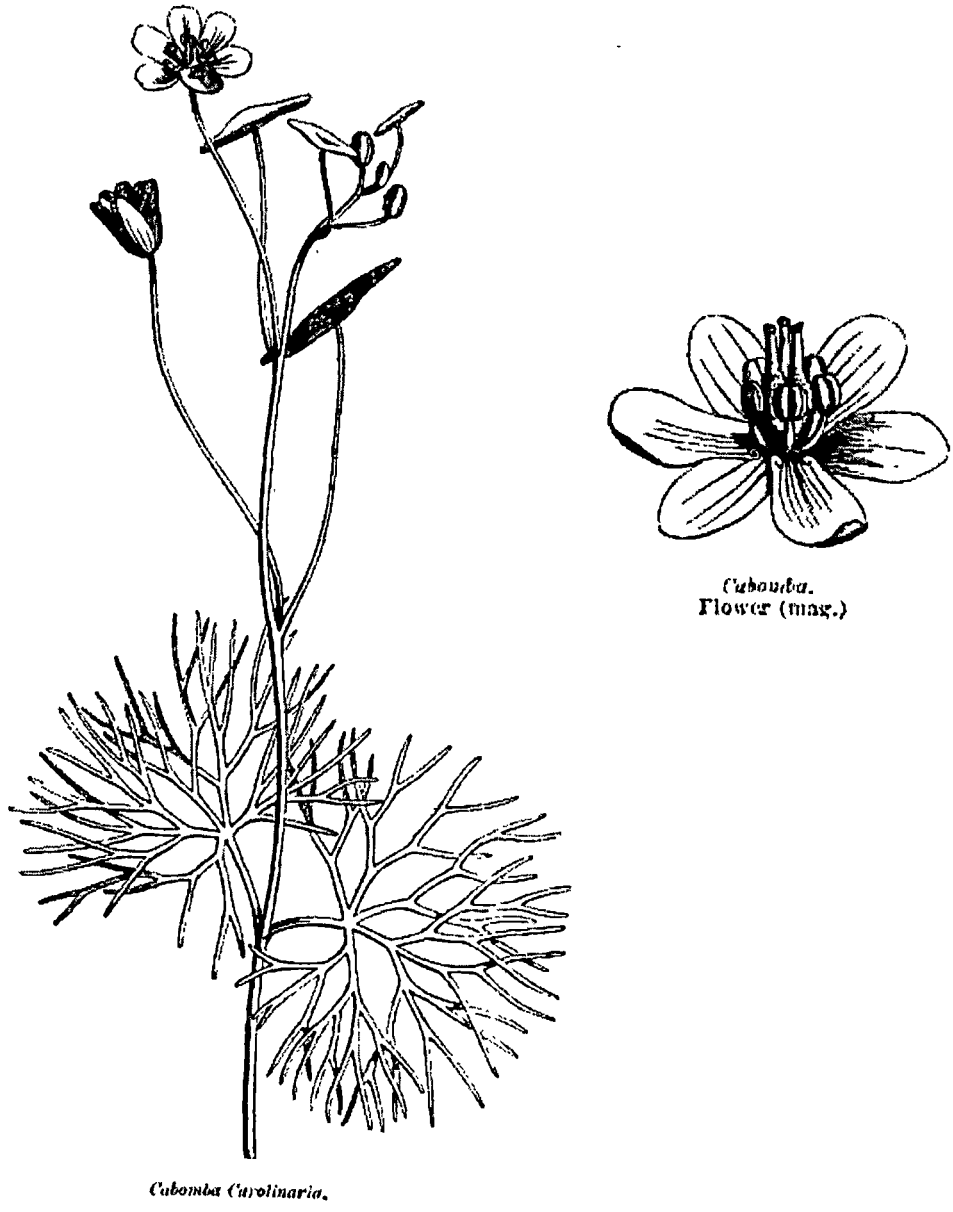


Figure I.1: The trimerous flower of *Cabomba* may represent the ancestral angiosperm flower. The flowers are bisexual, with three separate carpels, six stamens in a whorled arrangement, and a coloured perianth. The flowers are solitary, and produced in the axils of the leaves. The highly branched leaves of *Cabomba* are an adaptation to the underwater habit of this species, but the floating peltate leaves may be ancestral. *Cabomba* is a member of the Nymphales. This group, and members of the ancient group the Piperales, produce a perisperm (Doyle *et al.* 1994, Doyle 1994). The production of a perisperm may be a further ancestral characteristic. The survival of such an ancient group may be related to their aquatic lifestyle - the aquatic environment is more stable than the terrestrial.

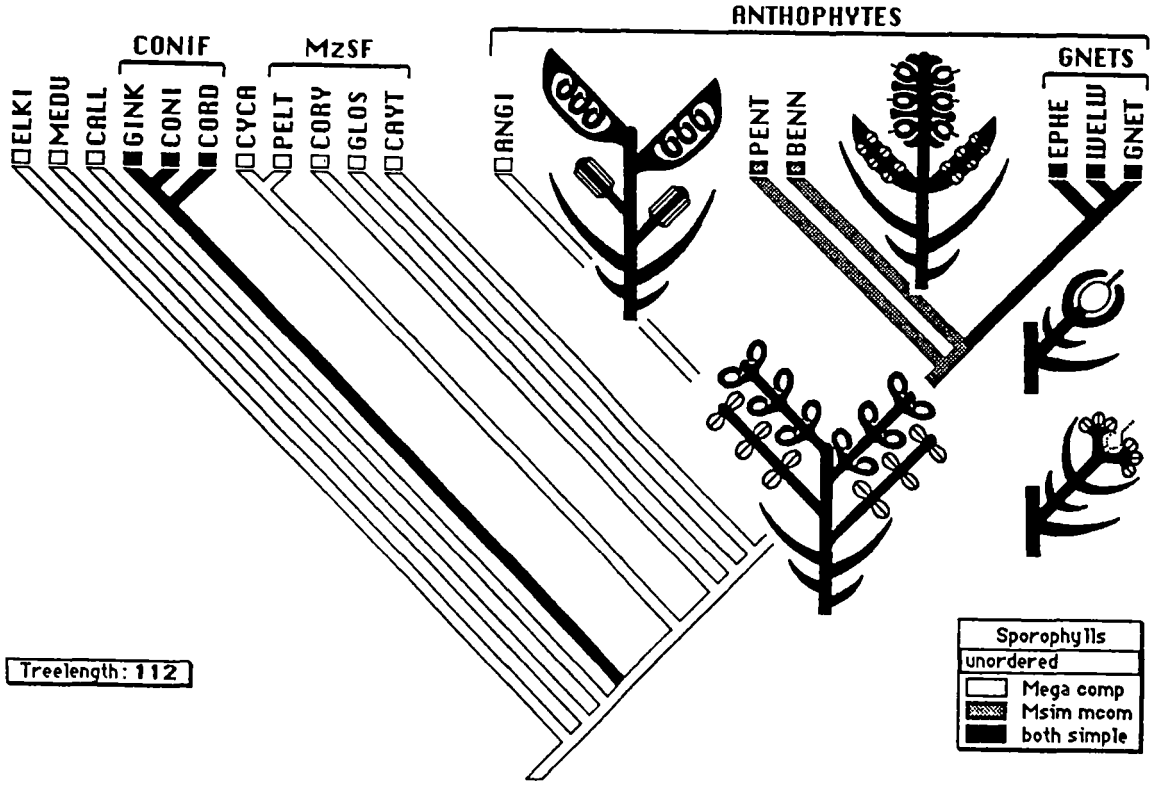


Figure I.2: Cladistic analysis of extant and fossil seed-bearing plants showing the hypothetical distribution of sporophyll characteristics for the anthophytes, and their common ancestor based on a euanthial hypothesis. ELKI, *Elkinsia*; MEDU, Medullosaceae; CALL, *Callistophyton*; GINK, Ginkgoales; CONI, Coniferales; CORD, Cordaitales; CYCA, Cycadales; PELT, *Peltaspermum*; CORY, Corystospermaceae; GLOS, Glossopteridales; CAYT, Caytoniaceae; ANGI, angiosperms; PENT, *Pentoxylon*; BENN, Bennittitales; EPHE, *Ephedra*; WELW, *Welwitschia*; GNET, *Gnetum*; CONIF, Coniferopsids; MzSF, Mesozoic 'seed ferns'; GNETS, Gnetales (from Doyle 1994). Similar cladograms were presented in Crane 1985, Doyle and Donoghue 1986, and Doyle *et al.* 1994 and are favoured by the support from stratiographic evidence of the timing of different groups origins.

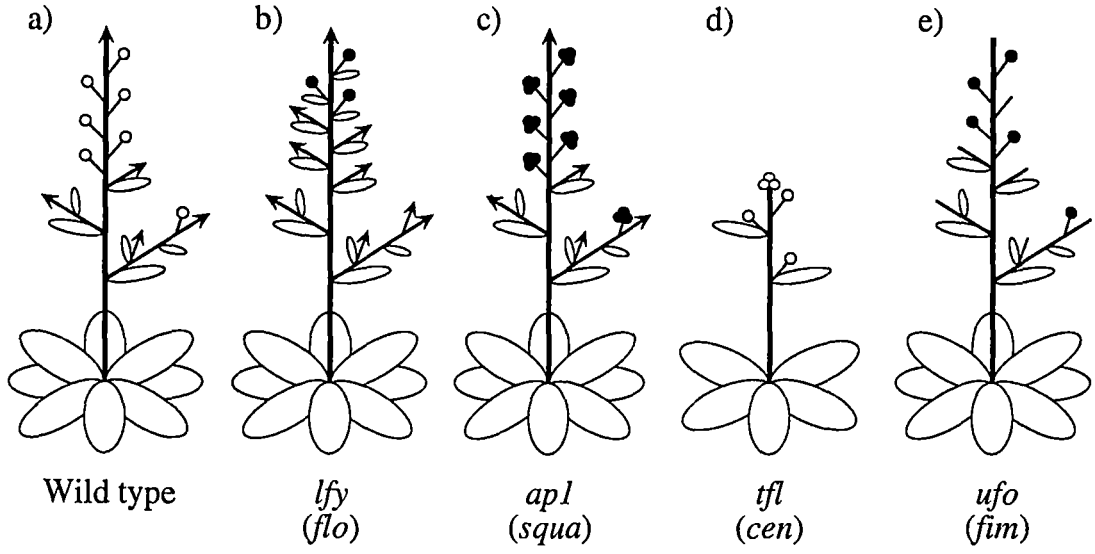


Figure I.3: Schematic representation of the floral and inflorescence meristem identity mutants of *Arabidopsis*. a) The wild-type *Arabidopsis* plant grown under typical (long day) conditions produces an indeterminate primary inflorescence upon floral induction. The inflorescence bears cauline leaves which subtend coflorescences (secondary inflorescences), and later produces solitary flowers. b) In the *leafy* mutant early flowers are replaced with coflorescences. The later formed flowers are abnormal and consist of sepals and carpels. All the flowers are borne in the axils of cauline leaves. c) The *apetala1* mutant produces flowers with indeterminate inflorescence-like characteristics; secondary flowers are borne in the axils of first whorl organs. The flowers also possess homeotic changes within the floral organs. d) The *terminal flower* mutant flowers early, and rapidly converts the primary inflorescence and coflorescence meristems into flowers. e) The *unusual floral organs* mutant produces abnormal flowers consisting primarily of sepals and carpels. Flowers may be replaced by filamentous structures, and the indeterminancy of the inflorescence may also be affected. The *Antirrhinum* homologue of these genes is given in parentheses under the *Arabidopsis* gene. leaf (cauline or rosette); flower; → indeterminate inflorescence; flower-like shoot or abnormal flower; proliferous flower; determinate inflorescence or filament.

Redrawn from Schultz and Haughn 1993, Haughn *et al.* 1995, Wilkinson and Haughn 1995, Bradley *et al.* 1997.

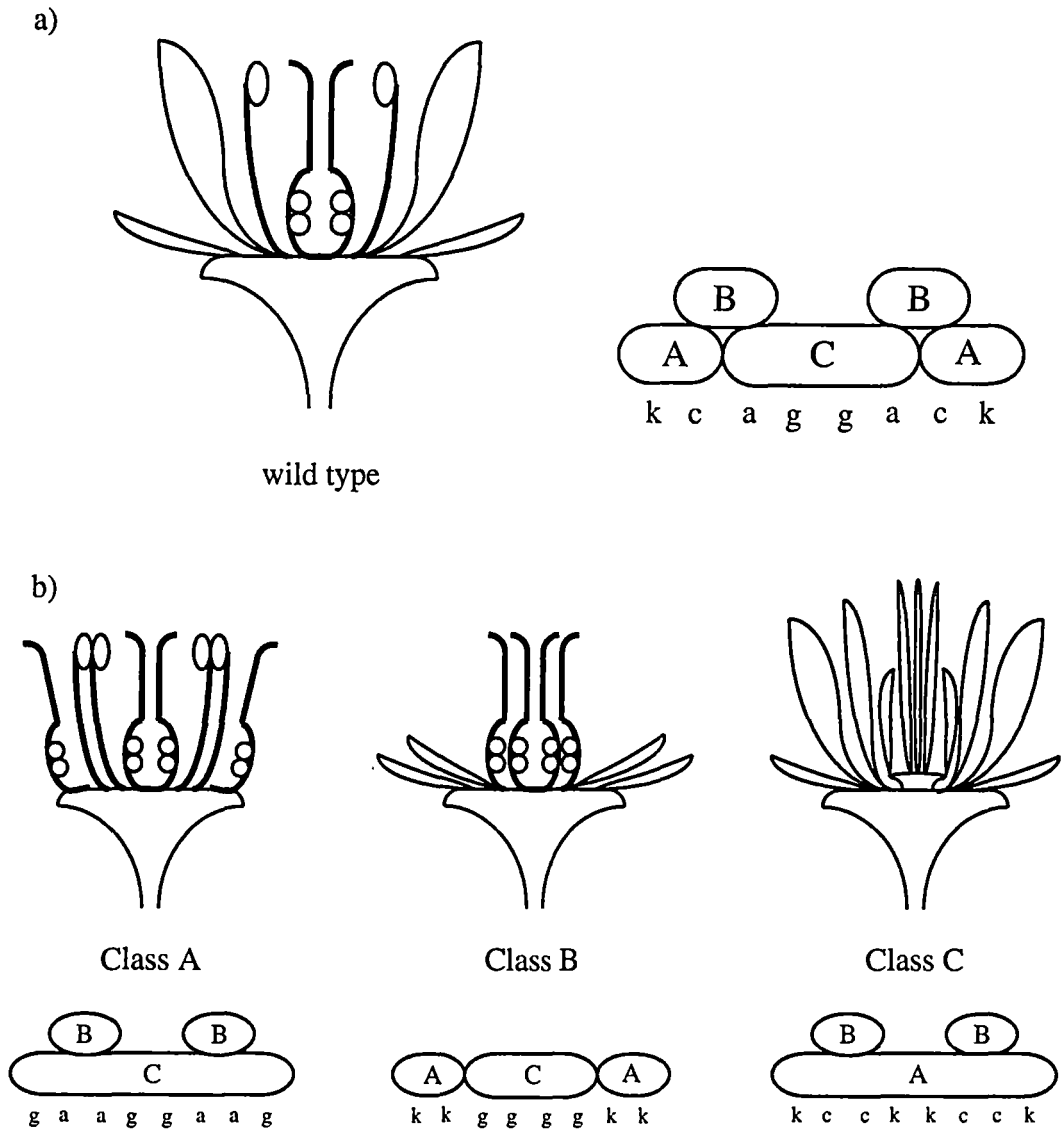


Figure I.4: The ABC model of flower development. Floral organ identity in the wild-type flower results from overlapping activities of three classes of floral homeotic genes. a) A cross section through a generalised flower, and the corresponding domains of activity of the three homeotic gene classes. b) The effect on flower phenotype of removing a single class of homeotic gene activity. k - sepals, c - petals, a - stamens, g - carpels. Redrawn from Schwarz-Sommer *et al.* 1990, Bowman *et al.* 1991.

Chapter II. General materials and methods

Phenology of the garden pea

The garden pea (*Pisum sativum* L.) is a caulescent, monocarpic, self-fertilising model legume with a long history of cultivation (Helbaek 1959, Zohary and Hopf 1973, Sutcliffe 1977, Marx 1977, Murfet 1985). The flowers are zygomorphic, pentamerous and encourage self-pollination. The sepals are green and fused at the base, forming a cup. The five petals are coloured and distinct. The vexillum or standard is largest (25 - 30 mm across in domestic cultivars) and surrounds the other four: two wings, and two fused petals that form the keel. Enclosed within the keel there are ten stamens – nine fused and one free – which surround the central single carpel (Makasheva 1983, Tucker 1989, Reid *et al.* 1996). Typically one or two flowers are borne on leafless secondary inflorescences that arise in the axils of the leaves on the primary inflorescence (Hole and Hardwick 1976, Tucker 1989, Singer *et al.* 1994, **Figure II.1**). Primary and secondary inflorescences are morphologically distinct and although both inflorescences are botanically indeterminate the secondary inflorescence typically terminates in a hairy stub after producing one or two flowers (Hole and Hardwick 1976, Tucker 1989, Singer *et al.* 1994). Secondary inflorescences arise over one of the two stipules (Makasheva 1983), in contrast to the vegetative lateral shoots which arise over the petiole of the leaf. The primary inflorescence bears normal leaves and except for the production of secondary inflorescences appears identical to the vegetative shoot from which it is derived (Makasheva 1983, Singer *et al.* 1994, **Figure II.1**).

Pea leaves are compound and show internal differentiation; the proximal structures are leaflets, the distal ones tendrils, and flanking the central rachis are two leafy stipules (Makasheva 1983, Young 1983, Gould *et al.* 1986, Marx 1987, **Figure II.1**). The pea leaf shows a consistent heteroblastic development (Gould *et al.* 1986, Wiltshire *et al.* 1994, Lu *et al.* 1996). The first two nodes possess rudimentary scale-like leaves. The first true leaf occurs at node three and possesses a single pair of leaflets. Leaf development occurs through the sequential addition of tendrils and leaflets at a steady rate until the most complex adult leaf form – typically with six leaflets and six tendrils – is formed (Wiltshire *et al.* 1994, Lu *et al.* 1996). Thus, the ontogenetic change in leaf form potentially provides an indication of the plants developmental age. The node at which changes in leaflet number occur is influenced by both the genotype and the environment (Wiltshire *et al.* 1994).

Pea lines used

Prof. I.C. Murfet supplied seed from a number of pure breeding pea lines that were used extensively in these analyses. The standard wild-type flowering genotype is *Lf E Sn hr* (Murfet and Reid 1993), and each line carries this genotype unless otherwise noted. This produces a late, quantitative long-day flowering phenotype. All other genes affecting flowering, and flower and inflorescence development do not appear to vary among domestic cultivars. In addition, each line carries the dominant *Tl* and *Af* required for normal leaf development unless otherwise specified. The standard lines HL2, HL24 (*e*), HL107, and Wt4042 all have a wild-type flowering phenotype. Mutant alleles of various flowering and leaf development genes were derived from HL7 (*lf-a tl*), HL31 (*Lf-d*), HL60 (*lf*), HL69 (*lf-a sn*), HL117 (*lf-a af tl*), HL172 (which segregates for *veg1*), HL216 (*det*), HL244 (*lf pim*), HL245 (*veg1 det*), M2/830 (*ppd-2*), MIII/122 (*aero*), Wt16005 (*veg2-1*), Wt16015 (*gi-2*), and Wt16123 (*veg2-2*). HL111 (Marx's multiple marker line A875-55-0, which carries the morphological markers *A b k st wb wlo gp tl r i le n te bt cp f s* and *fa*), HL59 (*lf sn*), HL34, and HL6 (which carries the wax gene *wa*) were used in linkage analyses (**Chapters III** and **VI**). In addition, Dr J.L. Weller provided the original population (M1a/324) that segregated for both a floral meristem identity and a *supaeromaculata* mutant (**Chapters III** and **VIII**), the population (M1a/224) segregating for what proved to be a *uni* mutant (**Chaper III**), and a single plant that was carrying a floral homeotic mutation (AF106) (**Chapter III**). These mutants were produced by James L. Weller in an EMS mutagenesis programme on the cultivar Torsdag (HL107) conducted in 1993 at the University of Tasmania in Hobart. Additional pure breeding type lines for uni^{tac} (JI1396) (**Chapter III**), *stp-1* (JI2163) (**Chapter III**), and *aero* (JI2767) (**Chapter VIII**) were provided from the John Innes *Pisum* Collection by Mr Mike Ambrose, John Innes Centre, Norwich. Specific details of the crosses used are described in the individual chapter methods.

Cultivation of plants

Plants were grown in Hobart at a density of one or two per 14-cm slim-line pot. Pots and labels were sterilised with 70% ethanol. The growth medium consisted of a 1 : 1 (v : v) mixture of 10-mm dolerite chips and vermiculite, topped with 2-4 cm of pasteurised peat / sand potting mixture. The potting mix included macronutrients which were supplied from 3-4 month Osmocote® (Scotts-Sierra Horticultural Products Co., USA). Pots were watered immediately prior to and after planting. For even and

maximal germination a small (2-3 mm^2) portion of the testa above one cotyledon was removed and the seeds were dusted with the fungicide Thiram. The seeds were planted at a depth of 2 to 4 cm.

After planting, pots were watered daily until signs of seedling emergence – approximately five days after planting – after which watering was stopped for approximately three days to avoid damage to the apical bud. Watering resumed three times per week when the plants had expanded their first leaf. After ten to twelve leaves had formed (approximately one months growth) the plants were watered daily. Plants were fed once a week throughout their entire growing period with Aquasol® (Hortico, Australia) or Total Growth Nutrient (R&D Aquaponics, Sydney). The main shoot was trained up a vertical string. Lateral shoots were excised regularly in short days (SD) until just prior to flowering, after which aerial laterals were left in order to increase the seed yield. Basal laterals (secondary stems) were generally not produced under Hobart standard 18-h long day (LD) conditions.

Plants grown at the John Innes Centre, Norwich, UK (JIC) were grown singly in 10 x 10-cm pots in John Innes No 1 potting mix containing 30% grit. Plants were watered daily and fertilised with Sangral soluble fertiliser 3: 1: 1 once a week (Richard Gould, Horticulturalist, JIC, pers. com.).

Photoperiod and temperature

Unless otherwise specified, plants were grown under 18-h LD conditions and received natural daylight extended with a mixture of fluorescent (Woton 40W cool white tubes, Italy) and incandescent (Thorn 100W pearl globes, Australia) lights providing 25 $\mu mols\ m^{-2}\ s^{-1}$ at pot top. Glasshouse temperatures ranged from 13 to 21°C during the winter months, and 17 to 30°C during summer. Daily maximum temperatures averaged approximately 25°C. Plants grown at the JIC received 16-h daylength with daylength extensions provided by Wotan Powerstar 400W/DH bulbs.

SD photoperiods consisted of 8 or 10 h of natural daylight after which plants were automatically transferred to night chambers maintained at 16°C.

Indices of growth and development

All plant characters were scored off the main stem. Node counts started at the first scale leaf as node one (the cotyledonary node as node zero). Flowering

characteristics recorded were: node of flower initiation (NFI), which is the node bearing the first secondary inflorescence; node bearing the first fully developed flower (NFD); flowering time (FT), the days between sowing and first open flower; and number of flowers produced per inflorescence (F/N). Nodes where the flower buds aborted or the plants reverted to vegetative growth were also noted. Vegetative plant growth was characterised by measures of the stem length between nodes *x* and *y* (L*x-y*), total stem length from node one to the apical bud (TL), total number of nodes produced on the main shoot (TN), and the nodes at which the number of leaflets-per-leaf increased to 3, 4, 5 or 6 (C-3, C-4, C-5, and C-6, respectively). In addition, other leaf characteristics, and floral organ number and identity, were recorded in specific experiments.

Isolation of genomic DNA

DNA was isolated from fully- and semi-expanded pea leaves (including stipules) following the procedure outlined by Ellis (1994). Approximately six to ten leaves were collected, frozen in liquid nitrogen, and ground to a fine powder under the liquid nitrogen using a mortar and pestle. After the tissue had warmed towards 0°C, 10 ml of extraction buffer (3x SSC, 0.1 M EDTA) was added and the tissue ground further. SDS (100 µl of 20%) was then slowly added while mixing to prevent SDS precipitation. This mixture was transferred to a 50-ml tube and extracted with 15 ml of chloroform / IAA (24 : 1; v : v) by mixing thoroughly. The tube was centrifuged (4000 rpm) for 10 min at room temperature, and the aqueous layer removed to a second 50-ml tube. The solution was overlaid with 20 ml of 96-100% ethanol. The DNA was spooled out using a pasteur pipette previously formed into a hook, then briefly air dried, and dissolved into 500 µl of 1x TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.4). The DNA was extracted with 500 µl of phenol equilibrated with TE (pH 8) (Sigma). The DNA / phenol mix was centrifuged at 10 000 rpm for 4 min and the aqueous layer removed to a clean 1.5-ml microfuge tube. The purified DNA was spooled off a second ethanol precipitation, rinsed in 70% ethanol, and air dried. Finally, the DNA sample was redissolved in a minimal volume of TE buffer and stored at 4°C.

DNA concentration was determined by measuring its absorbance at 260 nm in a DNA fluorometer (Hoefer Scientific Instruments) or a GeneQuant RNA/DNA calculator (Pharmacia). An absorbance of 1 at 260 nm indicates a DNA concentration of 50 µg/ml.

Isolation of plasmid DNA

Plasmid DNA was isolated from cultures of the specific *Escherichia coli* lines containing the desired plasmid grown overnight at 37 °C. These cells were grown in LB broth containing an appropriate antibiotic. Plasmid DNA required in high purity was isolated using Qiagen anion exchange minicolumns, and large amounts of plasmid were produced using a 5′-3′ Insta-midi-prep™ system.

Qiagen minicolumns required 3 to 4 ml of culture. Bacterial cells were pelleted by centrifuging for 4 min at 4 000 rpm, resuspended in TE buffer (pH 8) and lysed with an alkali / SDS containing solution. After addition of K acetate to lower the pH, the SDS and cellular debris were precipitated on ice and pelleted for 10 min at 10 000 rpm. The supernatant was added to a Qiagen-tip 20 and allowed to flow through by gravity. The bound plasmid DNA was washed four times at neutral pH, and medium salt concentration (1 M NaCl, 50 mM MOPS; pH 7.0) and eluted with 0.8 ml of buffer with higher salt and pH (1.25 M NaCl, 50 mM Tris HCl; pH 8.5). The purified plasmid preparation was precipitated with isopropanol. The pellet was washed with 70% ethanol and redissolved in a minimal volume of 1x TE. Plasmid DNA samples were stored at 4°C.

The Insta-midi-prep™ kit was used with 50 ml of overnight bacterial cell culture. The cells were pelleted and resuspended in 450 µl of ddH_2O, added to a prespun Insta-midi-prep™ tube (4000 g for 2 min), and lysed by the addition of 2 ml of phenol: chloroform: IAA (50: 49: 1) mixture. The organic layer was spun through the Insta-midi-prep™ gel at 7500 g for 1 min. The plasmid sample was extracted a second time with 2 ml of phenol: chloroform: IAA. Excess phenol was removed by adding 2 ml of chloroform: IAA (49: 1). The sample tubes were centrifuged between extractions. Excess impurities, including the remaining phenol, were removed by the activated charcoal present in the Insta-midi-prep™ filter tubes.

Isolation of total RNA

Total RNA was isolated from two to three grams of appropriate tissue (usually flowers or flowering apices) following the protocol outlined by Michael *et al.* (1996, modified from Verwoerd *et al.* 1989). Each sample was ground to a fine powder

under liquid nitrogen using a mortar and pestle and transferred while still frozen to a sterile 50-ml tube containing 10 ml of TLES buffer (100 mM Tris HCl pH 8, 100 mM LiCl, 10 mM EDTA, 1% SDS). 10 ml of phenol (equilibrated with citrate buffer pH 4.3) (Sigma) was added and the mixture vortexed for 30 sec. After this, 10 ml of chloroform: IAA (24: 1) was added and the sample vortexed for a further 30 sec. The sample was then centrifuged for 3 min at 3 000 rpm. The aqueous layer was removed to a clean 50-ml tube and re-extracted with an equal volume of phenol: chloroform: IAA (25: 24: 1). This was vortexed for 30 sec and centrifuged as above. The aqueous layer was transferred to a clean, sterile 50-ml tube and an equal volume of 4 M LiCl was added and mixed. The RNA was allowed to precipitate overnight at 4°C. The sample was then centrifuged at 10 000 rpm for 30 min. The RNA pellet was dissolved in 1 ml of RNase free water and reprecipitated overnight at 4°C by adding 100 µl of 2.0 M Na acetate (pH 4.5) and 2.5 ml of 100% ethanol. The sample was centrifuged for 20 min at 10 000 rpm, washed in 3 ml of 70% ethanol, and recentrifuged for 30 min at 10 000 rpm. The RNA preparation was resuspended in 500 µl of water and stored at -80°C.

Concentration and yield of the RNA samples were calculated from their absorbance at 260 nm using a GeneQuant RNA/DNA calculator (Pharmacia). For RNA quantification, an absorbance of 1 at 260 nm indicates a concentration of 40 µg/ml.

Restriction digestion

Prior to Southern analysis, DNA was digested by incubating an appropriate quantity (ca. 40 - 100 µg) of DNA with the required restriction enzyme (Promega, Nebb lab, or Progen) such that the number of units approximated the number of µg of DNA to be digested. Successful digestion was confirmed by electrophoresis and visualisation of a 5 to 10-µl aliquot on an agarose minigel stained with ethidium bromide.

Southern analysis and minigels

Size fractionation of the digested genomic DNA was performed using 1x TAE, 0.8 % agarose, vertical gels (JIC), or 1x TAE, 1.4 % agarose, horizontal gels (Hobart). Minigels (1x Tris Borate EDTA (TBE), 1 % agarose (JIC); or 1x TAE, 1 % agarose (Hobart) were used to examine PCR or digested plasmid DNA. The DNA

was visualised by staining with ethidium bromide either in an aqueous solution after running the gel, or by including ethidium bromide within the gel while running (at a final concentration of 0.5 μg/ml). Gels were visualised under UV light and photographed using a Polaroid camera or a gel documentation system (Mitsubishi).

Those gels required for Southern analysis were blotted onto Zetaprobe membranes (Bio-rad) after 20-min washes in denaturing (1.5 M NaCl, 0.5 M NaOH) and neutralising (3 M NaCl, 0.5 M Tris, pH 6.5) buffers. Gels were transferred to the Zetaprobe membranes overnight in 20x SSC (3 M NaCl, 0.3 M Na citrate.$2H_2O$) as described by Maniatis *et al.* (1989). After transfer, the filters were washed in 2x SSC and the DNA fixed to the filter by baking at 80°C for 30 min.

Northern analysis

Total or poly A^+ RNA was run on a denaturing (formaldehyde) gel as described by Fourney *et al.* (1988). The RNA was prepared by heating at 65°C for 15 min with 25 μl of electrophoresis sample buffer (stock solution contains 0.75 ml formamide, 0.15 ml 10x MOPS, 0.24 ml formaldehyde, 0.1 ml H_2O, 0.1 ml glycerol and 0.08 ml 10 % bromophenol blue) after which 1 μg of ethidium bromide was added. Samples were run on a 1.2% agarose gel containing 0.66 M formaldehyde and 1x MOPS (20 mM MOPS, 5 mM Na acetate, 1 mM EDTA), using 1x MOPS as a running buffer. Prior to transfer, the gel was prepared by soaking twice in 10x SSC for 20 min. The gel was transferred overnight using 10x SSC, and the filters baked at 80 °C for 30 min to fix the RNA to the membrane.

Preparation of a radioactive probe

Filters were dampened in 2x SSC and prehybridised in prehybridisation-buffer containing 1% SDS, 10% dextran sulphate, 50 mM phosphate buffer, 5x SSC, and 0.5 mg/ml salmon sperm DNA. The stringency of hybridisation was controlled by altering the proportion of formamide. 20% formamide at 37 °C was used in hybridisations with non-species-specific probes (low stringency), and 50% formamide at 42 °C was used for those with a species-specific probe (high stringency). Alternatively, filters were prehybridised in 'hybridisation juice' (4x SET, 10x Denhardt's, 0.1% SDS, 0.1% Na pyrophosphate, 50 μg/ml herring sperm DNA, 10% dextran sulphate) at 60 °C (JIC). Prehybridisation proceeded for one to three hours.

Radioactive DNA probes were prepared using an oligo-labelling/random priming system. Template DNA (25 to 200 ng, see specific chapter methods) was denatured by boiling for 2 min, and cooled on ice. Decanucleotides, nucleotides (dATP, dGTP, and dTTP), buffers and enzyme (Klenow fragment), and 3 to 5 μl of α^{32}P dCTP were added. The reaction was incubated at 37°C for 30 min to 2 h. The components of this reaction system were prepared specifically at JIC, and Gigaprime DNA labelling kits (Bresatec) were used in Hobart. Radioactive probes were desalted by running through Sephadex G50, equilibrated with TE buffer in glass pipettes at JIC or with ddH_2O in spin-columns (Promega) in Hobart. Probes were denatured by boiling for 5 min and cooling on ice, or alternatively by alkali denaturing with 1 M NaOH (to a final concentration of 0.2 M), after which the probe was neutralised with an equal volume of 1 M HCl and 1 M Tris buffer (pH 7.5). Hybridisation was allowed to proceed overnight at the same temperature used for prehybridisation.

Filters were washed three times for 30 min in 0.5% SDS and 2x, 0.2x or 0.1x SSC at 37, 45 or 65 °C depending on the required stringency (see specific chapter methods). The radioactive filters were exposed to autoradiography film (Kodak, Amersham) at -80°C. The time of exposure depended on the radioactivity of the filters as determined by a geiger counter. X-ray film was developed as described in Maniatis *et al.* (1989).

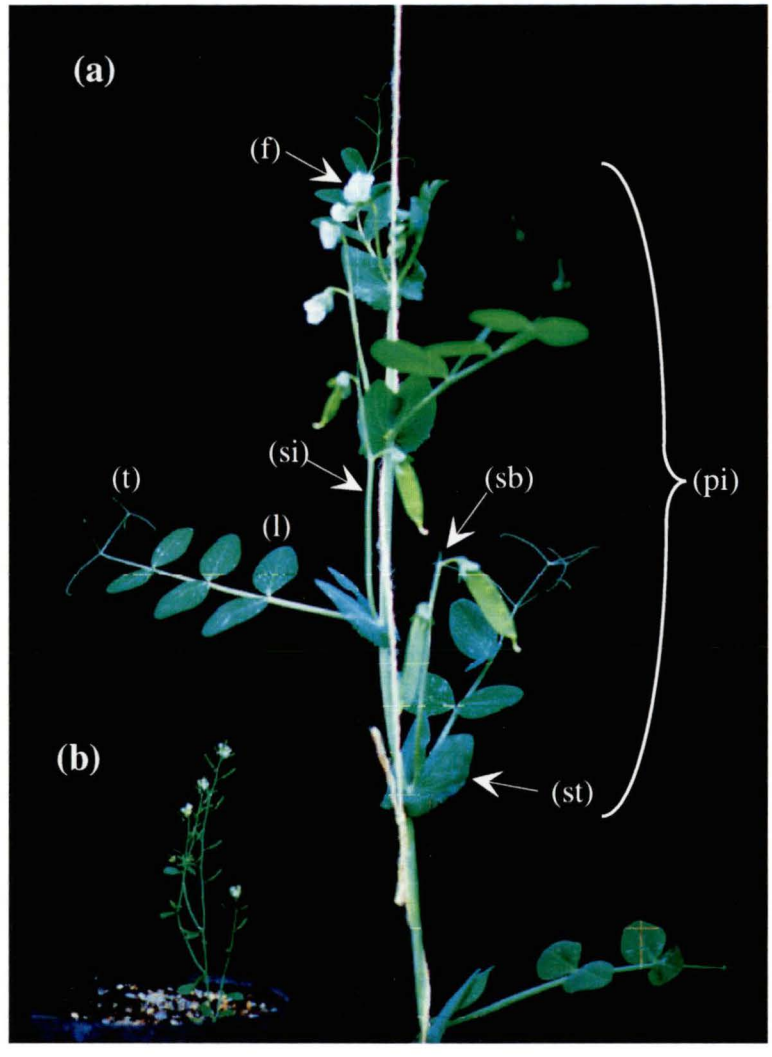


Figure II.1: The development of the garden pea.
The garden pea (*Pisum sativum*; a) possesses a more complex inflorescence and leaf structure than found in *Arabidopsis* (b). Papilionaceous flowers (f) are borne on leafless secondary inflorescences (si) that terminate in hairy stubs (sb). The secondary inflorescences arise from the axils of the leaves over one of the stipules (st). The indeterminate primary inflorescences (pi) is indistinguishable from the vegetative axis from which it is derived. Pea leaves are compound and show internal differentiation: distal structures are tendrils (t), and proximal structures leaflets (l). The pea and *Arabidopsis* plants shown were grown under an 18-h photoperiod and are at approximately the same developmental age.

Chapter III. The 'floral homeotic' mutation *stp* affects flower, leaf and inflorescence development

Introduction

The more complex leaf and inflorescence development seen in the garden pea (**Chapter II, Figure II.1**) provides additional opportunities to disrupt normal plant development when compared with the simpler leaves and inflorescences of *Arabidopsis* and *Antirrhinum*. Pea mutations that specifically affect either leaf (e.g. Marx 1987) or flower development (e.g. Reid *et al.* 1996, **Chapter I**) have been identified and characterised. In addition, a small class of pea mutations have pleiotropic effects on both leaf and flower development (Lamprecht 1958a, 1958b, Wellensiek 1959, Blixt 1972, Hofer *et al.* 1997).

Characterisation of the *stamina pistilloida* (*stp*) mutant by Monti and Devreux (1969) suggests that the *Stp* gene is specifically required for the normal identification of the flower. Mutant flowers contain petals with green sepaloid streaks and two of the stamens show partial conversion to carpels (Monti and Devreux 1969, **Figure III.1**). This phenotype is characteristic of a class-B floral homeotic mutation (Beltrán *et al.* 1996, **Chapter I**). An additional mutant, stp^{192}, also shows transformation of petals to stamens and stamens to carpels (Beltrán *et al.* 1996). In addition, stp^{192} flowers produce ectopic flowers from the common primordia, suggesting that *Stp* may play some role in the development of these primordia (Beltran *et al.* 1996). The common primordia normally produce petal and stamen primordia (Tucker 1989, Beltrán *et al.* 1996), and the proposed function of *Stp* in common primordia organisation may, in part, explain the phenotype of the *stp* mutants.

This chapter describes further characterisation of the original *stp* mutant. In addition, two new mutant alleles were identified and their effects on plant development characterised. The interactions between the severest identified mutant allele and other mutations affecting leaf, flower and inflorescence development were also examined. These studies show that the *Stp* gene is not only involved in the identification of the flower, but is also required for the normal development of the leaves and inflorescences.

Results

Inheritance and allelism of two new mutants at Stp

A single wild-type sibling from the M1a/324 mutant line was found to segregate for a floral meristem identity mutant characterised by a complete conversion of petals to sepals, and the presence of additional central carpels at the expense of stamens. However, the distinguishing feature was the production of secondary floral meristems on elongated pedicels within the primary flower (**Figure III.2a**). Allelism between this new mutant and the *stp* mutant described by Monti and Devreux (1969) was confirmed in a cross to line JI2163 (the type line for *stp*). Two F_1 plants produced from this cross possessed flowers with a phenotype somewhat intermediate between those of the original *stp* mutant and the new floral mutant (**Figure III.3a**). Thus, the new allele from the M1a/324 population was called *stp-2*, and the original mutant allele carried by JI2163, *stp-1*, following the trend in naming pea mutant alleles (Weller *et al.* 1995, Taylor and Murfet 1996, Reid *et al.* 1996).

As the fertility of a second floral homeotic mutant, AF106, was under question, the line was rescued by pollinating carpels with pollen from its initial line, HL107. Some selfed seeds were also produced following manual pollination. Early flowers from this mutant plant showed partial conversion of petals to sepals, and stamens to carpels (**Figure III.2b**), whilst flowers on lateral shoots often produced ectopic flowers within the primary flower. The similarity between the late flowers produced on mutant line AF106 and those produced in the M1a/324 mutant line indicated a possible relationship between the two mutants. Crosses between heterozygous *Stp / stp-2* plants and homozygous AF106 mutant plants produced 14 F_1 plants, of which 8 expressed a phenotype intermediate between the *stp-2* and AF106 mutant phenotypes (**Figure III.3b**). Thus, the AF106 mutation reveals a third allele at *stp*, *stp-3*.

Segregation analysis from F_2 populations from second backcrosses to their initial line, HL107, confirmed that both mutants showed single gene, recessive inheritance and segregated in accordance with a 3: 1 ratio (39 *Stp*: 14 *stp-2*; $\chi^2 = 0.06$, $P > 0.8$; 39 *Stp*: 10 *stp-3*; $\chi^2 = 0.55$, $P > 0.3$).

The phenotype of stp-1 *revisited*

Investigation of the *stp-1* mutant phenotype from the pure breeding type line JI2163 confirmed the analysis carried out by Monti and Devreux (1969). The primary effect of *stp-1* on flower development is the partial conversion of the two stamens either side of the free adaxial stamen to carpels (**Figure III.1**). This homeosis ranged from slightly carpelloid stamens, to almost fully formed, but unfused carpels, and was seen in all flowers examined. These carpelloid stamens were usually attached to the central carpel. In addition, wing, keel and, more rarely, standard petals contained green sepal-like tissue in streaks through the centre of the organ (**Figures III.1** and **III.4a**). Fusion of the two keel petals was often disrupted. The phenotype of *stp-1* mutant flowers also appears to be sensitive to environmental influences (Monti and Devreux 1969); more severe phenotypes, including the rare formation of ectopic flowers, were produced when the plants were grown in Hobart under high (29°C) temperatures (J. van de Kamp pers. com.).

The floral phenotype of stp-2 *and* stp-3

The *stp-3* mutant has a more severe phenotype than the *stp-1* mutant. Sepaloid streaks were always found through the centre of the petals (**Figure III.2b**), keel petals failed to fuse, and in the most severe cases all petals were completely green. Stamen to carpel transformations were also observed: the two adaxial stamens were converted to carpels or sepal/carpel chimeric organs. The free adaxial stamen was often completely converted to a carpel. Abaxial stamens formed groups of twos or threes connected by the tube tissue that joins the nine fused stamens in wild-type flowers (**Figure III.4b**). The central carpel was unaffected and able to produce seed following pollination. The floral phenotype of *stp-3* mutants was consistent within populations grown together, but differed in severity among populations grown at different times (e.g. compare **Figures III.2b** and **III.5a**) This may reflect an environmental effect on expression as noted for *stp-1* (Monti and Devreux 1969). Severity of petal and stamen transformation was related such that plants with more normal petal development also possessed more normal stamen development. The later produced flowers on the lateral shoots of *stp-3* mutant plants had a more severe phenotype and occasionally produced an ectopic flower on an elongated pedicel. The ectopic flowers were primarily composed of sepals and sepaloid carpels (**Figure III.5c**).

Primary flowers of the most severe mutant, *stp-2*, always contained five sepals in the first whorl, and a central carpel (**Figure III.2a, III.4c** and **III.5b**), which was able to produce seeds upon manual pollination. The second whorl was composed of four to eight sepals. Within these, and surrounding the central carpel, were a variable number of additional carpels, carpel-sepal chimeric organs, or sepals. There was always one, and occasionally more, ectopic flowers on elongated pedicels present in the primary flowers of the *stp-2* mutant (**Figure III.2a, III.4c** and **III.5b**).

An ectopic (secondary) flower of *stp-2* mutant plants was always found adaxially, and was contiguous to the base of the carpel. Where additional ectopic flowers were found, they surrounded the central carpels (**Figure III.5b**). The ectopic flowers had one to eight sepals outermost, which were usually in a whorled or partially spiralled arrangement. Within these, the flower was disorganised. Although occasionally following the pattern of the primary flower, these ectopic flowers often consisted of irregularly placed sepals, carpelloid sepals, and carpels. Petals, sepaloid petals, staminoid petals, and stamens (single or in pairs) were also found in the ectopic flowers in varying proportions (e.g. **Figure III.5d**), but were never seen in the primary flower. If stamens and petals were found, they usually occurred together, although rare stamens and/or petals were found in ectopic flowers that consisted mostly of sepaloid and carpeloid organs. Secondary flowers consisting entirely of spirally arranged sepals were also occasionally observed.

The secondary flowers often possessed their own ectopic (tertiary) flowers. By this stage the whorled arrangement of organs was completely abandoned. Outer organs of the tertiary flowers were generally sepaloid, inner organs were variable, and could be sepal, carpel, petal or stamen-like in appearance, or chimeras of these. Sepaloidy was most prevalent.

Dwarf (*le*) plants carrying *stp-2* (segregating in some of the crosses examined) showed a similar range of phenotypes, although pedicel elongation and thus the distinction between primary and subsequent flowers was reduced. Alterations in photoperiod (8 h vs 18 h) had little, if any, discernible effect on the *stp-2* flower phenotype (**Figure III.5d**).

Pleiotropic effects of stp-2 *and* stp-3

In addition to their profound effects on flower development, the *stp-2* and *stp-3* mutations both delayed the node of flower initiation (node bearing the first secondary

inflorescence; **Figure III.6a, Table III.1**). This delay was even more pronounced in *stp-2* plants grown under SD (**Figure III.6b**). The number of flowers borne on the first secondary inflorescence was also significantly reduced in *stp-2* plants in comparison with wild-type siblings (**Table III.1**). *stp-2* plants possessed secondary inflorescences bearing a single flower more often than their wild-type siblings, such that, on average, there was 0.4 'flowers' less on *stp-2* inflorescences. This effect was also found in subsequent inflorescences, although eventually both wild-type and mutant plants consistently bore single flowers. Determinancy of the primary inflorescence was also affected (**Table III.1**). Growth of the primary inflorescences of *stp-2* and *stp-3* plants was often arrested with an apparently terminal flower (**Figure III.7a**). Under an 18-h photoperiod, these 'terminal' flowers were produced after as few as five (although usually more) reproductive nodes had formed and were often associated with a simplification of the leaf from compound to unifoliate in *stp-2* mutant plants (**Figure III.7a**). Wild-type plants typically produced only four or five reproductive nodes before undergoing apical arrest, whereas *stp-2* and *stp-3* mutants not producing a 'terminal' flower often produced over twelve reproductive nodes and retained the potential for further growth (**Table III.1**). The *stp-2* mutation was also associated with a release from dormancy of the other two axillary buds present in the axils of the leaves on the primary inflorescence (**Figure III.7b**). These buds are normally suppressed in the primary inflorescence of wild-type peas so that only the secondary inflorescences develop.

The two new *stp* mutants also showed pleiotropic effects on leaf development. Normal heteroblastic development of the pea leaf occurs through an increase in the number of lateral structures up to a maximum of (typically) six tendrils and six leaflets (Wiltshire *et al.* 1994, Lu *et al.* 1996). Transitions from two to four leaflets-per-leaf, and four to six leaflets-per-leaf appear to be genetically controlled, and occur at specific nodes (Wiltshire *et al.* 1994). Both *stp-2* and *stp-3* delayed the production of the first foliage leaf from node 3 to node 4; wild-type plants normally possess two scale leaves, with the first true leaf at node 3 (see **Figure III.8** and **III.14a**). In addition, both *stp-2* and *stp-3* significantly delayed the transition from two to four leaflets-per-leaf (**Figure III.6, Table III.1**), and some *stp-2* plants never produced leaves bearing more than two leaflets. Neither *stp-2* nor *stp-3* plants ever produced leaves bearing five or six leaflets. The delay in heteroblastic leaf development seen under our standard LD conditions was even more pronounced in *stp-2* plants grown under SD conditions (**Figure III.6**).

Measurements of internode lengths revealed that *stp-2* and *stp-3* plants were also marginally, but significantly ($P < 0.001$), shorter than their wild-type siblings (**Table III.1**). **Figure III.9** illustrates a comparison of the first ten internodes from wild-type and *stp-2* plants and indicates that the 10 % reduction in L_{1-9} of the mutant plants was due to a small reduction in the length of each internode.

The observed pleiotropic phenotypes were found in both *stp-2* and *stp-3* plants, and appeared to be correlated with the severity of the floral phenotype (e.g. compare **Figure III.2** with **III.6**). These pleiotropic traits cosegregated with the floral phenotype in all crosses examined. Thus, it was concluded that the simplification of the leaf, the reduction in internode length, and the effects on inflorescence development were pleiotropic effects of the *stp* mutant alleles, and did not result from a second, unrelated mutation.

The effect of stp-2 *and* stp-3 *on cell division*

The effect of the *stp-2* and *stp-3* mutations on internode length provides a potential clue to the role of *Stp*. Internodes may be shorter for three reasons: cells within the internode may be smaller, there may be fewer cells present in each internode, or there may be fewer, smaller cells within the internode (e.g. see Murfet 1990). To differentiate between these possibilities epidermal cell lengths were measured from internodes and leaf rachises from *stp-2* and *stp-3* plants and their wild-type siblings. The results (**Table III.2**) suggest that the *stp* mutations specifically reduce the number of epidermal cells present within the internode and leaf petiole without affecting cell length.

A reinvestigation of the map location of Stp

The initial linkage analysis based on a cross between HL111 and the original mutant line (cross T21) failed to find any significant linkage between the *stp-2* mutant and the classical markers present in HL111. Isozyme analysis with a subset of this population did indicate weak linkage between the floral mutant and *Aat3* on the lower end of group VII (**Table III.3**). However, as only the fast *Aat3* form could be scored in this analysis the cross was in the inefficient repulsion phase for these two characters. Previously published results (Monti and Devereux 1969, Monti 1970) supported this linkage relationship as *stp-1* had been shown to be linked to *oh* and *ar*,

markers now assigned to linkage group VII (Weeden *et al.* 1996). To confirm these analyses, a cross between HL6, which carries the *wa* mutation affecting wax distribution, and a plant carrying the *stp-2* mutation (cross T46) was established. Segregation analysis of the F_2 indicated close linkage between *wa* and *stp-2* with no recombinants identified in 75 plants (**Table III.3**). A single *wa* F_2 plant was found to segregate *stp-2* plants in the F_3 generation and these double mutants were used in a cross (T74) to HL59 to test for linkage between *stp* and the group VII morphological markers *wa* and *sn*, and isozyme markers *Aat3* and *Skdh*. The data from cross T74 (**Table III.3**) confirm the *Stp* locus is in linkage group VII. *Stp* showed strong linkage ($P < 0.0001$) with both *Wa* and *Skdh* with a map sequence of *wa* 4 cM *stp* 10 cM *Skdh*. The full map derived from cross T74 is given in **Figure III.10**.

The interaction between stp *and* uni

The effect of *stp-2* on both leaf and flower development is similar to that seen in the *unifoliata* (*uni*) pea mutant (**Figure III.11**). Crosses between plants carrying the severe uni^{224} allele (see methods) and *stp-2*, and the linkage analyses (*uni* is located on the upper portion of linkage group III: Marx 1986c) indicate that the two genes are not allelic. Segregation of uni^{224} and *stp-2* in F_2 and di-hybrid F_3 populations was in accordance with a 9 wild type: 3 *stp*: 4 *uni* ratio (214 wild type: 72 *stp-2*: 89 uni^{224}; $\chi^2_{9\,3.4} = 0.33$, $P > 0.8$) indicating that uni^{224} is completely epistatic to *stp-2*. The epistatic relationship of uni^{224} to *stp-2* extended through most aspects of the plants development, and included leaf, flower and inflorescence structure, although *uni* and *stp* flowers are already very similar.

All *uni* mutant plants also produce small unifoliate leaf-like structures at the first two nodes replacing the scale leaves present in wild-type plants (**Figure III.8**). These leaf-like structures were also produced in all uni^{224} segregants, indicating that the loss of the first true leaf at node 3 in *stp-2* mutants is overridden in uni^{224} *stp-2* plants.

The epistatic relationship between the severe uni^{224} allele and *stp-2* raises the question regarding the interaction between weak alleles at each of the loci. The effect of *stp-1*, the weakest known mutant allele of *Stp*, appears to be confined to the flower (Monti and Devreux 1969, **Figure III.1**) and this allele has no obvious affects on leaf development. In contrast, the weakest described allele at *uni*, uni^{tac}, results in the production of a terminal leaflet with subterminal tendrils (**Figure III.12**). uni^{tac}

flowers are almost completely wild type, only occasionally showing some disruption of keel petal fusion (Marx 1986c). An F_2 population derived from the cross between the type lines carrying *stp-1* (JI2163) and *uni*^{tac} (JI1396) segregated into four distinct classes: 65 wild type: 16 *stp-1*: 10 *uni*^{tac}: 5 *stp-1 uni*^{tac}; corresponding to a 9:3:3:1 ratio ($\chi^2 = 6.19$, $0.2 > P > 0.1$). The double-mutant plants had flowers consisting entirely of sepals and carpels (**Figure III.12**), and this phenotype was confirmed in the F_3 derived from *stp-1* segregants. Sepal/carpel mosaic organs were noted, and ectopic flowers were also seen. This flower structure is highly reminiscent of that produced by both *stp-2* and *uni*^{224} mutants (**Figures III.5** and **III.11**). The leaf of *stp-1 uni*^{tac} plants was simplified further and rarely possessed the subterminal tendrils characteristic of *uni*^{tac}. *stp-1* and *uni*^{tac} single-mutant segregants in the cross produced typical flower and leaf phenotypes (**Figure III.12**).

Floral meristem identification: the interaction of stp *with* pim

To further investigate the apparent role of *Stp* in floral meristem development, plants carrying the *stp-2* mutation were crossed with lines homozygous for the *proliferating inflorescence meristem* (*pim*) mutation. *Pim* has recently been shown to be the homologue of *AP1* from *Arabidopsis*, and *Squamosa* from *Antirrhinum* (see **Chapter V**), and is essential for the identification of the floral meristem (Singer *et al.* 1994, **Chapter V**). The 59 plants in the F_2 population resulting from the cross between *pim-1* and *stp-2* segregated into four distinct classes: 26 wild type: 18 *pim-1*: 12 *stp-2*: and 3 *pim-1 stp-2*, which is in accordance with a 9: 3: 3: 1 dihybrid ratio ($\chi^2 = 6.11$, $0.2 > P > 0.1$). The phenotype of the double mutant combined characteristics of both the *pim* and *stp-2* mutants, although obvious ectopic flowers were not present. Flowers were formed from clusters of bract-like structures resembling those found in the outer whorl of *pim* mutants (**Figure III.13**). The effect of *stp-2* on leaf development was maintained in the *pim* mutant background.

Leaf development: the interaction of stp *with* afila *and* tendril-less

The simplification of the leaf caused by the *stp-2* allele was clearly expressed in the leaf homeotic mutants *afila* (*af*) and *tendril-less* (*tl*). Double mutant plants produced in F_2 and F_3 populations from the cross between HL117 (*af tl*) and an *stp-2* mutant (backcrossed once to HL107) showed characteristics of both mutants (**Figure III.14a**). The *af stp-2* double mutant plants showed a simplification of primary and

secondary ramifications of the typical *af* single mutant, such that the leaf consisted entirely of simple tendrils arising from the central rachis. In contrast, the *afila* (*af Stp*) leaf bears simple tendrils distally, and typically has compound proximal tendrils. Similarly, *stp-2 tl* mutant plants possessed fewer lateral structures (**Figure III.14b**) although the difference between *stp-2* and *Stp* segregants carrying *tl* was much less obvious than noted in the *af* segregants. Counts of lateral leaflet production in *stp-2 tl* and *Stp tl* plants confirmed this observation and indicated that the *stp-2* mutation reduced the maximum number of leaflets borne on a *tl* leaf (**Figure III.15**).

The effect of *stp-2* on leaf development was most pronounced in the double mutant *af tl* (pleiofila) leaf form (**Figure III.14c**). Compound lateral structures normally seen in the pleiofila leaf were severely reduced, and the leaves consisted of three similar units bearing leaflets smaller than those on wild-type plants, but larger than those normally present on pleiofila leaves.

Inflorescence development: the interaction of stp *with* det, veg2 *and* lf

The production of apparently terminal flowers on the primary inflorescence of *stp-2* plants suggests that the wild-type *Stp* allele may also be involved in maintenance of the unlimited growth habit of the primary inflorescence. This phenotype is somewhat similar to, although much weaker than, that of the inflorescence meristem identity mutant *determinate* (*det*). The *det stp-2* double-mutant phenotype was a simple addition of the two single-mutant phenotypes (**Figure III.16**). Although *det* reduces the complexity of the primary inflorescence, it appeared unable to affect the development of the ectopic flowers produced within the *stp-2* primary flower.

The *vegetative2-2* (*veg2-2*) mutation promotes indeterminacy in the normally determinate secondary inflorescences (Murfet and Reid 1993, Reid *et al.* 1996). However, addition of *veg2-2* to an *stp-2* background was unable to prevent the apical meristem being converted to a terminal flower. Rather, plants carrying both *stp-2* and *veg2-2* produced secondary inflorescences with characteristics of both mutations – that is the loss of normal secondary inflorescence characteristics caused by *veg2-2*, combined with the sepaloid terminal flowers of *stp-2* (**Figure III.17**). The simple additive nature of *stp-2* and *veg2-2* phenotypes suggests that the two genes are acting independently.

The delay in node of flower initiation noted in *stp-2* (and *stp-3*) single mutants (**Figure III.6** and **III.18**) was unaffected by the allele present at the *Late flowering* (*Lf*) locus. Double mutant plants carrying both *lf-a* and *stp-2* flowered significantly later than *lf-a Stp* siblings (2.21 nodes, $t = 3.59$, $0.01 > P > 0.001$). This delay is comparable to that seen between *Lf stp-2* and *Lf Stp* plants (**Figure III.18**). A SD (8 h) photoperiod delays flowering in *Lf stp-2* plants to a greater extent than in *Lf Stp* plants (**Figure III.19**). This probably results from the magnification of the small delay in flowering seen in *stp-2* plants under LD conditions. Whether this delay results from conversion of early secondary inflorescence meristems into primary meristems, or a delay in attaining competence to respond to the flowering signal is unknown.

Discussion

Although numerous mutations affecting flower development have been identified in pea (e.g. Blixt 1972, Reid *et al.* 1996), only three have been examined in detail: *stp* (Monti and Deveraux 1969), *veg1* (Reid and Murfet 1984), and *uni* (Hofer *et al.* 1997). However, the studies of mutations affecting flower development in *Antirrhinum* and *Arabidopsis* have led to a resurgence of interest in similar mutants from pea (e.g. Singer *et al.* 1994, Beltrán *et al.* 1996).

Among the mutations known to affect flowering and flower development in pea, three also show pleiotropic effect on the development of the pea leaf – *cochleata* (*coch*) on the stipules, *laciniata* (*lac*) on the leaflets and stipules, and *unifoliata* (*uni*), which disrupts the development of the compound leaf (Lamprecht 1958b, Wellensiek 1959, Blixt 1972, Hofer *et al.* 1997). The phenotype of the *stp-1* mutant has lead to the suggestion that *Stp* may represent a class-B floral homeotic gene of pea (Beltrán *et al.* 1996). However, the identification and characterisation of two, more severe alleles at *Stp* suggests that *Stp* plays a more basic role in identifying the floral meristem, and also belongs to the group of pea mutations with effects on both flower and leaf development.

The role of Stp *in flower, leaf and inflorescence development*

The disruption of floral meristem determinancy seen in the *stp-2* and *stp-3* mutants (e.g. **Figure III.2, III.4** and **III.5**) is atypical of floral homeotic genes [other than *agamous* homologues which reiterate the first three whorls (Bowman *et al.*

1989)]. This suggests that *Stp* acts earlier than the floral homeotic genes, and is involved in the identification of the floral meristem. However, even the more severe effects of *stp-2* and *stp-3* on flower development are consistent with a role of *Stp* in upregulating B-class activity in the floral meristem – the conversion of petals to sepals and stamens to carpels is consistent with a B-class mutation. A candidate downstream gene in pea is possibly revealed by the *multipistillate* mutant (Muehlbauer and Konzak 1973), which possesses a classic class-B mutant phenotype without the proliferation of the floral meristem. This regulation is not absolute and morphologically normal petals and stamens are produced even in the severest *stp* mutant, *stp-2* (e.g. **Figure III.5d**). Therefore, the known *stp* mutant alleles may not be completely null, and some residual *Stp* activity may permit the expression of B-class genes. Alternatively, there is some redundant pathway which partially replaces *Stp* activity, and allows the expression of B-class genes, albeit belatedly.

The production of mosaic organs within the flower suggests that *Stp* may also play a cadastral role in flower development by regulating the formation of the concentric A-, B- and C-class activities. Alternatively, the whorled placement of organ primordia may be disrupted, resulting in organ primordia arising across whorl boundaries. The disruption to the normal whorled floral phyllotaxy observed in the later ectopic flowers produced on *stp-2* mutant plants supports this suggestion.

In addition to its more generalised effect on flower development, *Stp* is also involved in other aspects of plant development. This is indicated by the pleiotropic effects of *stp-2* and *stp-3* on leaf and inflorescence development (**Table III.1** and **Figures III.6** to **III.9**). This would not be expected for a specific floral homeotic mutation. However, neither would it be expected from the mutant phenotypes of the floral meristem identity genes from *Arabidopsis* and *Antirrhinum*. As two independently isolated *stp* alleles were shown to affect both leaf and inflorescence development, it was concluded that these pleiotropic effects expose additional functions of *Stp*, rather than result from a mutation at a second gene. These observations suggest that the *Stp* gene plays an important and central role in the development of the flower, the identification and development of the secondary inflorescences, and in the normal heteroblastic development of the compound pea leaf. Thus, *Stp* appears to be involved in, or to influence several major aspects of plant development.

Although the flower, leaf and inflorescence phenotypes that result from the mutations at *Stp* are distinct, it is logical to assume that the *Stp* gene performs the same function in each organ. Thus, the different effects of the *stp* mutations on each organ

reflect differences between the development and final form of each organ. The role of *Stp* may involve the promotion of apical and suppression of lateral growth in the flower, leaf and inflorescence primordia (**Figure III.20**). Therefore, *stp* mutants may fail to produce leaves with as many lateral structures, as apical growth of the leaf rachis is limited. Similarly, fewer flower-bearing nodes are produced on the primary inflorescence of *stp* mutant plants as the growth of the apical meristem ceases after the production of relatively few secondary inflorescences. *stp* mutants may produce fewer flowers on their secondary inflorescences as limiting apical growth prevents the formation of additional floral meristems. The effect of *stp* on flower development is more complicated, as B-class gene activity is also affected. However, the effect of *stp* mutations on flower development may be explained in a similar manner. That is, apical growth is limited which limits the number of whorls produced before termination of the apex. The promotion of lateral development is only obvious in the flowers, the primary inflorescences, and in older leaves. Lateral growth in inflorescences is expressed by the release of axillary buds from dormancy, and in flowers by the production of ectopic floral meristems. Older leaves take on a form very similar to that seen in all leaves produced on *uni* mutants (e.g. compare **Figure III.7a** with **III.11b**), and a similar proposal was raised to explain the effect of the *uni* mutation on leaf and flower development (Hofer *et al.* 1997). A schematic representation of the effect of the *stp* and *uni* mutations on plant development is illustrated in **Figure III.20**.

The effects of *stp* may be mediated by influences on cell division. The internode lengths of the *stp-2* and *stp-3* mutants are approximately 10% shorter than those of wild-type plants (**Figure III.9**). This appears to be directly related to a reduction in the number of cells produced in each internode – epidermal cell lengths are unaffected (**Table III.2**). Similarly, the effect of *stp* on leaf development is also related to a reduction in the number of cells in the leaf rachis (**Table III.2**). Although cell numbers were not examined in the inflorescence and flowers, the effects of *stp* on flower and inflorescence development may also be related to this apparent effect on cell division. Assuming *Stp* promotes divisions parallel to the apex of the primordia (periclinal), or alternatively discourages divisions perpendicular to the primordia apex (anticlinal divisions), then a loss of *Stp* activity would result in greater anticlinal to periclinal divisions (see **Figure III.21**). Thus, *stp* mutants may possess broader, shallower primordia, which could result in the reduction of apical determinancy and gain in lateral indeterminancy as proposed in **Figure III.20**.

The Stp *pathway acts independently of* Pim, Veg2 *and* Det

Mutations at *Pim* affect the identification of the floral meristems, resulting in leafy, proliferous flowers (Singer *et al.* 1994, Reid *et al.* 1996). Similarly, mutations in both the *Det* and *Veg2* genes affect the identification of the primary and secondary inflorescences, respectively. Plants carrying the *det* mutation rapidly convert their primary inflorescence meristem to a hairy stub (Singer *et al.* 1990), whereas plants carrying *veg2-2* possess indeterminate leafy secondary inflorescences (Murfet and Reid 1993, Reid *et al.* 1996). Neither *pim*, *det* nor *veg2* affect the normal vegetative development of the pea plant (e.g. see **Chapters V** and **VI**), suggesting that *Pim*, *Det* and *Veg2* act in a distinct pathway (or pathways) from *Stp*. This proposal is supported by the additive phenotypes seen in plants carrying both the *stp-2* and *pim-1*, *det* or *veg2-2* mutations (**Figures III.13, III.16** and **III.17**).

The absence of identifiable floral organs in the *pim-1 stp-2* double mutant suggests that *Pim* and *Stp* act together to promote floral meristem, and floral organ identification (**Figure III.13**). Although *det* reduces the proliferation of the primary inflorescence meristem, the proliferation seen within *stp* mutant flowers was unaffected. However, the *det stp* double-mutant phenotype does not resolve the source of the terminal flower occasionally produced in *stp-2* and *stp-3* plants (**Figure III.7a**). This flower may result from a gradual decrease in wild-type *Det* activity, suggesting that *Det* activity is influenced by *Stp*. Alternatively, the invasion of the apical meristem by floral characteristics may be independent of *Det*. The production of the terminal flower in *stp-2* plants was also unaffected by mutations at *Veg2* (**Figure III.17**). This is more surprising, as the *det* mutant phenotype is dependent on wild-type *Veg2* activity (Murfet *et al.* 1995). This would suggest the terminal flower phenotypes of the *det* and *stp* mutants have different aetiologies.

Although *Pim*, *Det* and *Veg2* appear to act in a separate pathway to *Stp*, it is also obvious that these two pathways interact. *Pim*, *Det* and *Veg2* are essential for the normal identification of the flowers and inflorescences. Thus, they are involved in the differentiation between vegetative and reproductive growth. *Stp* appears to be required to control the development of the flower and inflorescence primordia or meristems. Normal development of the flowers and inflorescences requires a synthesis of the control of primordia development, and the determination of the primordias' fate. Thus activity of *Stp* must be regulated to coincide with that of *Pim* for flower development, and *Det* and *Veg2* for inflorescence development. Some of this co-ordination was apparent in the *lf-a stp-2* double mutants (**Figure III.18**). Although *lf-a stp-2* plants produced secondary inflorescences and therefore flowers at an earlier node and time

than their *Lf stp-2* counterparts, the effect of *stp-2* on development of these earlier produced flowers was similar to that seen in *Lf stp-2* plants. This would suggest that the interactions between *Stp* and *Pim*, *Stp* and *Det*, and *Stp* and *Veg2* occur whenever *Pim*, *Det* and *Veg2* are acting to determine meristem identity.

The effect of Stp *on leaf development is independent of* Af *and* Tl

Interactions between *stp* and the leaf homeotic mutants *af* and *tl* in pea indicate that *Stp* also acts independently from *Af* and *Tl* (see **Figure 14**). The effect of the *stp-2* mutation on the *afila*, acacia and pleiofila leaf forms can be interpreted in the same way; the *stp-2* mutation reduce the complexity of the leaf, and this effect is independent of leaf form. Thus, the role of *Stp* in leaf development does not aid in resolving the development and differentiation of the compound pea leaf or the origins of the pleiofila leaf form, and the models proposed to explain the activity of *Af* and *Tl* (e.g. Young 1971, Lu *et al.* 1996) are equally valid in an *Stp* or an *stp-2* background. However, these results are consistent with the proposal that the final leaf form (or that of any other multicellular plant structure) is independent of the regulation of cell division (e.g. Smith *et al.* 1996, Jacobs 1997, Poethig 1997).

However, the simplification of the pea leaf in *stp* mutants does indicate a role for *Stp* in leaf development. Presumably this underlies the identification of the proximal and distal structures by *Af* and *Tl* in much the same way as *Stp* activity underlies the identification of the primary and secondary inflorescences and the identification of the flowers. This is in agreement with the proposed role *Stp* plays in the development of meristems, with the identity of these meristems overlaying the activity of *Stp*. Thus, the activity of *Stp* may be ubiquitous.

Stp *and* Uni *act together to regulate plant development*

Each of the effects of *stp* on leaf, inflorescence and flower development are also seen in the *uni* mutant, often in a more severe form (**Figure III.11**). Thus petals and stamens are never seen in severe *uni* mutants, and the leaf is completely simplified. Effects of *uni* and *stp* on the inflorescence and on internode lengths are more difficult to compare, as these effects are more subtle, although evidence suggests that *uni* also affects cell division in the stem (**Table III.2**). The similarities between the *stp* and *uni* mutant phenotypes explain the epistasis of *uni* over *stp* – presumably *uni* represents the most severe reduction in flower and leaf complexity possible for mutations in this pathway in pea, and adding the *stp* mutation to a *uni* background does

little to influence the mutant phenotype. Formally, the epistasis of *uni* over *stp* mutations suggests that *Uni* function occurs before *Stp*.

It is possible that the most severe known allele at *stp*, *stp-2*, does not represent a complete null allele - the phenotypes of the leaf and flower of *stp-2* plants are less severe than those of severe *uni* mutants (e.g. uni^{224}, **Figure III.11**; or see Hofer *et al.* 1997). However, it would seem unlikely that a complete null at *stp* would affect leaf development in the same manner as uni^{224}. The weakest known allele at *uni* (uni^{tac}) (Sharma 1972, Marx 1986c, 1987) is noted primarily for its effect on leaves, yet *stp-2* plants, although possessing a severe flower phenotype, display only a relatively minor effect on leaf development. The difference in effect of the *uni* and *stp* mutations on the development of the pleiofila leaf type (Hofer and Ellis 1996, **Figure III.14c**) also suggests a fundamental difference in action of the two genes. However, the interaction between the weakest known alleles at *uni* and *stp* confirms the suggestion that *Stp* and *Uni* act together to regulate plant development (**Figure III.12**). The differences between the *stp* and *uni* mutants points to the presence of additional genes that interact with *Uni* to control the development of the compound leaf.

The effect of the *stp* and *uni* mutations on the juvenile plant, including organs laid down in the embryo (**Figure III.8**), suggests that these genes play little direct role in distinguishing between reproductive and vegetative growth. However, they certainly influence the timing of inflorescence identification and the development of the flower. An increase in cell division has been used as an early indication of the floral transition (Bernier 1988, Bernier *et al.* 1995) and it is possible that *Stp* and *Uni* are involved in this aspect of flower development. The similarity of *stp* and *uni* mutant phenotypes, the widespread activity of the two genes, and the interaction between *stp* and *uni* suggests that *Stp* and *Uni* define a generally acting pathway that is essential for meristem identification. This pathway is distinct from those controlling the development of particular structures, such as the *Af* and *Tl* interaction in the leaf, and the activities of *Pim* and *Veg2* in the flower.

A model for Stp *and* Uni *interaction*

The defined effect of *Stp* on cell division, and the homology between *Uni* from pea and *Flo* from *Antirrhinum* (Hofer *et al.* 1997), suggests a possible model for their interaction, which is outlined in **Figure III.21**. Evidence from chimeric *flo* mutants in *Antirrhinum* has suggested that *Flo* can act non-cell-autonomously, and *Flo* function

may be passed from outer to inner cells in a floral primordium through plasmodesmata (Carpenter and Coen 1995, Hantke *et al.* 1995, Mezitt and Lucas 1996). The homology between *Uni* and *Flo* suggests that *Uni* may also act non-cell-autonomously. Expression of *Uni* is greatest at the growing tips of the leaf and flower primordia (Hofer *et al.* 1997), and therefore the *Uni* product may provide positional information which is interpreted by *Stp* (**Figure III.21**). If *Stp* activity promotes periclinal divisions in cells of the primordia (perpendicular to the proposed gradient of *Uni* activity, and parallel with the apex of each primordium), then the net result would be apical growth. Loss of *Uni* function results in a loss of a functional gradient, and *Stp* function is disrupted (**Figure III.21**). Loss of *Stp* function results in the deregulation of cell divisions (as proposed above). The net phenotypes are virtually identical. This hypothesis implies that *Uni* and *Stp* expression is independent, but that the activity of the *Stp* product is dependent on the gradient of *Uni* activity. These considerations are important when pursuing the possible homologies between *Stp* and genes identified from *Arabidopsis* and *Antirrhinum*.

The search for homologies between genes affecting flower development in pea and those in *Arabidopsis* and *Antirrhinum* have so far yielded a single unexpected homology; that of *Uni* with *Flo* and *LFY* (Hofer *et al.* 1997). Further homologies have been proposed (Singer *et al.* 1994, Beltrán *et al.* 1996), and future work will determine the validity of these proposals. Such homologies provide an opportunity to compare pathways proposed to regulate flowering and flower development in pea, *Arabidopsis* and *Antirhhinum*. Therefore it is tempting to speculate on possible homologues between various characterised mutants. Many of the effects of the *stp* mutations on flower development in pea are also seen in *ufo* mutants from *Arabidopsis* and *fim* mutants from *Antirrhinum*. Details of the analysis of the potential homology between *Stp* and *UFO / Fim*, and the conclusions drawn from these studies, are presented in **Chapter IV**: The molecular nature of *Stp*.

Methods

Sources of the mutant lines

Two previously uncharacterised mutations affecting the development of the flower were isolated from an EMS mutagenesis programme on HL107 (cv. Torsdag) conducted by J.L. Weller at the University of Tasmania in Hobart. The first, segregating from the M1a/324 population grown under SD (8 h) conditions, resulted in a severe disruption to normal flower development. The second was isolated as a 'large-scale-leaf' mutant from a seedling phenotype screen under far-red conditions and was given the number AF106. Subsequently, this mutant plant was also found to produce flowers with homeotic conversion between whorls of the flower. Seed from the M1a/324 population and the single AF106 plant were supplied by J.L. Weller.

A mutant with a *uni*-like phenotype was also isolated from this mutagenesis programme (mutant line M1a/224), and seed from this line was again supplied by J.L. Weller. This mutant was found to be allelic to *uni* by crossing with JI1396 (uni^{tac}) - the two F_1 plants possessed the uni^{tac} phenotype. As yet, a numbering system has not been established for the known *uni* alleles. Therefore this mutant allele was identified as uni^{224} for convenience. The phenotype of plants carrying uni^{224} was similar to that of plants with the severe *uni* allele from JI2171 (Hofer *et al.* 1997).

The type lines for *stp-1* (JI2163) and uni^{tac} (JI1396) were supplied by Mike Ambrose (John Innes Centre, UK). All other lines used were from the pea germplasm collection at Hobart.

Characterisation of the stp *mutants*

Characterisation of the two new *stp* mutant phenotypes was carried out using mutant plants backcrossed at least once to the initial line cv. Torsdag (HL107). These analyses were confirmed using a smaller number of plants from a second backcross to HL107. Epidermal strips were taken from *stp-2* and *stp-3* plants and compared with their wild-type siblings in the same second backcross population [Crosses T61 (*stp-2*) and T62 (*stp-3*)]. Flower number per inflorescence was scored from two separate populations used in the analysis of interactions between *stp-2* and *pim*, and *stp-2* and *veg2-2*. *veg2-2* and *pim* segregants were not included in this analysis. Unfortunately, a line carrying the fourth mutant allele at *Stp*, stp^{192}, was unavailable and therefore could not be included in these analyses.

The characterisation of the *stp-2* and *stp-3* mutants was presented in September 1996 at the Australian Society of Plant Physiologists combined conference, Canberra (SYM-09-05). Thus, the naming of the *stp-2* and *stp-3* alleles takes precedence over stp^{192} , which was published in the Flowering Newsletter, November 1996 (Beltrán *et al.* 1996). The stp^{192} allele should now be called *stp-4*.

Epidermal strips

Epidermal strips were taken from internode 7-8 and the petiole (between the stem and first leaflet pair) from the leaf at node 8 from six *stp-2* and *stp-3* plants, and six of their wild-type siblings segregating in backcrosses to HL107. Cell numbers were calculated for each plant by dividing the internode or petiole length by the average of ten cell lengths measured at random from that structure.

Linkage analysis of stp

The initial linkage analysis was conducted in a cross (T21) between HL111 and the original *stp-2* mutant line (M1a/324). To confirm the map position of *Stp*, a cross (T46) was made between HL6 (*Stp wa Sn*) and a mutant plant (*stp-2 Wa Sn*) from the F_2 of the first backcross of the original mutant line to its progenitor (HL107). *stp-2 wa Sn* F_3 recombinants derived from a single *Stp wa* F_2 plant were crossed with HL59 (*Stp Wa sn*) to confirm and clarify the linkage relationships of *stp* (cross T74). The isozymes *aspartate amino transferase-3* (*Aat3* or *Aat-m*), *aldolase* (*Aldo*), *shikimate dehydrogenase* (*Skdh*), *4-methylumbelliferyl-ß-D galactosidase-2* (*ß-Gal2*), and *6-phosphogluconate dehydrogenase-1* (*Pgd1*), were examined as described below. These isozyme loci are all present on linkage group VII (Weeden *et al.* 1996). Only *Skdh* and *Aat3* were found to segregate in cross T74.

Recombination fractions were calculated using the product ratio method (Stephens 1939) and joint segregation Chi-squares (χ^2) were calculated using a 2 x 2 contingency table.

Isozyme analysis

The isozyme phenotypes of parental and F_2 populations (in cross T74) were determined using horizontal starch electrophoresis. Isozymes were extracted from leaf

samples using a 0.2 M Tris HCl, 10% (v:v) glycerol and 10% (w:v) PVP-40 (pH 8.0) extraction buffer containing 20 µl of Triton-X-100, and 10 µl of mercaptoethanol (per 10 ml). Ground samples were absorbed onto filter-paper wicks. Samples were loaded onto three gels, each specific for the particular enzyme under consideration. Electrophoresis was performed for five to eight hours at 5°C.

Segregation of *Aat3* was examined using a 'Standard' gel system (Tris citrate buffer: 6.2 g Tris in 1 L, pH 8.4 with citric acid; lithium borate buffer: 47.7 g boric acid, to pH 8.1 with lithium hydroxide in 4 L). The gel was prepared using 10: 1 Tris citrate: lithium borate buffers with 10% starch. Lithium borate was used as the running buffer. *Aat* was stained in the dark at room temperature with L-aspartic acid (100 mg), α-ketoglutarate (50 mg), Fast Blue BB (50 mg) and piridoxal phosphate (trace) dissolved in 50 ml of 0.1 M Tris HCl buffer at pH 8.0.

A Histidine gel was used for *Skdh*, *Aldo* and *Pgd1*. Histidine gels contain a $^1/_4$ dilution of the histidine buffer (10.088 g L-histidine in 1 L, brought to pH 6.5 with approximately 1.5 g citric acid) and 10% starch. The samples were run in undiluted histidine buffer. *Skdh* was visualised by overlaying the gel slice with 30 mg shikimic acid, 8 mg NADP, 10 mg EDTA previously dissolved in 50 ml of 0.1M Tris HCl pH 8.5 containing MTT and MB. *6-Pgd* was stained at 37°C with 50 ml of 0.1M Tris HCl pH 8.5 containing 6-phosphogluconic acid (20 mg), NADP (8 mg), MTT and MB. Visualisation of *Aldo* required 50 ml of 0.1M Tris HCl pH 8.5 containing D-fructose 1,6-diphosphate (50 mg), arsenic acid (300 mg), NAD (30 mg), 80 µl glyceraldehyde 3-phosphate dehydrogenase, MTT and MB.

Analysis of the segregation of *ß-Gal2* required a C-gel system. As *ß-Gal2* runs towards the cathode, tissue samples were loaded into the middle of the gel. C-gels contain a 1: 10 dilution of C-gel buffer (8.2 g citric acid, to pH 6.1 with N-(3-aminopropyl) morpholone) with 10% starch and are run in undiluted C-gel buffer. *ß-Gal2* segregation was assayed using a fluorescent overlay. 4-methylumbelliferyl-ß-D galactosidase was dissolved into 2 ml of dimethyl formamide, diluted with 10 ml of 0.1M sodium citrate buffer (pH 4.5) and stained in the dark at 37°C for 15 min. The stain was visualised under UV light.

The interactions between stp-2 *and* uni, pim, af, tl, lf-a, det *and* veg2-2

Crosses between homozygous *stp-2* segregants and heterozygous *Uni/uni* plants from mutant line M1a/224 were made to determine the relationship between uni^{244} and *stp-2* (cross T27). The uni^{244} allele results in a severe phenotype.

The interactions between *veg2-2* and *stp-2* were examined in the cross T42 between line Wt16123 (*veg2-2*) and homozygous *stp-2* segregants from the original mutant line. Interactions between *stp-2* and *af*, *tl*, and *lf-a* were examined in a single cross (T44) between HL117 (*af tl lf-a*) and an *stp-2* plant from the first backcross. The *pim stp-2* double mutant was isolated from a similar cross (T64) with HL224 (*lf pim*). The interaction between *stp-2* and *det* was examined in cross T23 between HL245 (*r det veg1*) and an *stp-2* plant from the original mutant line. The *det* and *r* loci are tightly linked (Marx 1986b) and to save space only the wrinkled (*r*) F_2 seed were sown in cross T23. Most plants were grown two-per-pot in Hobart under the standard 18-h photoperiod conditions. For the examination of the SD phenotype, five populations segregating for either *uni* or *stp-2* were grown one-per-pot under an 8-h photoperiod.

Table III.1: Characteristics of *stp-2* and *stp-3* plants and their wild-type siblings from a second backcross to HL107. Data are shown as mean ±SE. Photoperiod 18 h. Students t-values were calculated between wild-type and their mutant siblings ($P < 0.001$ in all cases).

Character	*Stp*[a]	*stp-2*	t	*Stp*[a]	*stp-3*	t
L_{1-9} (cm)	50.44 ±0.39	45.20 ±0.59	7.41	49.44 ±0.65	43.41 ±0.98	5.13
C-3	11.48 ±0.11	20.60 ±0.51[b]	17.48	11.27 ±0.11	15.73 ±0.38	11.27
NFI	16.29 ±0.08	20.09 ±0.21	16.84	15.95 ±0.06	18.73 ±0.20	13.31
TN	20.56 ±0.14	31.73 ±0.68	16.09	20.24 ±0.14	31.18 ±0.62	17.21
Flowers[c]	1.69 ±0.06	1.28 ±0.09	3.92	–	–	–

a: *Stp* values are derived from wild-type siblings from populations segregating for either *stp-2* or *stp-3*; wild type: 41 individuals, *stp-2* or *stp*-3: 11 individuals

b: only five *stp-2* plants reached C-3; neither *stp-2* nor *stp-3* plants attained C-5

c: number of flowers present on the first secondary inflorescence scored from wild-type and *stp-2* segregants from two separate populations; wild type: 55 individuals, *stp-2*: 29 individuals

Table III.2: Epidermal cell lengths (µm) and numbers measured from internode 7-8 and the leaf rachis from node 8 for *stp* mutants and their wild-type siblings (mean ±SE). Cell numbers were calculated by dividing the length of the internode or rachis by the average of ten cell lengths measured from that internode/rachis.

Tissue	Cell lengths			Cell numbers		
	Stp[a]	*stp-3*	t	*Stp*	*stp-3*	t
Internode[b]	452.5 ±15.0	467.2 ±9.0	0.84	240.6 ±12.2	187.3 ±5.8	3.94**
Leaf rachis[b]	376.6 ±8.0	369.8 ±8.0	0.60	170.4 ± 3.8	139.0 ±2.9	6.57***
	Stp[a]	*stp-2*	t-value	*Stp*	*stp-2*	t-value
Internode[b]	468.2 ±9.0	453.7 ±14.0	0.87	219.6 ±5.0	194.1 ±8.0	2.69*
Leaf rachis[b]	372.5 ±5.4	361.3 ±5.8	1.41	171.6 ±5.3	131.0 ±4.0	6.10***

a: *Stp* values are derived from wild-type siblings from populations segregating for either *stp-2* or *stp-3*.

b: 10 cell lengths from six individuals of each genotype were measured for each tissue.

*, **, *** $P < 0.05$, 0.01, and 0.001, respectively

Table III.3: F_2 joint segregation data for *stp-2* and several linkage group VII markers.

Loci		Cross[a]	Phase[b]	Phenotype[c]						Total	Single Seg. χ^2		Joint Seg. χ^2	Prob.	Recomb. Fraction
				DD	DR	RD	RR				Locus 1	Locus 2			
Stp	*Aat3*[d]	1	Rep	13	14	11	2			40	1.20	3.33	4.86	0.05	26.5 ±14.5%
Stp	*Wa*	2	Rep	35	18	22	0			75	0.75	0.04	9.83	0.01	~6.6 ±11.5%
Stp	*Wa*	3	Cou	88	2	4	24			118	0.10	0.55	86.65	0.0001	4.4 ±1.95%
Stp	*Aat3*	3	Rep	52	38	25	3			118	-	5.98*	9.36	0.01	27.2 ±8.4%
Stp	*Sn*	3	Rep	73	17	22	6			118	-	1.91	0.08	0.9	52.2 ±6.7%
Wa	*Sn*	3	Rep	75	17	20	6			118	-	-	0.27	0.5	53.9 ±6.6%
Wa	*Aat3*	3	Rep	53	39	24	2			118	-	-	10.75	0.002	22.3 ±8.7%
Sn	*Aat3*	3	Cou	65	30	12	11			118	-	-	2.14	0.1	40.5 ±6.2%
				DF	DH	DS	RF	RH	RS						
Stp	*Skdh*	3		8	40	42	24	4	0	118	-	9.32**	84.68	0.0001	10.1 ±3.0%
Wa	*Skdh*	3		11	39	42	21	5	0	118	-	-	50.19	0.0001	13.8 ±3.5%
Sn	*Skdh*	3		26	36	33	6	8	9	118	-	-	0.16	0.9	47.3 ±6.7%
Aat3	*Skdh*	3		30	34	13	2	10	29	118	-	-	36.07	0.0001	8.4 ±4.3%

a: Cross: 1) T21 (M1a/324 x L111; *stp-2* $Aat3^F$ x *Stp* $Aat3^S$)
2) T46 (M1a/324 x L6; *stp-2 Wa* x *Stp wa*)
3) T74 (T46- *stp-2 wa* F_3 x L59; *Sn stp-2 wa* $Aat3^F$ $Skdh^F$ x *sn Stp Wa* $Aat3^S$ $Skdh^S$)

b: Phase: Rep = repulsion, Cou = coupling

c: Phenotype: D= dominant, R= recessive, F= homozygous fast, H= heterozygous, S = homozygous slow. The first named locus is shown first.

d: Only the presence of the fast form of *Aat3* could be scored, therefore fast and heterozygous classes have been grouped as D, the slow form as R.

*, **: significant at the 0.05 and 0.01 levels, respectively.

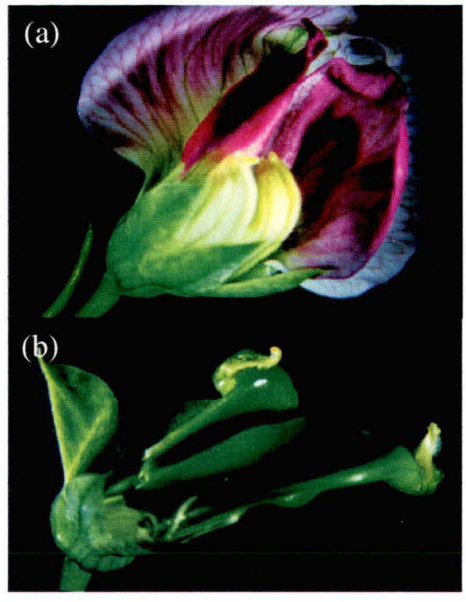


Figure III.1: The original *stp-1* mutant described by Monti and Devreux (1969). This mutant has characteristics of a class B floral homeotic mutation.
(a) Petals contain green sepaloid streaks and fusion of the keel is disrupted. (b) Two of the stamens are partially transformed into carpels.

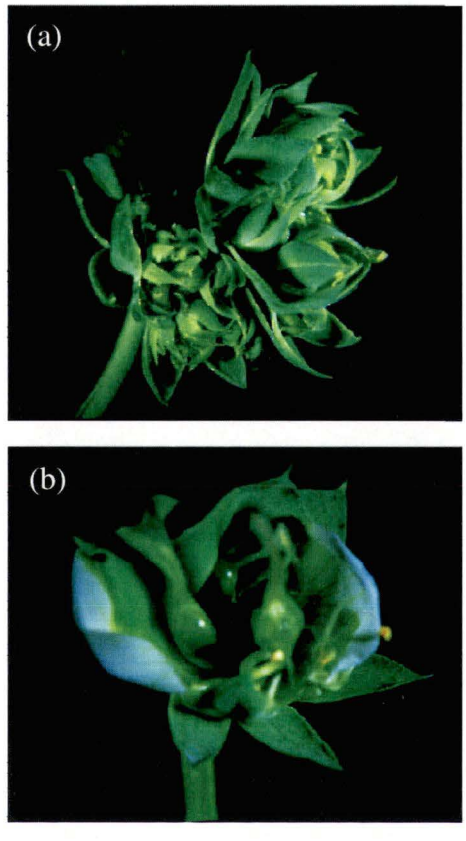


Figure III.2: The *stp-2* and *stp-3* mutants were isolated from an EMS mutagenesis programme at Hobart. (a) The *stp-2* mutant shows complete transformation of petals into sepals and stamens into carpels. In addition, the floral meristem proliferates; secondary and tertiary flowers are produced on pedicels within the primary flower.
(b) The severity of the *stp-3* allele lies between that of *stp-2* and *stp-1*.

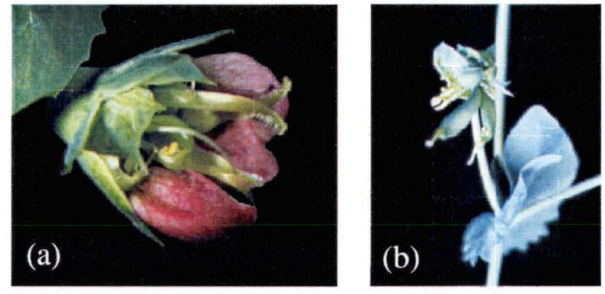
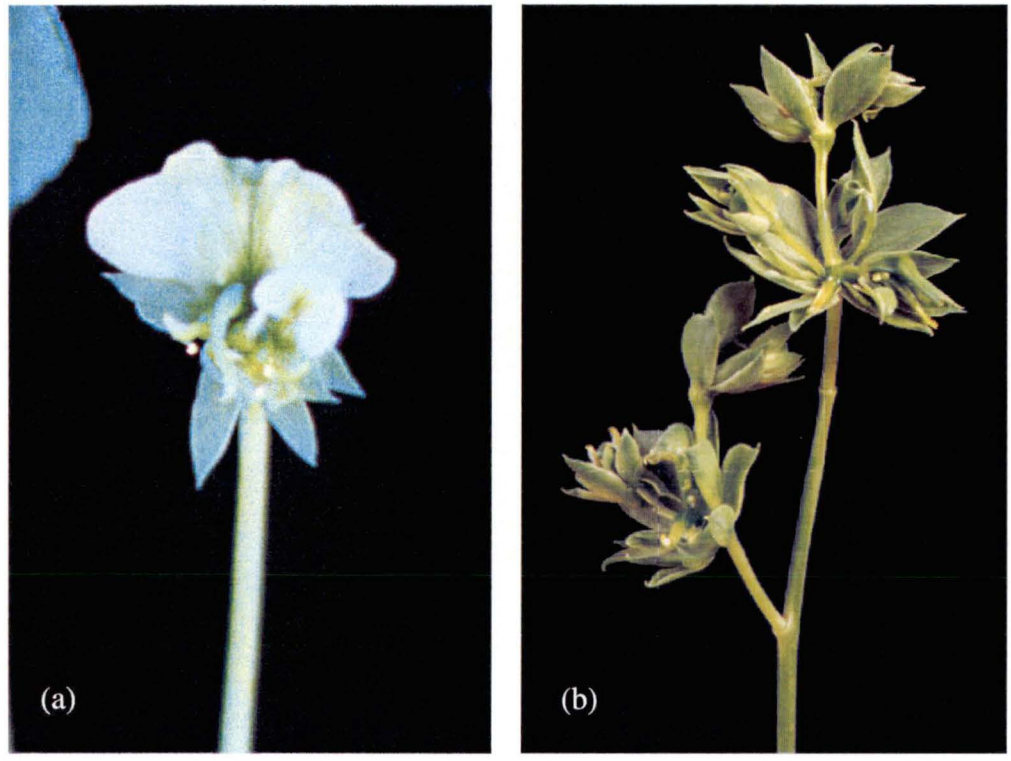
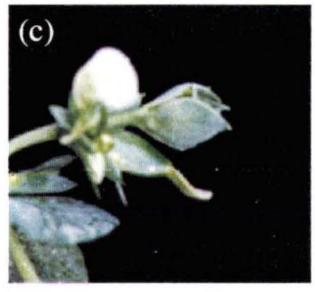


Figure III.3: The phenotype of the F_1 flowers in crosses between *stp* mutants. (a) A flower from an *stp-1 stp-2* heterozygote. (b) A flower from an *stp-2 stp-3* heterozygote. The phenotype of the F_1 flowers lies between that of each homozygous parent (see **Figures III.1**, **III.2** and **III.4**).

Figure III.4: Dissection of *stp-1* (a), *stp-3* (b) and *stp-2* (c) mutant flowers. (a) The sepaloid streaks in the petals, and the conversion of stamens to carpels, can be seen in *stp-1* mutant flowers. (b) *stp-3* causes the complete conversion of some petals into sepals and some stamens into carpels, although normal stamens and petals do form. (c) The *stp-2* mutant shows complete conversion of all petals to sepals and stamens to carpels. The numbers of these organs are also reduced. In addition *stp-2* mutants always produce at least one secondary ectopic flower (arrowed).





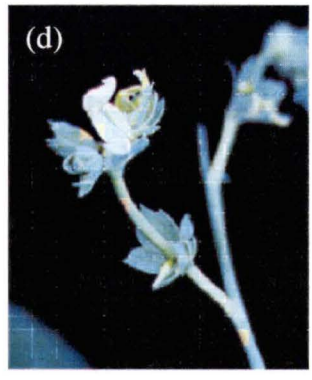


Figure III.5: *stp-2* and *stp-3* mutant flowers. (a) A weaker *stp-3* mutant flower; in this case the standard has not been severely affected by the *stp-3* mutation. Despite this, petal-to-sepal and stamen-to-carpel transformations are still more severe than those caused by the *stp-1* mutation (see **Figure III.1**). (b) Proliferation of the *stp-2* mutant flower; four ectopic flowers in the upper flower, and three in the lower flower. Tertiary flowers are also partially visible. (c) Ectopic flowers were also found in *stp-3* mutant flowers that developed late on lateral shoots. (d) The *stp-2* mutant phenotype was unaffected by growth under a SD (8 h) daylength. Both petal and stamen production were also found under LD (18 h) conditions.

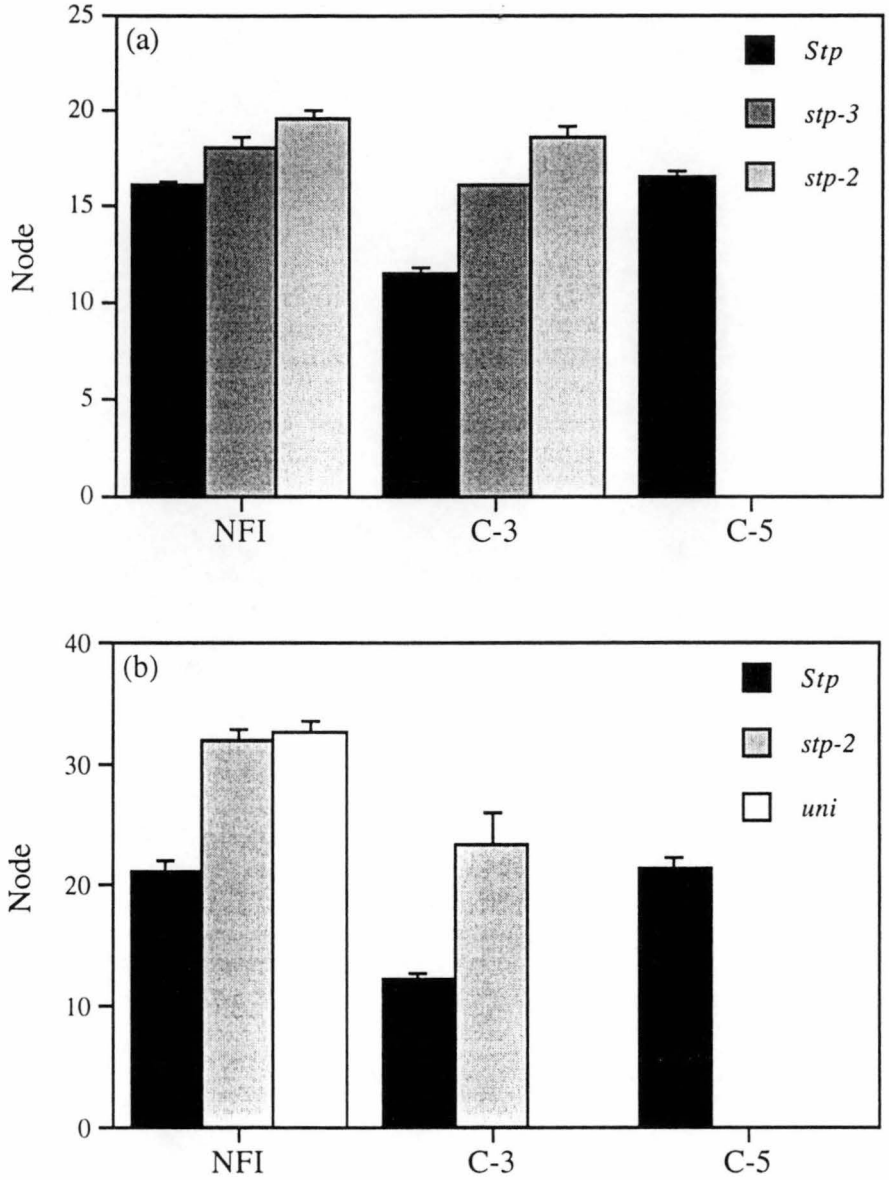


Figure III.6: Characteristics of *Stp*, *stp-3* and *stp-2* plants grown under an 18-h photoperiod (a) and *Stp*, *stp-2* and *uni* plants grown under an 8-h photoperiod (b). NFI, node of flower initiation; C-3, node bearing the first leaf with three or more leaflets; C-5, node bearing the first leaf with five or more leaflets.

Figure III.7: Inflorescence development in the *stp-2* mutant.
(a) The apex of an *stp-2* mutant plant that has prematurely arrested in an apparently terminal flower. This was often associated with the complete simplification of the leaf, similar to that produced by *uni* mutant plants (see **Figure III.11**). (b) The production of more than one axillary shoot was also associated with the *stp-2* mutation. (c) Wild-type plants only release one axillary bud from dormancy. In contrast, *stp-2* mutant plants typically released three in their primary inflorescence. Although one was the secondary inflorescence and the other two vegetative laterals, the three lateral shoots were often very similar in appearance.

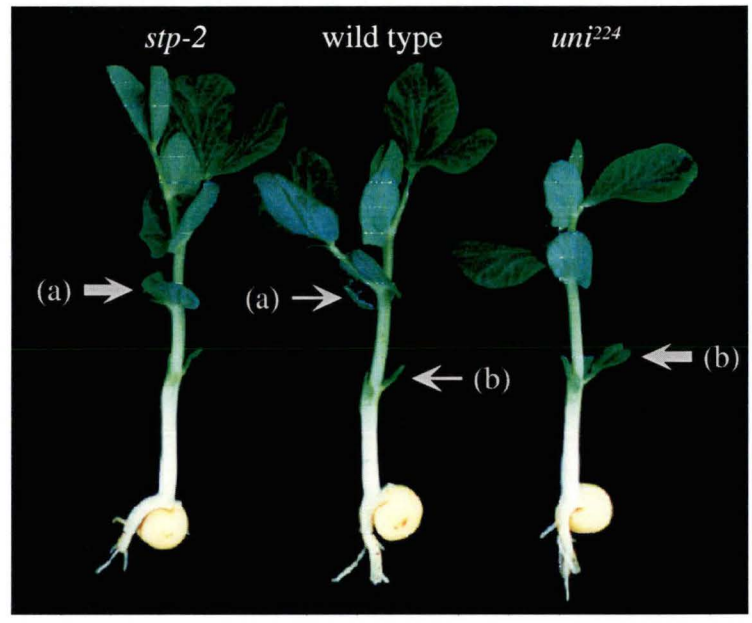


Figure III.8: Seedling phenotypes of *stp-2*, wild type and *uni*^{224}. Both *uni*^{224} and *stp-2* can be identified on the basis of specific seedling characteristics. *stp-2* mutant plants lack the foliage leaf normally present at node three (a). *uni*^{224} and *uni*^{224} *stp-2* double mutant plants are unifoliate and also possess small leaf-like structures at node two (b). These three plants were 11 days old when photographed. The three genotypes were segregating in F_3 populations from the cross between *stp-2* and *uni*^{224}.

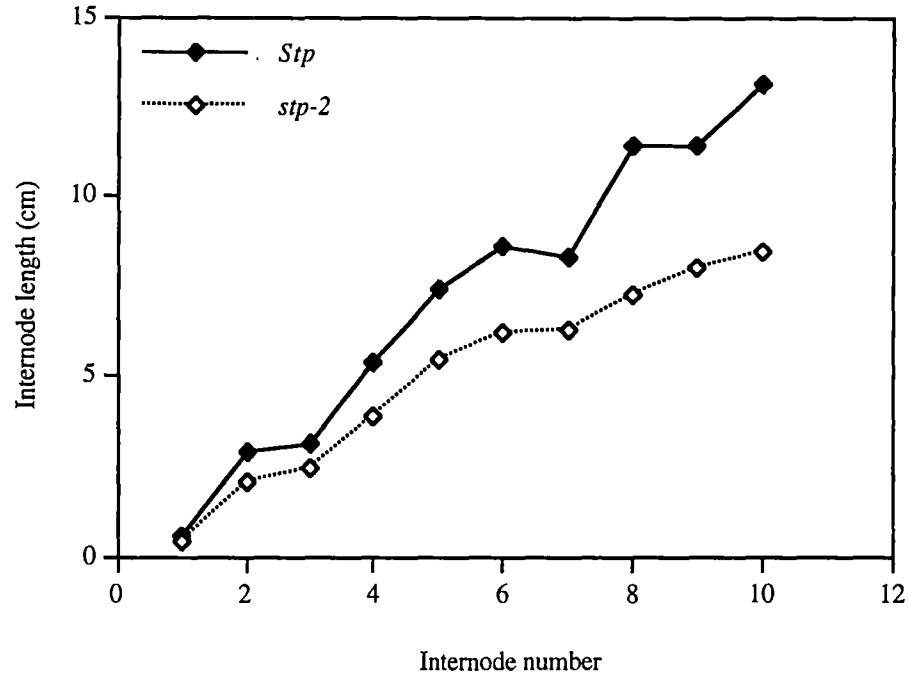


Figure III.9: Effect of the *stp-2* mutation on internode length. Each internode of the *stp-2* mutant plants was marginally (although not always significantly) smaller than the corresponding wild-type internode. The sum total of these slight differences results in an approximately 10% reduction in the stem length between nodes one and nine (see **Table III.1**). Photoperiod 18 h.

Figure III.10: Summary of linkage results from cross T74 for segregation of *stp-2* and group VII markers (**Table III.3**). Linkage between *Sn* and the other markers examined was not significant (**Table III.3**). Recombination values between pairs of loci are expressed in cM.

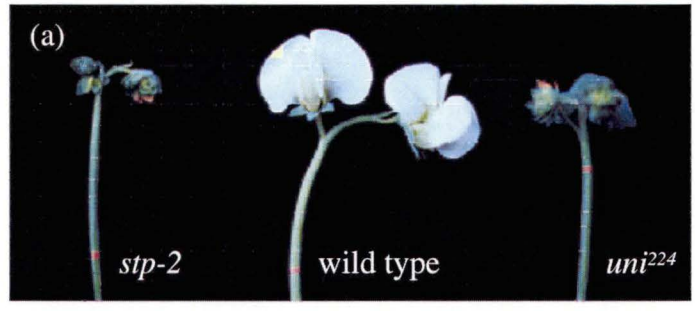


Figure III.11a: Secondary inflorescences from *stp-2*, wild-type and *uni*^{224} plants. The *uni* and *stp-2* mutations have a very similar effect on the flower phenotype.

Figure III.11b: Primary inflorescence from a *uni*^{224} plant. *uni* results in the complete simplification of the compound pea leaf. The effect of *uni*^{224} on leaf development is more severe than that of *stp-2*. However, the simpler *stp-2* leaf may represent an intermediate between the complex wild-type and the simple *uni* leaf forms.

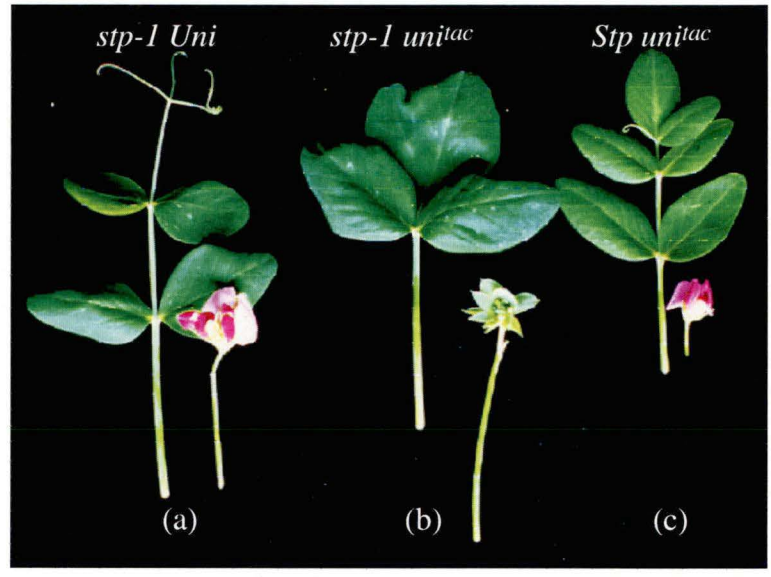
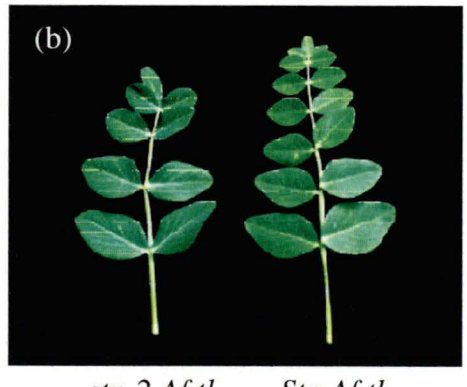


Figure III.12: Interaction between *stp-1* and *uni*^{tac}. Although the weakest mutant alleles at *Stp* (*stp-1*, a) and *Uni* (*uni*^{tac}, c) only affect the development of the flower or the leaf, respectively, the double mutant (b) shows severe disruption of both flower and leaf development. The double mutant phenotype resembles that of a severe *uni* mutant.

stp-2 Pim *stp-2 pim-1* *Stp pim-1*

Figure III.13: Interaction between *stp-2* and *pim-1*.
Three plants, representing the genotypes *stp-2 Pim*, *stp-2 pim-1* and *Stp pim-1*, are shown (a). The *stp-2 pim-1* double mutant possesses characteristics of both parental mutants (b). Flowers are defined, but consist entirely of bract like organs. Thus, *Stp* and *Pim* act together to identify the normal organs within the flower, but are not essential for floral meristem identification.

stp-2 af Tl *Stp af Tl*



stp-2 Af tl *Stp Af tl*

stp-2 af tl *Stp af tl*

Figure III.14: Interactions between *stp-2* and the leaf homeotic mutations *af* and *tl*. (a) Interaction between *stp-2* and *af*. The *af* mutation converts all leaflets to tendrils; addition of the *stp-2* mutant allele simplifies the *af* leaf at each comparable node. (b) Interaction between *stp-2* and *tl*. The *tl* mutation replaces all tendrils with leaflets; *stp-2* reduces the number of leaflets borne on each leaf (see also **Figure III.15**). (c) The phenotype of the triple mutant clearly indicates a role for *stp-2* in leaf development. Leaves in (b) and (c) were from equivalent nodes.

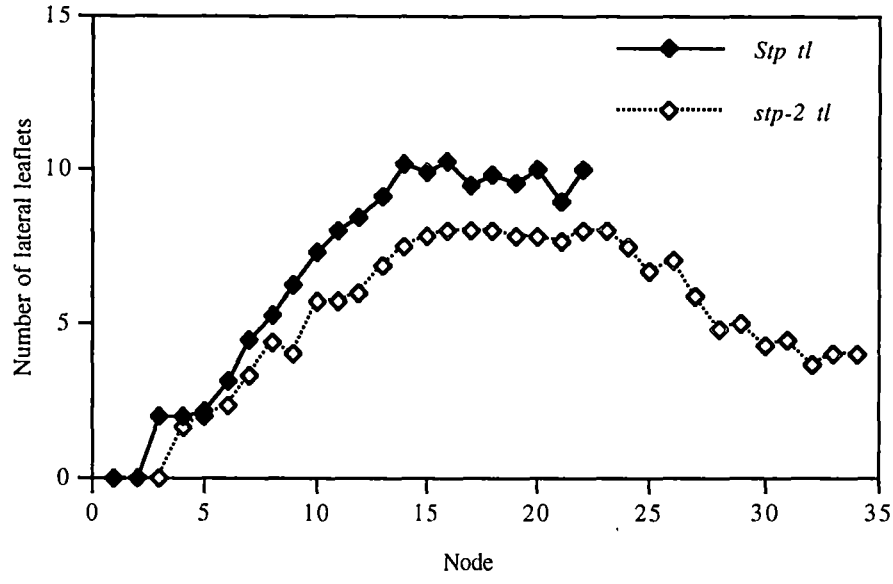
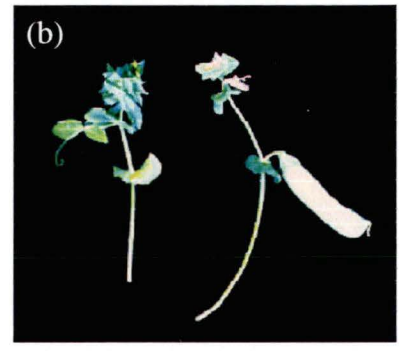


Figure III.15: Number of lateral leaflets borne on leaves at all nodes of *stp-2* and *Stp* siblings carrying the recessive *tl* mutation (see **Figure III.14b**). The *stp-2* mutation reduces the total number of structures (leaflets or tendrils) borne on each leaf when compared with a wild-type leaf at the same node. However, the sequential addition of lateral structures during ontogeny is largely unaffected by *stp-2*. Note also that *stp-2* plants do not produce their first true leaf (bearing two leaflets) until node four. Photoperiod 18 h.

stp-2 det *Stp det*

Figure III.16: The 'determinate' inflorescence structure caused by *det* is unaffected by the *stp-2* mutation and the proliferation of the *stp-2* mutant flower is unaffected by the *det* mutation. These older mutant flowers also illustrate the extent of floral proliferation that may be reached on *stp-2* mutant plants.

stp-2 veg2-2 *Stp veg2-2*



stp-2 veg2-2 *Stp veg2-2*

Figure III.17: Primary inflorescences (a) and secondary inflorescences (b) from *Stp veg2-2* and *stp-2 veg2-2* F_2 segregants. The double mutant phenotype is a simple addition of the two single mutant phenotypes. The proliferous secondary inflorescences characteristic of the *veg2-2* mutant express typical characteristics of the *stp-2* primary inflorescence in the double mutant and terminate in a cluster of sepal and carpel chimeric organs (compare (b) with **Figure III.7**).

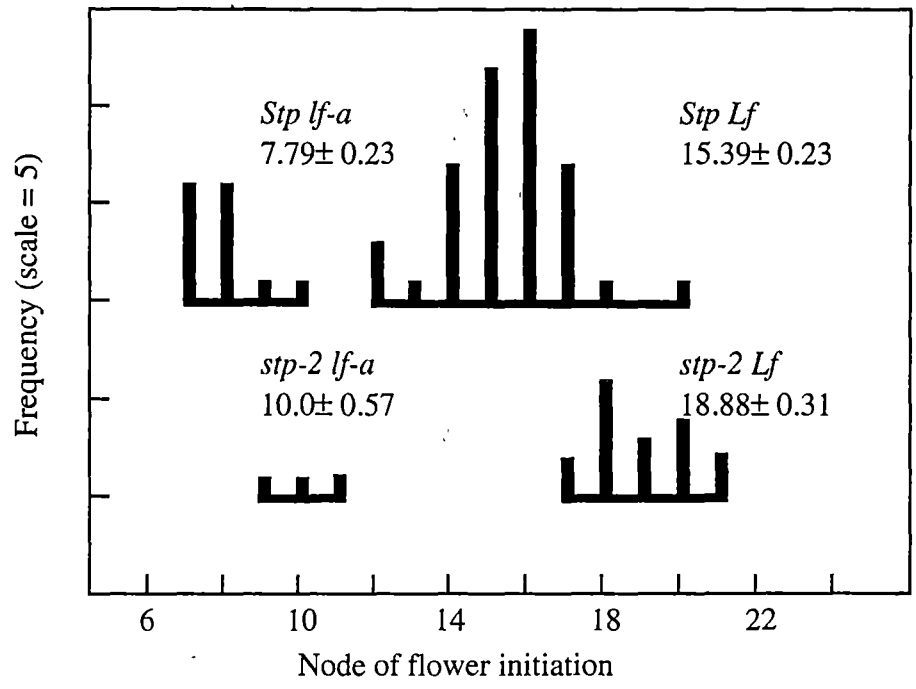


Figure III.18: Node of flower initiation of the four classes segregating in the cross between *lf-a Stp* and *Lf stp-2*. The four genotypes and the mean (± SE) are given above the distributions of node of flower initiation. The *stp-2* mutation was able to significantly delay ($P < 0.001$) the node of flower initiation in both *Lf* and *lf-a* plants.

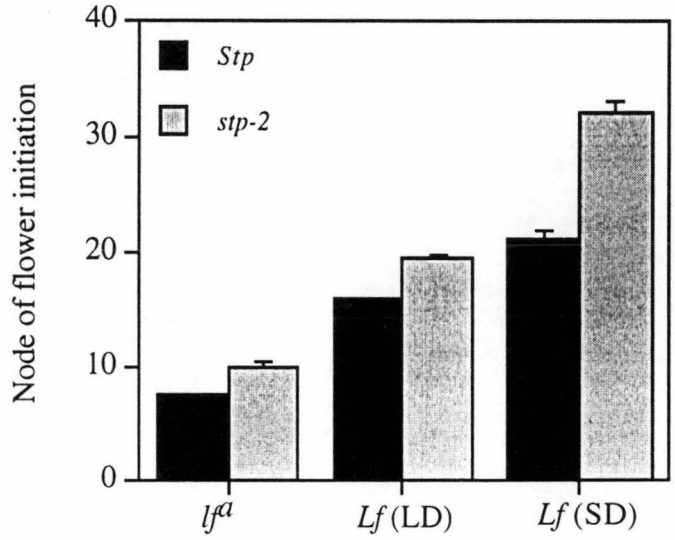


Figure III.19: Effect of *stp-2* on node of flower initiation. The delay in node of flower initiation caused by the *stp-2* mutation is expressed in both *lf-a* and *Lf* plants. In *Lf* plants, the delay is enhanced under a short day photoperiod.

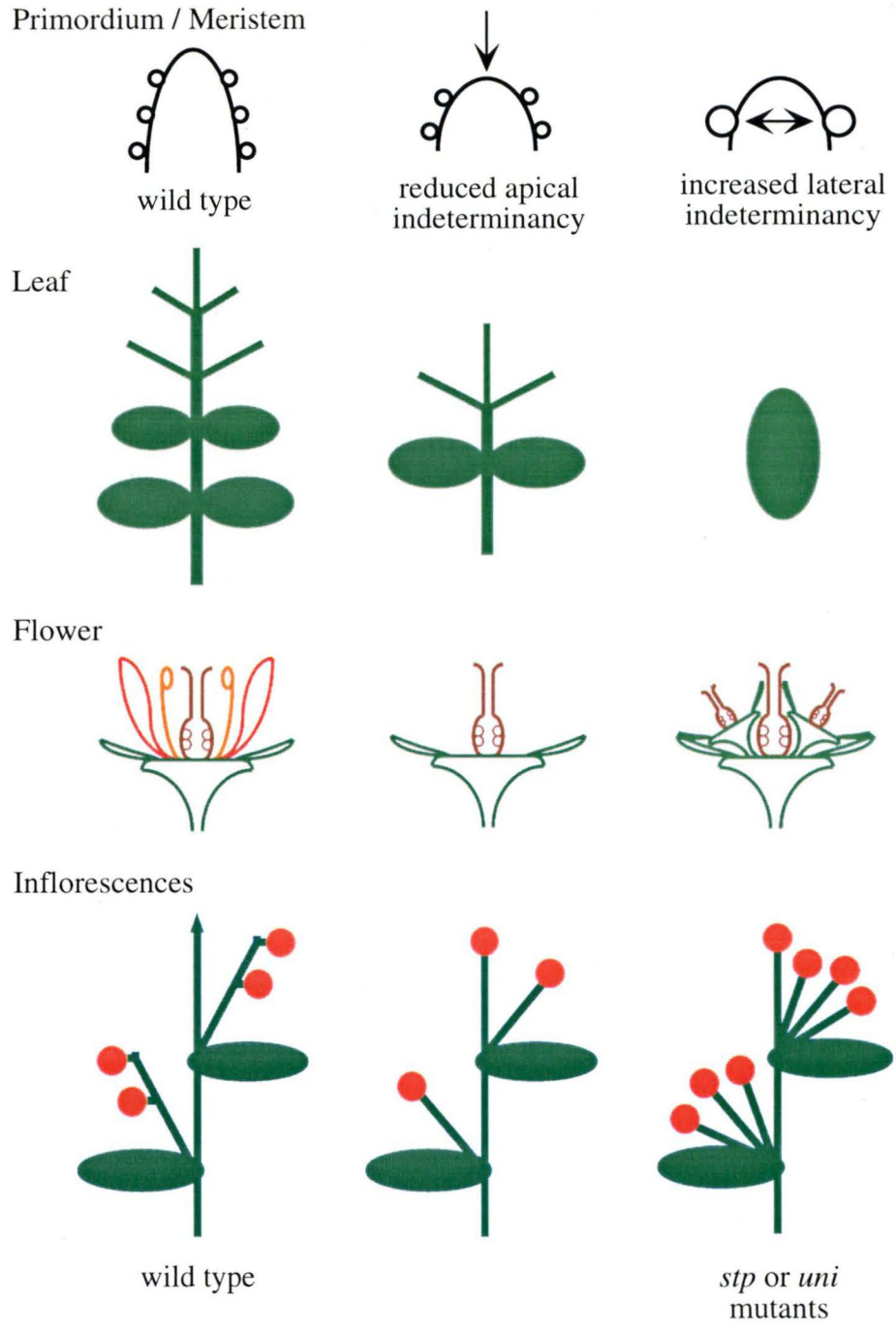


Figure III.20: The effect of the *stp* and *uni* mutations on plant development may result from direct effects on primordium or meristem growth. It is proposed that the *stp* and *uni* mutations reduce the apical growth of the meristems or primordia and increase lateral indeterminancy (shown here as a two-step process).
The general effect on the primordium / meristem is given first.
In the leaf, this results in a reduction in length and eventual production of a single lamina. In the flower, with the associated effect on Class B floral homeotic gene activity, petals and stamens are lost, and lateral shoots (ectopic flowers) are produced. In the inflorescences, a decrease in apical indeterminancy results in the production of a terminal flower and the reduction of secondary inflorescences whilst an increase in lateral indeterminancy promotes outgrowth of axillary shoots.

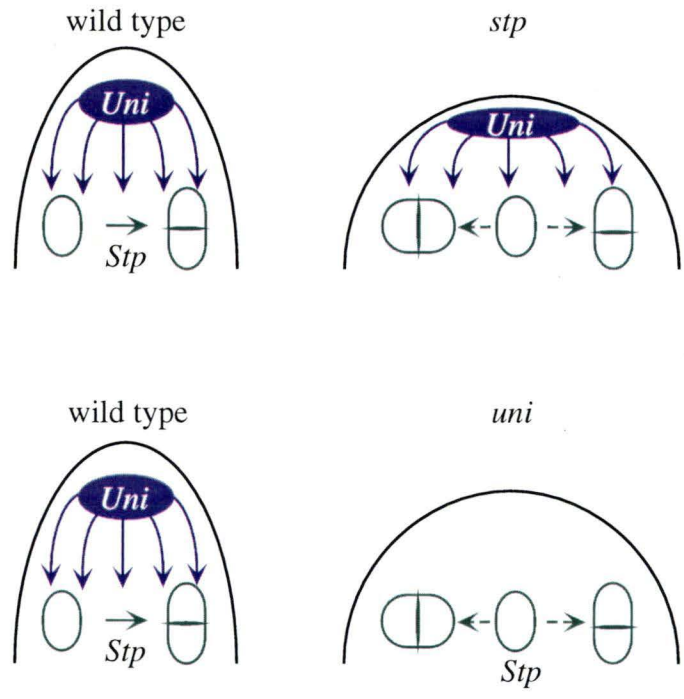


Figure III.21: Possible interaction between *Uni* and *Stp*.
In this model, the development of a wild-type primordium or meristem requires the interaction between *Uni* and *Stp*. It is proposed that *Uni* activity results in a non-cell-autonomous signal that provides positional information. *Stp* responds to the gradient in this signal by favouring periclinal divisions (or suppressing anticlinal divisions). Plants carrying an *stp* mutation are unable to read the positional information provided by *Uni*, or correctly direct cell divisions. Plants carrying a *uni* mutation cannot provide the positional information. The effect of either mutation is a relative increase in anticlinal division, reducing apical growth and promoting lateral growth of the primordium / meristem. This role is non-specific; thus *uni* and *stp* mutations affect a wide range of developmental processes (e.g. **Figure III.20**).

Chapter IV. The molecular nature of *Stp*

Introduction

The characterisation of the two newly identified alleles at *stp* has revealed a third gene (in addition to *Pim* and *Uni*) involved in the identification of the floral meristem in pea (**Chapter III**). The *Stp* gene functions in combination with *Pim* to correctly identify the floral meristem. *Stp* also interacts with *Uni*; the two genes may partner each other in floral meristem identification, or alternatively, the function of *Uni* may be mediated through *Stp* activity. In addition, the phenotypes of the more severe *stp-2* and *stp-3* mutants have indicated that *Stp*, like *Uni*, is also involved in leaf and inflorescence development. The pleiotropic effects of the *stp* mutations may result from the influence of *Stp* on cell division (**Chapter III**). Together, *Stp* and *Uni* define a developmental pathway that may regulate the growth pattern of all shoot-derived meristems.

The phenotypes of *stp* mutant flowers are remarkably similar to those of the *fimbriata* mutant of *Antirrhinum*. Severe mutations at these two genes result in sepaloidy and production of ectopic flowers within the primary flower (**Chapter III**, Carpenter and Coen 1990, Simon *et al.* 1994, Ingram *et al.* 1997). Intermediate alleles of *stp* or *fim* result in chimeric organs – primarily petal/sepal mosaics – and the mutants again possess a remarkably similar phenotype. *Fim* is thought to act after *Floricaula* (*Flo*, the *Antirrhinum* homologue of *Uni*; Hofer *et al.* 1997), and severe *flo* mutations are completely epistatic to mutations at *fim*. *Fim* also acts in combination with *Squamosa* to identify the floral meristem. *Fim* is thought to regulate the activity of the floral homeotic genes *Deficiens* (class B) and *Plena* (class C) (Simon *et al.* 1994). Thus, mutants in *Fim* and *Stp* share many of their morphological characteristics and genetic interactions.

The relationship both phenotypically and genetically between *Uni* and *Stp* is also mirrored in that between the *LFY* and *UFO*, the *Arabidopsis* homologues of *Flo* and *Fim* from *Antirrhinum* (Weigel *et al.* 1992, Ingram *et al.* 1995). The *uni* and *stp* mutants share a number of characteristics; they have simpler leaves, their primary inflorescence often terminate with a flower, and they have similar floral morphologies (**Chapter III**, Hofer *et al.* 1997). Similarly, both *lfy* and *ufo* mutants in *Arabidopsis* possess cauline leaves or filaments subtending the flowers, and they have remarkably similar floral morphologies (Lee *et al.* 1997). Severe *uni* mutations reduce the complexity of the pea compound leaf to unifoliate, whereas *lfy* mutations in

Arabidopsis 'increase' the complexity of the leaf; from the lack of a structure subtending each flower to the production of cauline leaves on the inflorescence (Schultz and Haughn 1991, Huala and Sussex 1992, Weigel *et al.* 1992). Mutants *stp* from pea and *ufo* from *Arabidopsis* form intermediate structures: *stp* plants have simpler leaves but the compound nature is retained (**Chapter III**) and *ufo* mutants often have filamentous structures subtending the flowers (Wilkinson and Haughn 1995, Levin and Meyerowitz 1995). Although these phenotypes are opposite in effect, the progression from wild type to *stp / ufo* to *uni / lfy* implies a similar relationship underlies the *Uni – Stp* and *LFY – UFO* interactions.

Furthermore, the observed effects of the *stp* mutations on cell division (**Chapter III**) are in agreement with the proposed role of *UFO* and *Fim* in cell proliferation (Wilkinson and Haughn 1995, Ingram *et al.* 1997, Meyerowitz 1997).

The phenotypic similarities between the *stp*, *fim* and *ufo* mutants may indicate that the three mutations are in homologous genes. *UFO* and *Fim* have already been shown to be *Arabidopsis* and *Antirrhinum* orthologues (Ingram *et al.* 1995). This chapter describes work at the molecular level aimed to determine the relationship between the *Stp* gene of the garden pea, *Fim* from *Antirrhinum*, and *UFO* from *Arabidopsis*.

Results

Isolation of the pea homologue of Fimbriata / UNUSUAL FLORAL ORGANS

Although the existence of a pea homologue of *Fim / UFO* was expected, heterologous probing of a pea genomic Southern blot with the full length *Arabidopsis UFO* gene failed to detect any bands. Attempts to isolate a fragment of the pea homologue of *Fim / UFO* using PCR (Polymerase Chain Reaction) on wild-type pea genomic DNA also proved unsuccessful. Therefore, a 580-bp PCR-derived fragment from a conserved, 3′ region of the *UFO* gene was used to screen a phage cDNA library produced from flowering apices of JI813 (HL51y). This library was kindly supplied by Dr Julie Hofer (JIC, UK). Screening of the library resulted in the identification of a single clone (GoC.1) found to cross-hybridise with the *Arabidopsis* probe. A restriction map of this 1.9-kb clone is presented in **Figure IV.1**. Both strands of the cDNA insert were sequenced using ABI prism dye terminator chemistry (Applied Biosystems) and a series of specific oligonucleotides as primers. GoC.1 contained the complete open reading frame as a number of possible start methionine

and stop codons were present, flanked by putative untranslated 3′ and 5′ regions and a polyA tail (**Figure IV.2**). The protein produced from GoC.1 would be expected to be 443 aa in length if translated from the first start methionine (**Figure IV.2**), with a mass of ~49 kD. Database searches revealed significant similarity between the deduced amino acid sequence of the pea clone and the *Antirrhinum* and *Arabidopsis Fim* and *UFO* sequences. No significant homology with other sequences present in the GenBank database was found. Therefore this cDNA clone was named *peafim* and it is likely to represent the pea homologue of *Fim / UFO*.

Alignment of the deduced amino acid sequences revealed large areas of conservation between the pea, *Antirrhinum* and *Arabidopsis* sequences (**Figure IV.3**), and a high overall amino acid identity between the *peafim* clone and *Fim* (63.7%) and between *peafim* and *UFO* (61.1%). These percentages compare favourably with the 60% sequence identity found between *UFO* and *Fim* (Ingram *et al.* 1995) and similarities between other known pea, *Antirrhinum* and *Arabidopsis* gene orthologues (**Table IV.1**).

Phylogenetic analyses indicate that pea and *Arabidopsis* are the more closely related of these three species; both belong to the subclass Rosidae (Chase *et al.* 1993, Soltis *et al.* 1997). However, the consistently higher similarity between the pea and *Antirrhinum* sequences would suggest that these two are the more closely related of the three species. This is also reflected in the nucleotide sequence comparisons among pea, *Antirrhinum* and *Arabidopsis* – *peafim* sharing 67.6% sequence identity with *Fim* and 65.6% with *UFO*. The *Fim* and *UFO* nucleotide sequences share 63.9% sequence identity. The apparent insertion present in the *Arabidopsis UFO* gene (**Figure IV.3**) does not affect this similarity.

Cosegregation of peafim *and* stp-2

A search for a polymorphism between wild-type (*Stp*) and mutant (*stp-2*) plants revealed a clear RFLP between Hobart lines 111 and 107 for the *peafim* gene (**Figure IV.4**). This polymorphism was followed in 50 F_2 plants from the cross between lines HL111 and M1a/324 (*stp-2*; M1a/324 is a mutant line derived from HL107). Analysis indicated that each of the 10 plants homozygous for *stp-2* was also homozygous for the 8-kb HL107 polymorphism, whereas the 40 wild-type plants each carried the ~5-kb polymorphism from HL111 (**Figure IV.5**). Thus, *Stp* and *peafim* are closely linked.

To support this cosegregation analysis, *peafim* was mapped on the pea molecular map from the recombinant inbred lines derived from the cross JI281 x JI399. This was done by Drs Noel Ellis and Julie Hofer in the Applied Genetics Department of the John Innes Centre. The *peafim* clone showed strong linkage to characters on pea linkage group VII, and lies between *Cab/1* (*chlorophyll a/b binding protein*) and *Rrn2* (*ribosomal DNA cluster 2*) (**Figure IV.6**). This is the same region where the *Stp* gene is found (Monti and Devreux 1969, Monti 1970, **Chapter III**) and provides an additional bridge between the pea classical and molecular linkage maps (**Figure IV.6**). Therefore, these results agree with the cosegregation analysis and support the hypothesis that the *stp* phenotype results from mutations in the *peafim* gene.

Expression of peafim *in the* stp *mutants*

To examine the possibility that one or all of the *stp* mutations affect the size or production of the *peafim* transcript, total RNA was isolated from flowering apices of wild-type, *stp-1*, *stp-2* and *stp-3* plants. Under highly stringent conditions, a single transcript was found to cross hybridise with the *peafim* probe (**Figure IV.7**). The size of the *peafim* transcript was expected to be approximately 1.9 kb, which is also the size expected from the lower ribosomal band. Thus, it was unclear whether this represented the true expression pattern of *peafim* in these mutants, or if it resulted from cross hybridisation with the lower ribosomal band. Northern analysis of poly A^+ RNA isolated from the total RNA samples and probed with *peafim*, failed to detect any transcript, even under lower stringency. This would indicate that the bands produced from the total RNA northern resulted from cross hybridisation with ribosomal RNA. It would also indicate that the *peafim* transcript is sufficiently rare as to place it below the sensitivity of the detection system used.

Reverse Transcription-PCR (RT-PCR) was performed on the poly A^+ RNA using oligonucleotides specific to the *peafim* sequence to identify the effect of the *stp* mutations on *peafim* expression. The presence of a PCR product from wild-type and the three mutant lines, each product the expected size of the cDNA (**Figure IV.8a**), clearly indicates that the three mutants are indeed able to produce RNA transcripts of an appropriate size. However, the amount of product produced in the *stp-2* and *stp-3* reactions at higher dilutions ($^1/_{1000}$) appeared to be lower than that for *Stp* and *stp-1* (**Figure IV.8a**). All three *stp* mutants are in tall (*Le*) backgrounds. Therefore a second PCR reaction using the $^1/_{100}$ and $^1/_{1000}$ cDNA dilutions and oligonucleotides

specific for the pea *Le* gene (Lester *et al.* 1997) was used to confirm the effect of *stp-2* and *stp-3* on the *peafim* transcript. This analysis revealed a similar decrease in the amount of *Le*-PCR product (**Figure IV.8b**). Although it is recognised that informative quantitative PCR requires the use of an internal control, these results suggest that the reduced level of product are due to initial template quantities and not from differences in levels of *peafim* expression in *stp-2* and *stp-3* mutants.

PCR of peafim *from genomic DNA*

In an effort to resolve the relationship between *peafim* and *Stp*, specific oligonucleotides were designed to amplify by PCR the *peafim* gene from total genomic DNA isolated from HL107, AF106 (*stp-3*) and an *stp-2* F_2 segregant (T21a/1/23) from cross M1a/324 x HL111. The *stp-2* plant used was considered acceptable as it was shown to possess the HL107 polymorphism when probed with *peafim* (**Figure IV.5**) and thus should contain the *peafim* sequence originally derived from HL107. PCR was initially unsuccessful. Eventually, 1.9 kb PCR products with restriction patterns expected from the restriction map of the *peafim* clone were produced from wild-type and each of the mutant plants (**Figure IV.9**). The size of the genomic *peafim* PCR product indicates that, like *UFO* and *Fim* (Simon *et al.* 1994, Ingram *et al.* 1995), *peafim* does not contain any introns. A single PCR product from each line was inserted into the pGEM T-Vector (Promega) and sequenced as described above.

The lack of *Eco*RI and *Bam*HI restriction sites in the PCR products derived from HL107 genomic DNA (**Figure IV.9**) and the presence of two bands for these digests in the Southern analysis (**Figure IV.1**) indicates that a second, closely related *peafim*-gene is present in the pea genome. This was also found in *Arabidopsis* where nine classes of *Fim*-like clones were found in a genomic library (Ingram *et al.* 1995).

A comparison between the HL107 *peafim* amino acid sequence and the sequence of the original cDNA from the library derived from HL51y (JI813) revealed a number of minor differences. Two lysine to arginine changes and three asparagine to serine changes were identified in HL107 compared with HL51y which result from five A to G changes in the nucleotide sequence. The result is a *peafim* sequence from HL107 that is marginally closer to that of *Fim* and *UFO* compared with the *peafim* cDNA clone from HL51y (64.6% vs 63.7% and 63.1% vs 61.1%, respectively). Two additional A to G changes which have no effect on the amino acid sequence were also present in the HL107 sequence. Cultivars Torsdag (HL107) and HL51y have completely distinct origins (Torsdag is a Swedish tall combining cultivar used for the

production of split peas, whereas HL51y is a selection from Vinco, a cultivar from middle Europe which may have been used for animal feed) and the two lines differ in a number of major genes (I.C. Murfet pers. com.).

Sequence comparison between the single HL107, *stp-2* and *stp-3* PCR products revealed a number of differences which may have resulted in the mutant phenotypes. However, the sequence from a single PCR product is not ideal as it is subject to PCR artefacts. Therefore regions with sequence differences were resequenced from two additional PCR products produced from wild-type, *stp-2* and *stp-3* plants (either from the same genomic DNA, or from cDNA). All PCR products from the *stp-2* mutant plants possessed a G to A change at base 1111 resulting in a shift from a tryptophan residue to a stop codon at position 252 in the deduced amino acid sequence (**Figure IV.2**). This would result in a truncated protein of approximately 250 aa compared with the 443 aa wild-type sequence. The three PCR products from *stp-3* plants also had a G to A change at base 1051, which would result in a non-conservative alanine to threonine substitution at position 232 in the predicted amino acid sequence (**Figure IV.2**). This change is present in a region that is highly conserved between pea, *Arabidopsis* and *Antirrhinum* (**Figure IV.3**) and is therefore likely to be important for normal function of the *peafim* protein.

Discussion

The evidence presented strongly supports the hypothesis that the *Stp* gene from pea is the homologue of *Fim* from *Antirrhinum* and *UFO* from *Arabidopsis*. The evidence is threefold. First, the pea cDNA homologue of *Fim / UFO* (*peafim*) maps to the same region of linkage group VII where *Stp* has been mapped (**Chapter III, Figure IV.6**). Second, an RFLP analysis shows that the *peafim* clone always cosegregated with the *stp* mutant phenotype (**Figure IV.5**). This supports the linkage analysis, and provides a further bridge between the molecular and classical linkage maps. Third, two independently isolated mutant alleles at *stp* contain separate nucleotide substitutions in the open reading frame of the *peafim* sequence (**Figure IV.3**). The effect of the *stp-2* mutation would be expected to drastically alter the gene product. The effect of the predicted amino acid substitution in the *stp-3* mutation would be more subtle, but occurs in a highly conserved region of the *Stp* gene (**Figure IV.3**). The resultant changes to the predicted amino acid sequence specified by the *stp-2* and *stp-3* mutant alleles reflects the relative severity of the two mutant phenotypes (see **Chapter III**). Formal complete proof of the relationship between *peafim* and *Stp* requires complementation of the mutant phenotype with the *peafim*

clone. However, although transformation protocols for pea have been established (Schroeder *et al.* 1993, 1994, Grant *et al.* 1995, Bean *et al.* 1997), they are not yet widely performed, and have not been undertaken at the University of Tasmania in Hobart.

Southern analysis indicates that there is at least one related gene present in the pea genome (**Figure IV.4**). However, the similarity between the *peafim* sequence and that of *Fim* and *UFO* suggests that *Stp* represents the pea orthologue of *Fim* and *UFO*. The nature of this second gene remains undetermined, but it may be involved in the partial redundancy of the *stp* mutant phenotypes suggested in **Chapter III**. *UFO*-related sequences were also found in *Arabidopsis* (Ingram *et al.* 1995) suggesting that *Stp* and *UFO* belong to a small gene family.

The Fimbriata *homologues interact with genes involved in cell cycle regulation*

The predicted amino acid sequences of *Stp*, *UFO* and *Fim* show a high degree of conservation in two regions. The first region of homology occurs around the only identified motif present, the F-box (Bai *et al.* 1996, Meyerowitz 1997; **Figure IV.3**). Of the 39 amino acids comprising the F-box, 28 of these are conserved between pea, *Arabidopsis* and *Antirrhinum* (71.8% identity). The F-box motif is present in a number of genes connected to the cell-cycle machinery (Bai *et al.* 1996) and may suggest a similar role for *Stp / Fim / UFO*. The specific effect of the *stp-2* and *stp-3* mutations on cell division in the internode and leaf rachis (**Chapter III**) supports this proposal, as does the role *UFO* and *Fim* play in cell proliferation in the floral meristem (Wilkinson and Haughn 1995, Meyerowitz 1997). Further supporting the role of *Fim* in cell-cycle regulation is the identification of three genes, *Fim-associated-proteins1* to *3* (*FAP1* to *3*), whose protein products interacts with Fim and show sequence similarity to the human and yeast cell cycle control proteins $p19^{skp1}$ and SKP1 (Ingram *et al.* 1997). Similar results have been described for *Arabidopsis*; *UFO-Interacting Protein1* (*UIP1*), identified from an *in vitro* screen using UFO as bait, also displays significant sequence similarity to p19 (George Haughn, described in Somerville and Somerville 1996). Thus, the functions suggested by the molecular analyses of *UFO*, *Fim* and *Stp* support the mode of action of *Stp* proposed in **Chapter III**.

The second region of homology occurs towards the carboxyl end (residues 223 to 342 of the *peafim* sequence in **Figure IV.3**) in which, although covering almost a quarter of the protein, over 70% of the amino acids present are identical in all three sequences. In addition, both *stp* mutant alleles and four of the published sequences

from mutant *ufo* alleles result from mutational events in this region (Ingram *et al.* 1995, Lee *et al.* 1997), which also suggests that this region is important for *Fim*-homologue function. This region may provide some of the specificity of function of the Fim-protein in regulating cell cycle (or transcription), or enable the Stp / Fim / UFO protein to interact with other proteins such as Uni / Flo / LFY. There is currently no significant homology between this region and other sequences present in the GenBank database, so the possible function of this region of the protein remains unknown.

Compound leaf development and the overexpression of UFO

The potential to overexpress *Stp* in a wild-type plant provides an opportunity to further dissect the roles of *Stp* in meristem identification. Overexpression of *UFO* does not promote early flowering. However, it does show *LFY*-dependent effects on wild-type leaf development (Lee *et al.* 1997). The lobing produced on plants overexpressing *UFO* is similar to that produced by overexpression of *KNAT1* in *Arabidopsis* (Chuck *et al.* 1996, Lee *et al.* 1997). This supports the role of *UFO* in meristem identification, and reconciles the effect of *Stp* on leaf development in pea with its homology with *UFO*. Overexpression of *Stp* in pea may result in 'super-compound' leaves. This phenotype could potentially resemble those produced in *af tl* double-mutant plants (the 'pleiofila' leaf phenotype; see **Figure III.14c**) as *Stp* function is partially required for this phenotype (**Chapter III**). This possibility is supported by the higher expression of *Uni* in *af tl* leaflets in comparison with those from wild-type plants (Hofer *et al.* 1997). If this should be the case, then the pleiofila leaf phenotype may result from the deregulation of *Stp* and/or *Uni* function, implicating *Af* and *Tl* in the regulation of *Uni* and/or *Stp* activity. Alternatively, as both *Af* and *Tl* appear to act in separate pathways to *Stp* and *Uni*, overexpressing *Stp* in a plant bearing wild-type leaves may result in the production of many more leaflets and tendrils per leaf, or such a plant may produce bipinnate leaves. In either case, these examinations will provide valuable insight into the regulation of the compound leaf development in pea, and the roles played by *Af*, *Tl*, *Uni* and *Stp*, while also providing insight into the evolution of the compound pea leaf.

Such studies would also provide similar advances in understanding aspects of flower and inflorescence development in pea. Overexpression of *UFO* slightly increased the number of organs present in each floral whorl (Lee *et al.* 1997), a phenotype not seen in *LFY*-overexpressing lines (Weigel and Nilsson 1995, Lee *et al.* 1997), and this effect provided part of the evidence suggesting *LFY* and *UFO* had related but separate functions (Lee *et al.* 1997). Similar results would be expected in

the flowers of pea. However, the more complex development of the pea inflorescence potentially provides an additional point of *Stp* influence. Whether overexpression of *Stp* could indeed influence inflorescence and leaf development depends on the degree of redundancy present in the development of these structures. It is possible that *Stp* expression is not a rate limiting step in flower, inflorescence and/or leaf development, and overexpressing *Stp* will do little to influence the plants phenotype. However, this possibility is counter-intuitive as *UFO* overexpression is able to affect leaf and flower development in *Arabidopsis* (Lee *et al.* 1997). Thus, similar effects would be expected in pea.

Evolutionary implications of the Stp *homology*

The broader effects of the *stp* and *uni* mutations in pea suggests these genes have been recruited into the development of the compound leaves and inflorescences of pea. Alternatively, the ancestral function of the interaction between *Uni* and *Stp* was to regulate cell proliferation in primordia and this role has been maintained in the development of complex organs such as the flowers, and also the compound leaf in the case of pea. The fact that the *ufo* and *fim* mutations do not affect development of the simple leaf of *Arabidopsis* and *Antirrhinum* may result from the differing constraints on the development of simple versus compound leaves (Harevern *et al.* 1996, Sinha 1997).

The only other *Fim*-homologue currently identified at the molecular level is from *Impatiens* (Pouteau *et al.* 1995). However, this gene has not been identified from a mutant. *Impatiens-Fim* also shows vegetative expression (Pouteau *et al.* 1995). Thus, like the *Flo* homologues, vegetative expression of the *Fim*-homologues may not be uncommon. The role of moss-*Flo* in the transition from the protonemal to leafy gametophyte stage suggests an ancient role of *Flo* in meristem identification (M. Leech pers. com.). A similarly ancient role for the *Fim* homologues can also be envisaged, and this possibility should be examined further. In fact, the simpler moss system may prove ideal for dissecting the respective roles of the *Flo* and *Fim* homologues in meristem identification.

Methods

Screening of a peashoot cDNA library

Two hundred thousand plaques of a Lambda Zap cDNA library (Stratagene), prepared from RNA isolated from flowering shoot apices of JI813 (HL51y), were screened with a 580-bp PCR fragment spanning nucleotides 244 to 824 of the *Arabidopsis UFO* gene (Ingram *et al.* 1995). This fragment was produced from the plasmid PJAM 180, which contains a 3.4-kb genomic insert containing the entire *UFO* coding region (kindly supplied by Ms Gwyneth Ingram from Dr E. Coen's group in the Department of Genetics, JIC, UK), using two primers specific to the *Arabidopsis* sequence (5′ Fim oligo: TTC TCC AAC ACC TTC CTC GA; 3′ Fim oligo: ACG CTA AAA GGG CTA TAG TTC AT). The library was plated and screened following the procedure described in the Stratagene manual supplied with the library and summarised as follows. The phage were incubated for 30 min with 200 µl XL-Blue cells (OD_{260} = 0.5) and plated onto 150-mm NZY plates in 6 ml of Top-agar. The XL-Blue cells had been resuspended in 10 mM Mg SO_4 after overnight growth at 30°C. The plated phage were grown for six to ten hours at 37°C. Duplicate filters (Colony/Plaque Screen™ Hybridisation Transfer membrane, NEN™ Life Science Products) taken from this library were hybridised with the radiolabelled PCR product overnight at 37°C in 20% formamide, 5x SSC, 1% SDS, 50 µM phosphate buffer, 10% dextran SO_4 and 0.5 mg/ml salmon sperm DNA. Filters were washed at 50°C in 1x SSC, 0.5% SDS. Plaques cross-hybridising with the *UFO* fragment were isolated and resuspended in 400 µl of SM buffer and 20 µl of chloroform (the SM buffer contains 5.8 g/L NaCl, 2 g/L $MgSO_4.7H_2O$, 0.05 M Tris-HCl pH 7.5, and 0.05% gelatin). These were replated and screened until plaque-pure.

The pBluescript phagemid containing the GoC.1 cDNA was excised from the phage by co-infecting XL-Blue cells with the purified plaque and ExAssist™ helper phage (Stratagene) for 20 min at 37°C, after which 3 ml of 2x YT media was added. The cells were incubated for a further 2.5 hours, then killed by heating to 70°C for 20 min. After this, the cellular debris were pelleted by centrifuging at 4 000 g for 15 min. A 1, 10, or 50-µl aliquot of the supernatant was added to 200 µl of an overnight culture of SOLR cells (grown in LB broth containing 50 µg/ml of kanamycin) and incubated for 20 min at 37°C, after which 100 µl of the cells were plated onto LB-ampicillin plates (all LB-ampicillin plates and broth contained 50 µg/ml of ampicillin) and grown overnight at 37°C. Colonies produced from this process resulted from SOLR cells containing the GoC.1 phagemid.

The phagemid was isolated from overnight cultures grown in LB-ampicillin broth using the plasmid preparation methods outlined in **Chapter II.**

Sequencing and sequence analysis

Plasmid and PCR derived fragments were sequenced using dye terminator technology (Applied Biosystems International) according to manufacturers instructions in the Perkin Elmer 9600 thermal cycler. Sequencing template consisted of 1.2 to 1.5 μg of plasmid or 10 to 100 ng of purified PCR product. Sequencing was performed on an ABI 370 automated sequencer (Applied Biosystems) by Dr Peter Grewe at the CSIRO Marine Laboratories, Hobart. Sequences obtained were compared with those in GenBank (world wide web address: http://golgi.harvard.edu/genbank.html) using a BLAST search (Altschul *et al.* 1990) and examined using Sequence Navigator (version 1.0, Applied Biosystems 1989-94), SeqVu (version 1.0.1, Garvan Institute 1992-95) and Clustalw (version 1.5).

Southern and northern analysis

Total genomic DNA was isolated from HL107, HL111 and 50 F_2 plants from cross T21, which was used in the initial linkage analysis of the *stp-2* mutant (see **Chapter III**), following the method of Ellis (1994) outlined in **Chapter II.** DNA from both wild-type lines and a single mutant (*stp-2*) F_2 plant, T21a/1/23, was digested with *Eco*RI, *Eco*RV, *Hin*dIII and *Bam*HI. An equal amount of the digested DNA was size fractionated on a 1.4% agarose / 1x TAE gel and blotted onto nitrocellulose membrane. After air-drying and baking at 80°C for 30 min, the membrane was probed with the *peafim* PCR product (see below and **Chapter II**). The filter was washed at high stringency (0.1x SSC, 0.5% SDS at 65°C) and exposed to X-ray film (Kodak) at -70°C. The polymorphism identified between HL111 and HL107 was followed in the F_2 population using *Eco*RI digests of the DNA extracted from these plants following a similar protocol.

Total RNA was isolated from pea-shoot apices from HL107 (*Stp*), JI2163 (*stp-1*), AF106 (*stp-3*) and *stp-2* segregants from cross T46 (see **Chapter III**) using the method outlined in **Chapter II.** Poly A^+ RNA was purified from this using an mRNA isolation kit (Boeringer Mannheim, 1 741 985) using biotin labelled oligo $(dT)_{20}$, and streptavidin magnetic particles, as described in the protocol. Total or A^+ RNA was size fractionated on a denaturing formaldehyde gel following the protocol of

Fourney *et al.* (1988, **Chapter II**), blotted onto Zetaprobe (Biorad), and probed as above (and see **Chapter II**).

A 2-kb PCR product, which was derived from the *peafim* cDNA clone using 25 pmol of standard Reverse and Universal (M13) primers (Bresatec), was used as a probe for the cosegregation and northern analyses. A sample of this PCR product and the original *peafim* plasmid were sent to Dr Julie Hofer (Department of Applied Genetics, JIC) for use in the molecular mapping of *peafim*. The radiolabelled probe was made using Gigaprime DNA random priming system (Bresatec) and ^{32}P dCTP (Bresatec) and was desalted using Sephadex G50 spin columns (Promega) as described in **Chapter II**.

Reverse transcription

1 µl of the isolated polyA$^+$ RNA was used in the reverse transcription analysis. This gave a similar quantity of RNA for each sample: HL107, 424 ng; JI2163, 344 ng; AF106, 456 ng; and *stp-2* segregants from cross T46, 376 ng (the concentrations of the RNA samples were calculated from their absorbance at 260 nm using a GeneQuant RNA/DNA calculator; Pharmacia). cDNA was synthesised using GIBCO BRL Superscript™ preamplification system (Life Technologies) for first strand cDNA synthesis following the manufacturers instructions, except that 0.5 µM of the oligonucleotide SKTTT (CGC TCT AGA ACT AGT GGA TCC TTT TTT TTT TTT TTT TT) was used as a starting point for cDNA synthesis. The SKTTT primer was incubated with the A$^+$ RNA for 10 min at 70°C, then cooled on ice for 1 min. 7 µl of a master mix containing dNTP (1 µl of 10 mM), $MgCl_2$(2 µl of 25 mM), PCR buffer (2 µl of 10x) and DTT (2 µl of 0.1 M) was added and then incubated for 5 min at 42°C. After annealing, 200 units of Superscript II reverse transcriptase was added, and the tubes incubated for a further 50 min (at 42°C). The reaction was terminated by heating to 70°C for 15 min. RNA was removed by adding 1 µl of RNase H (Life Technologies) and incubating for 20 min at 37°C.

PCR of peafim *from genomic and cDNA*

Oligonucleotides designed specifically to match regions of the 5′ and 3′ untranslated region of the *peafim* sequence were used in the PCR analysis of total genomic DNA and cDNA from mutant and wild-type lines. These were made by Bresatec. Primer sequences 5′ to 3′ : 5′ oligonucleotides: 5′ peafim 2b - CAC TTT GAT CTC TTC TTG CAT ATC CCT C (33-60); peafimV.1 - CTG AAA ATG AGG AAC AGA

ATC TAC C (98-122). 3′ oligonucleotides: 3′ peafim a - ATA CAC AAC ACG AGA CAT TCA TTC AAA TAC (1780-1809); peafimIII.1 - CAT ATT AAT CAC TAC ACA TAC CAT ACC (1754-1780); the numbers in parentheses refer to nucleotide positions from **Figure IV.2**; the 3′ oligonucleotides were the reverse complement of the sequence given in **Figure IV.2**. The oligonucleotides 5′ peafim 2b, and 3′ peafim a were found to produce primer-dimer artefacts which interfered with the PCR reaction and subsequent ligations. Thus, these were replaced by peafimV.1 and peafimIII.1.

PCR was performed using the KlenTaq enzyme (Clontech) in a 20-µl-reaction containing 0.5 µM of the primers peafimV.1 and peafimIII.1 (2 µl of a 5 µM solution) and 8 ng of genomic DNA from *Stp*, *stp-2* and *stp-3* plants or 2 µl of a $^1/_{10}$, $^1/_{100}$, $^1/_{500}$ or $^1/_{1000}$ dilution of the cDNA preparations (above). The reactions were performed in a PTC-100™ programmable thermal cycler (MJ-Research Inc.) held at 94°C for 1 min, then through 35 cycles of 94°C (30 sec), 60°C (1 min) and 68°C (2 min). This was followed by 3 min at 68°C and then a continuous 15°C soak. PCR products were visualised on a 1.4% agarose / 1x TAE minigel and stained with ethidium bromide as described in **Chapter II**. Marks' it™ (AMRESCO) was used as a size marker. Analysis of *Le* expression in the $^1/_{100}$ and $^1/_{1000}$ cDNA preparation was performed using oligonucleotides specific to the *Le* sequence (Lester *et al.* 1997).

PCR products were purified for sequencing using a QIAquick™ Gel extraction kit (Qiagen). The single band produced in the PCR was excised from the gel, the gel fragment solubilised at 50°C into buffer QX1 supplied with the kit, and spun through a QIAquick column as described in the protocol supplied. The column was washed with buffer PE, and the DNA was eluted using 50 µl of sterile MilliQ water. DNA concentration was determined using a DNA fluorometer (Hoefer Scientific Instruments) and the DNA was used directly for sequencing.

Ligation of PCR products and identification of plasmid inserts

2 µl of the unpurified PCR products produced from genomic DNA from *Stp*, *stp-2* and *stp-3* plants were ligated into 100 ng of pGEM-T vector (Promega) using 1 µl of T4 DNA ligase (Promega) and an overnight incubation at 4°C. 4 µl of these ligation products were transformed into 100 µl of JM109 high efficiency competent cells (Promega) by incubating the cells with the ligation products on ice for 20 min, and then heat-shocking at 42°C for 50 sec. The cells were allowed to recover on ice for a further 2 min after which 500 µl of S.O.C. media was added and the cells allowed to recover at 37°C for 90 min. These cells were plated over two LB-ampicillin

plates previously spread with IPTG (15 µl of a 0.1 M solution) and X-Gal (40 µl of 2% 5-bromo-4-chloro-3-indolyl-ß-D-galactoside in N,N′-dimethylformamide). These were grown overnight at 37°C. Positive (white) colonies presumed to contain inserts were replated onto a single LB-ampicillin plate. Transformation efficiency was calculated to be approximately 11% across the four ligation reactions, and no white colonies were found on the control (no PCR product in ligation) plates. Replated (presumed positive) colonies were screened by boiling a scraping of the colony streak in 20 µl of water, and 2 µl of this was used to amplify the insert using PCR and primers specific to the *peafim* sequence. A single positive colony containing the correct insert from each of the wild-type (A2) and two mutant lines (D6 and G2) was replated out onto a LB-ampicillin plates for further analysis. Plasmid DNA was isolated from colonies grown overnight in LB-ampicillin broth using the 5′-3′ Insta-midi-prep™ method outlined in **Chapter II**.

Table IV.1: predicted amino acid sequence identity between orthologues of pea, *Antirrhinum* and *Arabidopsis* genes

Orthologue	pea x *Antirrhinum*	pea x *Arabidopsis*	*Arabidopsis* x *Antirrhinum*
peafim / Fim / UFO	63.7 %	61.1 %	60.0 % [a]
peasqua / Squa / AP1	76 % [b]	76 % [b]	67 % [c]
peaflo / Flo / LFY	76 % [d]	71 % [d]	71 % [e]
Le / GA4	—	53 % [f]	—

References - a: Ingram *et al.* 1995; b: Hofer J.L.M. pers com.; c: Mandel *et al.* 1992; d: Hofer *et al.* 1997; e: Weigel *et al.* 1992; f: Lester *et al.* 1997.

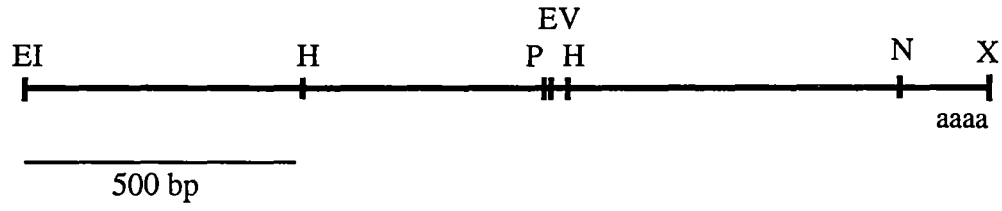


Figure IV.1: Restriction map of the *peafim* cDNA insert. Total length 1.9 kb. Only the sites for specific restriction enzymes are shown. aaaa - poly A tail. EI - *Eco*RI, H - *Hin*dIII, P - *Pst*I, EV - *Eco*RV, N - *Nde*I, X - *Xho*I.

```
1       CTTTGTTCCATTTCTGGTTATGAAACCGATCACACTTTGATCTCTTCTTGCATATCCCTCCTCTCTCTCTCTCCAATAT  81
82      CTTTCTCCAAATCCCACTGAAAATGAGGAACAGAATCTACCATCTCTTCCCAGGTGATTTTTAGCATTCACCACCAACTAT  162
163     CCTTCCAAAAACCACCTTTTTCACCTTCTTCCAACCCTACACTACAAACACTCCTTTCCCCTCTTAGGTGCTTCCCTAATA  243
244     GCGTTCTTTTCCACAAATATTTTCTTTCTCAAGTTACAAAACATAACCATAACCTTTCCTTTATCTTTAAAGCCAACCCTA  324
325     GCTAAAAAAAGAACCATATATACATAAATATTTATGGAAGGTTTTCACCCATCTATGAATATGAGCATGAGCATGAGCATG  405
                                                M  E  G  F  H  P  S  M  N  M  S  M  S  M  S  M

406     AACATGAACCCTTCTTTTTCATATACTTTCCCTATCACTGCTACTGCTTCTGGTGCTATCACAAATATTACTACTACCACC  486
         N  M  N  P  S  F  S  Y  T  F  P  I  T  A  T  A  S  G  A  I  T  N  I  T  T  T  T

487     ACCACTTACAACACTACCAGTACTACTCCATGGATGAACAGCAGGATATGGAGTAAGCTTCCTCACAGACTACTTGATCGC  567
         T  T  Y  N  T  T  S  T  T  P  W  M  N  S  R  I  W  S  K  L  P  H  R  L  L  D  R

568     ATCATTGCTTTCCTTCCTCCACCTGCTTTCTTCCGTGCACGTGCTGTCTGCAAGAGATTTTACTCTCTTCTCTTTTCCAAC  648
         I  I  A  F  L  P  P  P  A  F  F  R  A  R  A  V  C  K  R  F  Y  S  L  L  F  S  N

649     TCTTTCCTTGAATTATACCTTCAAGTTTCACCTCGTTTCCACTGGTTCATATTCTTCAAACATAAAACCAGATCAAAAACC  729
         S  F  L  E  L  Y  L  Q  V  S  P  R  F  H  W  F  I  F  F  K  H  K  T  R  S  K  T

730     CACATCTACAAAAACAACACCATCACTGATAATAATTCCTTTGAAGGCTACATCTTTGATCCTAATGAAGTGGCATGGTAC  810
         H  I  Y  K  N  N  T  I  T  D  N  N  S  F  E  G  Y  I  F  D  P  N  E  V  A  W  Y

811     CGTATTTCCTTCGCTTTAATCCCTTCTGGTTTCTCACCATCTTCTTCATCTGCTGGTTTACTATGCTGGGTTTCTGATGAG  891
         R  I  S  F  A  L  I  P  S  G  F  S  P  S  S  S  S  A  G  L  L  C  W  V  S  D  E

892     TCCGGTCCAAAAACAATGCTTCTCTCAAACCCGATCCTCGGTTCCATTACTCAGTTACCACCAACACTAAGACCAAGACTC  972
         S  G  P  K  T  M  L  L  S  N  P  I  L  G  S  I  T  Q  L  P  P  T  L  R  P  R  L

                                                                                  (stp-3) A
973     TTCCCTTCAATAGGTTTAACCATTACTCCATCTTCCATAGATGTTACTGCTGCAGGTGATGACATGATATCACCTTATGCA  1053
         F  P  S  I  G  L  T  I  T  P  S  S  I  D  V  T  A  A  G  D  D  M  I  S  P  Y  A

                                                                (stp-2) A
1054    GTCAAAAACCTATCATCTGAAAGCTTTCATATAGATGCAAGTGGGTTTTACTCCATCTGGGGAACAACCTCTTCATTACCA  1134
         V  K  N  L  S  S  E  S  F  H  I  D  A  S  G  F  Y  S  I  W  G  T  T  S  S  L  P

1135    AGACTGTGTAGTCTTGAATCAGGTAGAATGGTTTATTCACAAGGGAAATTTTACTGCATGAACTGTAGTCCTTTCAGTGTT  1215
         R  L  C  S  L  E  S  G  R  M  V  Y  S  Q  G  K  F  Y  C  M  N  C  S  P  F  S  V

1216    TTAGCTTATGATATAGCTACAAACACCTGGTTCAAAATTCAAGCTCCTATGAAGAAGTTTTTAAGGTCACCTAACTTGGTT  1296
         L  A  Y  D  I  A  T  N  T  W  F  K  I  Q  A  P  M  K  K  F  L  R  S  P  N  L  V

1297    GAATGCAATGGAAAATTGTTGCTTGTTGCTGCTGTTGAAAAAAATAAGCTGAATGTTCCAAAGAATTTGAGGGTCTGGAGT  1377
         E  C  N  G  K  L  L  L  V  A  A  V  E  K  N  K  L  N  V  P  K  N  L  R  V  W  S

1378    TTGCAAGGCTGTGGAAATGTGTGGGTGGAAACGGAGAGAATGCCACAACAACTTTATGTTCAGTTTGCTGATATGGAAAAT  1458
         L  Q  G  C  G  N  V  W  V  E  T  E  R  M  P  Q  Q  L  Y  V  Q  F  A  D  M  E  N

1459    GGGAATGGATTTGAGTGTGTTGGGAATGGTGAGTTTATTGTGATAATGATAAAAGGGAGTGATAAGGGTTTGGTGTATGAT  1539
         G  N  G  F  E  C  V  G  N  G  E  F  I  V  I  M  I  K  G  S  D  K  G  L  V  Y  D

1540    ATAGGAAGGAAGAGGTGGCAGTGGATTCCACCATGTCCTTATGCTGGTTATGATGGGTTTGAGTTGCATGGTTTTGCTTAT  1620
         I  G  R  K  R  W  Q  W  I  P  P  C  P  Y  A  G  Y  D  G  F  E  L  H  G  F  A  Y

1621    GATCCTAGGTTGGCAACACCTGTTACTGCTCTACTTGATCAGTTGGCAATGCCTCTTCCGCAGTTTTAATTCACAAATGCT  1701
         D  P  R  L  A  T  P  V  T  A  L  L  D  Q  L  A  M  P  L  P  Q  F  *

1702    TCTGAAATAGTATAGGGGTTATCATATGAAAGTATAATGTTTAATTAATGATGGTATGGTATGTGTAGTGATTAATATGTA  1782
1783    TTTGAATGAATGTCTCGTGTTGTGTATTAGCAATGATCAGTTTAATAACTTAAACTGTTAATCTTGGTTGTTTTGAATCTT  1863
1864    AATGCAATATGATTTTATAAAAAAAAAAAAAAAAAA                                                1899
```

Figure IV.2: *peafim* cDNA sequence and predicted amino acid sequence. Also shown are the positions of the single base changes present in the *stp-2* and *stp-3* mutants. The G to A substitution in *stp-2* replaces the tryptophan (W) with a stop codon (*). The replacement of G with A in *stp-3* results in the non-conservative substitution of the alanine (A) with a threonine (T) residue.

```
peafim      1  MEGFHPSMNMSMSMSMNMNPSFSYTFPITATASGAITNITTTTTTYNTTS  50
Fimbriata   1  MEAFQT------------PIFNLPLPYGFTTTPNTINLQN---TMIMST  34
UFO         1  MDSTVFINN----------PSLTLPFSYTFTSSSNSSTTTS---TTTDSS  37

peafim     51  TTPWMNSRIWSKLPHRLLDRIIAFLPPPAFFRARAVCKRFYSLLFSNSFL  100
Fimbriata  35  TNPWMDCRIWSRLPQKLIDRIIACLPPPAFFRSRSVCKRWYSLIFSTTFL  84
UFO        38  SGQWMDCRIWSKLPPPLLDRVLAFLPPPAFFRTRCVCKRFYSLLFSNTFL  87

peafim    101  ELYLQVSPRFH-WFIFFKHKTRSKTHIYKNN-TITDNNS-FEGYIFDPNE  147
Fimbriata  85  ELHLQASPIRH-WFMFFKQQS-IKHHIYNNNSTNARPTN-YEGYLFDPQT  131
UFO        88  ETYLQLLPLRHNCFLFFKHKT-LKSYIYKRGGTNDDDSNKAEGFLFDPNE  136

peafim    148  VAWYRISFALIPSGFSPSSSSAGLLCWVSDESGPKTMLLSNPILGSITQL  197
Fimbriata 132  LKWYRISFPLIPPGFSPASSSGGLICWVSEDSGPKNILLSNPLTNTAIQL  181
UFO       137  IRWYRLSFAYLPSGFYPSGSSGGLVSWVSEEAGLKTIRKALALILVATDS  186

peafim    198  PPTLRPRLFPSIGLTITPSSIDVTAAGDDMISPYAVKNLSSESFHIDASG  247
Fimbriata 182  PSTLEPRLCPTIGLTITNSSIDISFAGDDLISPYAVKNLTSESFHIDVGG  231
UFO       187  RIRQEGYTRPSIGLSVTPTSIDVTVAGDDLLSPYAVKNLSSESFHVDAGG  236

peafim    248  FYSIWGTTSSLPRLCSLESGRMVYSQGKFYCMNCSPFSVLAYDIATNTWF  297
Fimbriata 232  FYSIWNTTSSLPRLCSLESGRMVHVQGRFYCMNYSPFSVLSYDISLNQWC  281
UFO       237  FFSLWAMTSSLPRLCSLESGKMVYVQGKFYCMNYSPFSVLSYEVTGNRWI  286

peafim    298  KIQAPMKKFLRSPNLVECNGKLLLVAAVEKNKLNVPKNLRVWSLQGCGNV  347
Fimbriata 282  KIQAPMRRFLRSPTLVESKGKLILVAAVEKSKLNVPKSLRLWALQECGTI  331
UFO       287  KIQAPMRRFLRSPSLLESKGRLILVAAVEKSKLNVPKSLRLWSLQQDNAT  336

peafim    348  WVETERMPQQLYVQFADMENGNGFECVGNGEFIVIMIKGS-DKGLVYDIG  396
Fimbriata 332  WVEIERMPQQLYIQFAEIEGGRGFSCVAHAEFVVILIRGSYDKAVMFDFC  381
UFO       337  WVEIERMPQPLYTQFAAEEGGKGFECVGNQEFVMIVLRGT-SLQLLFDIV  385

peafim    397  RKRWQWIPPCPYAGYDG---------FELHGFAYDPRLATPVTALLDQLA  437
Fimbriata 382  RKQWVWVPPCPYVGGD----------DELHGFAYEPRLAVPITPLLEQLT  421
UFO       386  RKSWLWVPPCPYSGSGGGGSSGGGSDGEVLQGFAYDPVLTTPVVSLLDQLT  435

peafim    438  MPLPQF                                              443
Fimbriata 422  LPFQSFTA                                            429
UFO       436  LPFPGVC                                             442
```

Figure IV.3: Alignment of the deduced amino acid sequences from *peafim* (pea), *Fimbriata* (*Antirrhinum*) and *UFO* (*Arabidopsis*). Identical amino acid residues are shaded, residue numbers are given at both the left and right of the amino acid sequence. Gaps produced during alignment are represented by a hyphen. The F-box motif is enclosed in a rectangle. (*Fimbriata* sequence from Simon *et al.* 1994, *UFO* from Ingram *et al.* 1995).

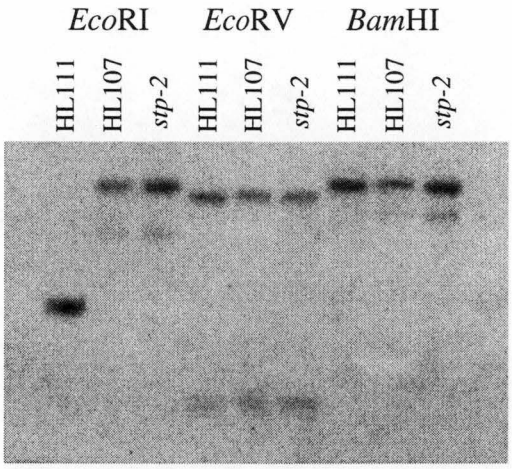


Figure IV.4: RFLP analysis of HL111, HL107 and an *stp-2* plant from cross HL111 x M1a/324 probed with the *peafim* cDNA.

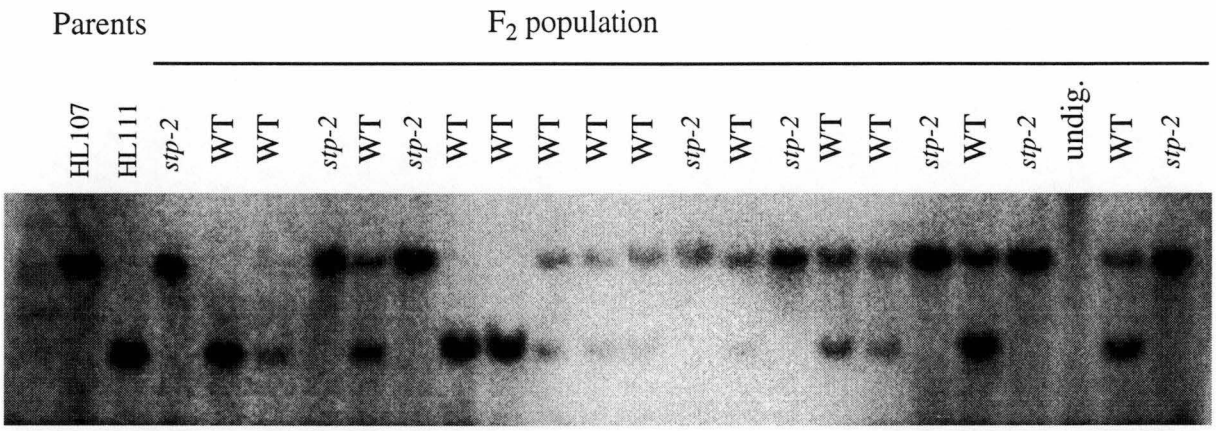


Figure IV.5: Cosegregation between the *stp-2* mutation and the 8 kb HL107 *peafim Eco*RI fragment identified in the RFLP analysis (**Figure IV.4**). Each wild-type plant carries the smaller fragment derived from the HL111 parent. This Southern represents a subset of 50 F_2 plants from the cross HL111 x M1a/324 analysed. WT - wild-type phenotype; undig. - undigested DNA.

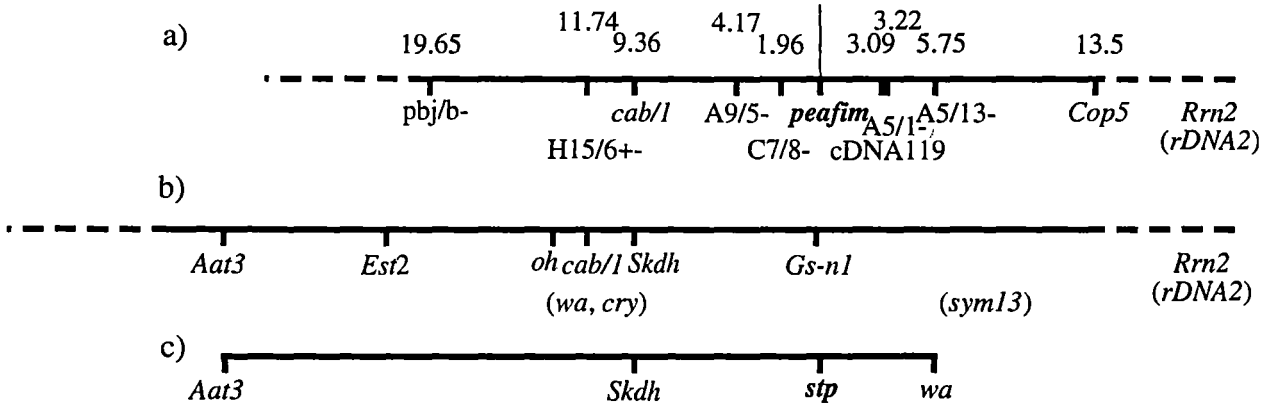


Figure IV.6: Comparison between RFLP map position of *peafim* (a), the middle region of linkage group VII from the published pea linkage map (Weeden *et al.* 1996) (b), and the map position of *stp* from **Chapter III** (c).

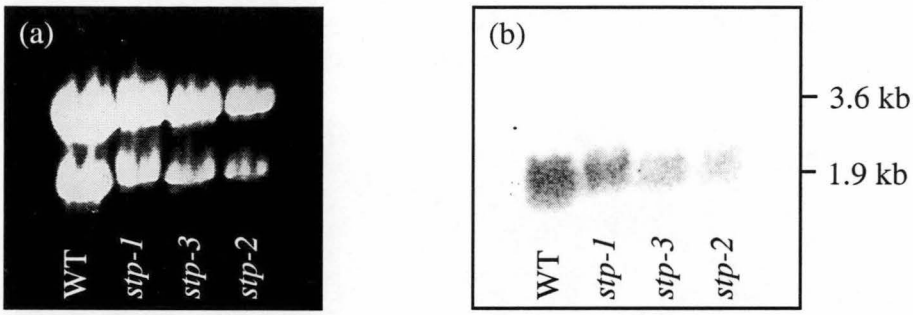


Figure IV.7: Expression of *peafim* in the *stp* mutants was examined using a northern blot of total RNA isolated from flowering apices of wild-type (WT), *stp-1*, *stp-2* and *stp-3* plants (a - ethidium bromide stained gel). Probing the northern with *peafim* resulted in a band approximating the size and shape of the 1.9 kb ribosomal band (b). Expression of *peafim* was too low to be detected on a polyA+ northern blot.

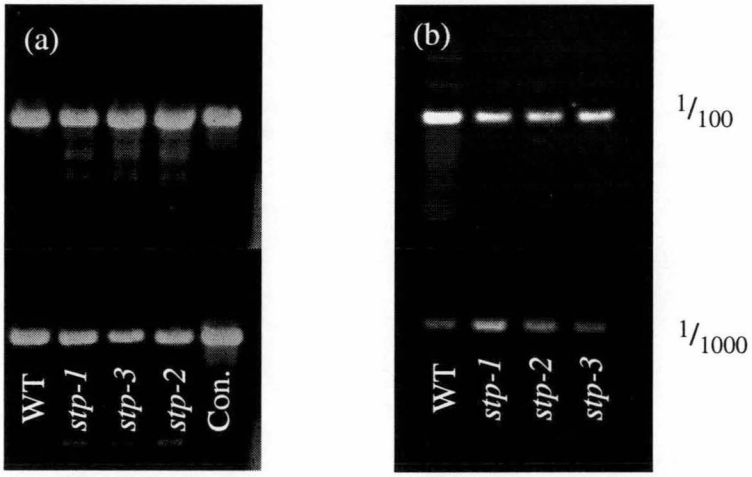


Figure IV.8: RT-PCR. cDNA synthesised from polyA+ RNA isolated from flowering apices of wild-type (WT), *stp-1*, *stp-2* and *stp-3* plants was subjected to PCR using oligonucleotides specific for the *peafim* sequence (a) or the *Le* sequence (b). Differences between product levels in (a) are not readily apparent in the copy of the polaroid, but were reflected in the difference in product produced in (b). These results suggest that the *stp-1*, *stp-2* and *stp-3* mutations do not affect the size or level of the *peafim* transcript. The *peafim* plasmid was used as a control (Con.); this produces a 1.9-kb band.

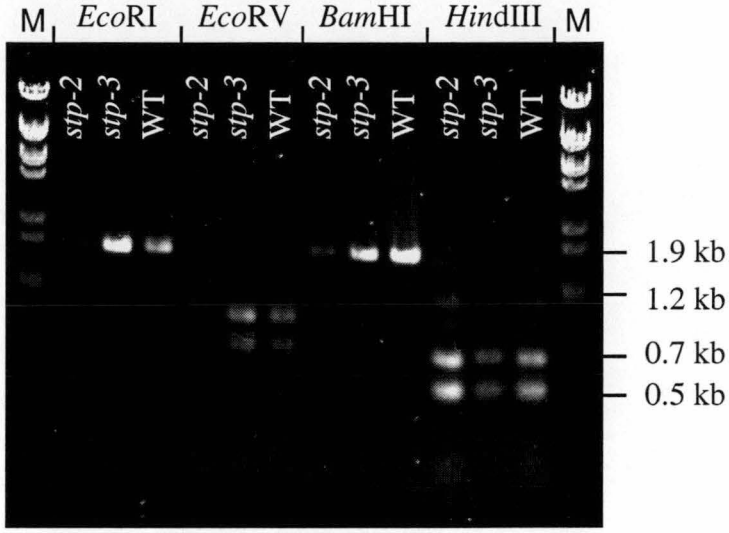


Figure IV.9: Restriction digestion of genomic PCR products from wild-type (WT), *stp-2* and *stp-3* plants visualised by ethidium bromide staining of the agarose gel. Oligonucleotides specific to the *peafim* sequence were used. The restriction pattern for each PCR product is as predicted by the restriction pattern of the original cDNA clone (**Figure IV.1**). The size of the genomic PCR products suggests that *peafim* contains no introns. The amount of product in each lane varies and bands are not visible in some lanes. M = size marker (Marks' it™, AMRESCO).

Chapter V. The floral meristem identity mutant *pim*

Introduction

At least two of the genes involved in floral meristem identification in pea, *Uni* and *Stp*, are homologous to genes found in *Antirrhinum* and *Arabidopsis* (Hofer *et al.* 1997, **Chapter IV**). This conservation of the control of floral meristem identification also extends to other species, and genes corresponding to *Flo/LFY* and *Fim/UFO* have been identified in *Impatiens* (Pouteau *et al.* 1995, 1997). *Flo/LFY* sequences have also been found in tobacco, cauliflower, tomato, *Eucalyptus* and aspen (Anthony *et al.* 1993, Coen and Nugent 1994, Kelly *et al.* 1995, Southerton *et al.* 1995, Strauss *et al.* 1995), and also non-flowering plants such as *Ginkgo*, *Pinus radiata* and the moss *Physcomitrella patens* (Weigel and Meyerowitz 1993, Leech *et al.* 1995. Mouradov *et al.* 1997a). Homologues of *Squa/AP1* have also been identified in a range of species, which suggests that this independent pathway has also been conserved (Kempin *et al.* 1995, Mena *et al.* 1995, Kyozuka *et al.* 1997, Pouteau *et al.* 1997). Potential mutant phenotypes for the *Flo/LFY* or *Squa/AP1* homologues have only been described in tomato, cauliflower and pea.

The phenotype of the *proliferating inflorescence meristem* (*pim*) mutant from pea was described as somewhat similar to that seen in *squa* and *ap1*, and it was suggested that *Pim* may correspond to *Squa* and *AP1* (Singer *et al.* 1994). The proposed homology of *Pim* and *Squa/AP1* was strongly supported when a Southern analysis indicated that *pim* mutant plants contained a complete deletion of the pea homologue of *Squa/AP1* (*peasqua*; Susan R. Singer, Julie M. Hofer, Scott A. Taylor, Noel Ellis and Margaret Knox, unpublished). This chapter examines the *pim* mutant and its relationship to *peasqua* and considers the role of *Pim* in the transition to flowering.

Results

Identification of a second mutant allele at Pim

During the investigation of the interaction between *uni* and *stp-2* (**Chapter III**) a third, distinct mutant phenotype appeared in a single F_3 population. Flowers on these plants possessed bract-like sepals, fewer petals that were often malformed, a reduction in the number of stamens, and occasionally the central carpel was unfused.

Floral meristem identification was also disturbed and multiple 'flowers' arose where a single flower would be found on a wild-type plant. This phenotype, which is distinct from *stp-2* and *uni*, was strongly reminiscent of that produced by plants carrying the *pim-1* mutant allele (Singer *et al.* 1994). The progeny from test-crosses between plants heterozygous for this new mutation and *pim-1* segregated two wild-type and five mutant-flowered plants indicating that the new mutant was an allele of *pim*. *pim-1* was not present in the original investigation of the interaction between *uni* and *stp-2*. Therefore, the new mutation was a unique event, and the new allele was called *pim-2*.

The F_2 from the backcross of an original *pim-2* mutant plant to its wild-type progenitor (HL107) segregated in a Mendelian fashion for the recessive *pim-2* allele ($\chi^2_{(3.1)} = 0.22$; $0.5 < P < 0.7$); *stp-2* and *uni* mutants were not found. A comparison between the *pim-2* plants and their wild-type siblings failed to find any significant differences between NFI, $L_{1\text{-}9}$ and C-3 ($P > 0.5$ for all), supporting the flower-specific role of *pim*.

The phenotype of the pim *mutants*

Although the secondary inflorescence was correctly identified in both the *pim-1* and *pim-2* mutants, the transition from inflorescence to flower morphology was delayed. Additional secondary inflorescences bearing two or more abnormal flowers were produced in place of the flowers normally present on wild-type inflorescences (**Figure V.1**). *pim* mutants can also show complete floral reversion, with inflorescences often bearing a morphologically normal leafy shoot replacing one of the flowers. This occurred more commonly in *pim-2* than in *pim-1* plants, and more often in early rather than later formed flowers. These shoots bore typical *pim*-like flowers. Floral morphology was also aberrant (**Figure V.1**). First whorl sepals were replaced by leafy bract-like structures, and early flowers on *pim-1* plants consisted of these bracts surrounding reduced reproductive organs (**Figure V.1a**). Later flowers on *pim-1* and all flowers on *pim-2* produced varying numbers of completely normal standard and wing petals, although keel petals were never seen (**Figure V.1b**). Placement of the petals was irregular, and some flowers contained more than one standard, or more than two wing petals. Wild-type flowers only ever contain a single standard and two wing petals. Normal stamens and a central carpel were seen in many flowers, and self-pollination was possible, although not common, in both *pim-1* and *pim-2* mutant plants. However, flowers consisting of either outer bracts, petals and a cluster of central stamens or outer bracts, stamens and a single central carpel were also

noted. Complex proliferous flowers composed of combinations of these simpler flower types were also found. Mosaic organs containing either petal and bract or petal and stamen tissues were sometimes found in later formed flowers on early inflorescences. Carpel fusion was not always successful.

pim-1 and *pim-2* flowers produced late on the primary inflorescence, or on lateral shoots produced after flowering, often had a nearly wild-type appearance. Although larger and leafier, the outer whorl was clearly formed from five sepal-like organs. Fewer petals and fewer stamens were produced. The central carpel appeared to be normal.

Unfortunately, the *pim-1* and *pim-2* mutations arose in markedly different backgrounds and thus a direct comparison between the two phenotypes is not strictly valid. However, the new *pim-2* allele appears to have as severe an effect on flower development as the original *pim-1* mutation.

Southern analysis of pim-2

Unlike the complete deletion of *peasqua* in *pim-1* (which suggests that *pim-1* is a null mutation), the molecular lesion in the *pim-2* allele does not result in any identifiable RFLP compared with HL107 (the progenitor of the *pim-2* mutant allele) for the four restriction enzymes examined (**Figure V.2**). The effect of *pim-2* on expression of *peasqua*, or on any of the other flowering genes cloned in pea, was not determined. Identification of a mutant lesion in the *peasqua* gene from the *pim-2* mutant plants would lend support to the proposed homology between *Pim* and *Squa* (and *AP1*).

Expression of peasqua *in floral mutants of pea*

The effect of various flowering mutations on *peasqua* (*Pim*) expression was examined using total RNA isolated from wild-type, *pim-1*, *stp-2* and *uni* flowers, and vegetative laterals from *veg1* mutant plants taken from sites normally occupied by flowers in wild-type plants. To examine the role of *Gi* in *peasqua* regulation, total RNA was also isolated from apices of vegetative wild-type and *gi-2* plants grown under long days (18 h) and vegetative and flowering *gi-2* plants grown under short days (8 h).

peasqua expression was only detected in tissue samples containing flowers or floral primordia (**Figure V.3**). However, *pim-1* flowers did not express *peasqua*. *peasqua* expression was not detected in *veg1* plants, the non-flowering wild-type and *gi-2* plants under long days, and the non-flowering *gi-2* plants under short days. Flowering *gi-2* plants did appear to have some *peasqua* expression, although this was at a much lower level than that present in wild-type flowers (this weak expression is not visible in *gi*-2 plants in the autorad in **Figure V.3**). This may result from the apex versus flower origin of the *gi-2* versus wild-type RNA samples. *stp-2* and *uni* mutant flowers expressed *peasqua* to the same level, or greater, than did immature wild-type flowerbuds (**Figure V.3**). There also appeared to be reduced levels of *peasqua* expression in older (fully developed) wild-type flowers in comparison with immature flower buds. The absence of *peasqua* expression in *pim-1* flowers provided one of the stimuli leading to the examination of the relationship between the *pim* mutant and the *peasqua* clone.

Expression of peasqua *in a wild-type shoot apex*

In situ hybridisation analyses indicate that *peasqua* has a clearly delineated pattern of expression within the developing wild-type flowers (**Figure V.4**). Expression was completely restricted to the floral primordia and flower, and hybridisation was not observed in vegetative or mature inflorescence tissue (**Figure V.4a**). No signal was detected with the control, sense probe.

Expression of *peasqua* occurred throughout the entire wild-type floral primordium from a very early stage, as indicated by the youngest flower initial present on the *in situ* (**Figure V.4b**). However, hybridisation of the *peasqua* probe appeared to be reduced in the centre of the floral primordium, where the carpel would eventually arise, indicating specialisation of the flower at this early stage. The outcome of this specialisation was illustrated by the second youngest flower present in the section (**Figure V.4c**). Expression of *peasqua* was limited to the outer three whorls: sepal, common petal/stamen and stamen primordia. The central carpel dome was free from hybridisation, and this pattern extends downwards into the pedicel of the developing flower (**Figure V.4c**)

Late expression of *peasqua* was restricted to the inner surface of the sepals, their vascular tissue, and the petals (**Figure V.4d**). Weak expression in the filaments of the stamens was also noted (**Figure V.5**). *peasqua* expression within the petals

appeared to be maintained at the high level seen in the early floral primordium. This suggests a late role of *peasqua* in the regulation of sepal and petal development, and possibly also the stamen filaments. The reduced expression of *peasqua* in older flowers reflects the results seen in wild-type flowers in the northern analysis (**Figure V.3**).

Discussion

Unlike *uni* and *stp*, the *pim* mutant phenotype is completely restricted to the flower (**Figure V.1**). This is reflected in the specific expression of *peasqua* in the floral primordia and floral organs in the *in situ* hybridisation and northern analyses (**Figures V.3** and **V.4**). The proposed homology between *Pim* and *peasqua* is also in agreement with the flower specific role the *Squa* homologues appear to play in *Impatiens*, *Eucalyptus*, *Arabidopsis* and *Antirrhinum* (Huijser *et al.* 1992, Mandel *et al.* 1992, Bowman *et al.* 1993, Gustafson-Brown *et al.* 1994, Pouteau *et al.* 1995, 1997, Kyozuka *et al.* 1997). This would suggest that the principal function(s) of these homologues has remained conserved during the divergence of angiosperms.

Expression of Pim *differs from that of* AP1 *and* Squa

Early expression of *peasqua* occurs throughout the floral primordium (**Figure V.4b**), indicating that *peasqua* plays an early role in identification of the floral meristem. This is reflected in the *pim-1* and *pim-2* mutant phenotypes. During the later development of the flower, the expression of *peasqua* becomes restricted to the two outer whorls (**Figure V.4c** and **V.4d**). Both the early and late expression patterns of *peasqua* in the wild-type pea flower is essentially the same as that described for *Squa* in *Antirrhinum* (Huijser *et al.* 1992), *AP1* in *Arabidopsis* (Mandel *et al.* 1992, Gustafson-Brown *et al.* 1994), and *EAP1* and *EAP2* from *Eucalyptus* (Kyozuka *et al.* 1997). Late expression of *peasqua* in the petals is also similar to that described for *Imp-squa* (Pouteau *et al.* 1997).

An important difference between pea and *Arabidopsis*, *Antirrhinum* and *Impatiens* is the weak expression of *peasqua* within the developing third whorl primordia and the stamen filament (**Figure V.4c** and **V.5**). Petal and stamen development appear to be closely related in the garden pea as the five petals and five of the ten stamens arise from four common primordia (Tucker 1989). This may account for the distinct *peasqua* hybridisation pattern seen in pea. However, one of the *Squa*

homologues from *Eucalyptus*, *EAP1*, is also expressed in the anthers (Kyozuka *et al.* 1997). *Eucalyptus* and pea belong to the same subclass (Rosidae) of the Magnoliaphytes (Cronquist 1981), and the differences in expression may be specific to this subclass. However, *Impatiens* also belongs to the Rosidae (Cronquist 1981) and the *Squa*-homologue from *Impatiens* is not expressed in the stamen primordia (Pouteau *et al.* 1997). Whether the expression of the *Squa*-homologues from *Eucalyptus* and pea reflects a common ancestral shift in *Squa* activity, or if they represent convergences in expression pattern is unknown. The function (if any) of *peasqua* in the filaments also remains unknown.

Pim *and the identification of the flower*

The flower-specific nature of the *pim* mutant phenotype indicates that the *Pim* gene belongs to a pathway which is central to flowering. This has also been suggested for *AP1* from *Arabidopsis*, which is thought to regulate flower development in a pathway distinct from *LFY* (Shannon and Meeks-Wagner 1993, Schultz and Haughn 1993). Therefore *Pim* may belong to the group of genes that differentiates between reproductive and non-reproductive growth. However, the production of some almost completely normal flowers in the presumably null *pim-1* mutant plants suggests that *Pim* is not an essential requirement for floral meristem identification, particularly later in plant development. These redundancies may result from interactions between *Pim* and earlier acting genes, for example *Veg1* and/or *Veg2*, which are involved in the identification of the secondary inflorescences and flowers (see **Chapter VI**). As both the *pim-1* and *pim-2* mutants retain the ability to produce each of the four types of floral organs (although the production of the sepals is severely disrupted), the late, floral-homeotic role proposed for *AP1* activity (Bowman *et al.* 1993) may also be subject to redundancies in pea. Presumably therefore *Pim* also interacts with later acting, floral homeotic genes.

The possibility exists that other genes act specifically with *Pim* to identify the floral meristem. The interaction between *pim*, and a mutant tentatively named *broccoli* (*broc*; Singer *et al.* 1995) is similar to that seen in the *AP1 / CAULIFLOWER* (*CAL*) interaction (Bowman *et al.* 1993). Although *broc* has no phenotype in a wild-type (*Pim*) background, in the *broc pim* double mutant the floral meristem is completely converted into meristematic, cauliflower-like inflorescences. *Broc* may well represent the pea homologue of *CAL* (Singer *et al.* 1995).

Pim also interacts with *Stp* to identify the floral meristem (**Chapter III**), although these two genes act in distinct pathways. A more detailed understanding of the role that *Pim* plays in the identification of the flower will require a more detailed analysis of the interactions between *Pim* and other known genes involved in the transition to flowering (such as *Veg1*, *Veg2* and *Gi*), and the characterisation of further genes involved in the identification of the floral meristem.

Methods

Investigation of the pim-2 *mutant*

Three spontaneously arising *pim-2* mutant plants were found in a single F_3 population from the cross between *stp-2* (M1a/324) and *uni* (M1a/224) used in the examination of the interaction between *uni* and *stp-2* (see **Chapter III**). The mutant was rescued by crossing with HL107 pollen. The resultant F_1 plants, heterozygous for the new mutation, were test-crossed with HL244 which carries *pim-1*. The phenotype of *pim-1* and *pim-2* was compared using HL244 (*pim-1*) and an F_3 progeny from a single *pim-2* plant from the F_2 of the backcross to HL107. These plants were grown under LD conditions. Wild-type (HL107) plants were grown as controls.

Total genomic DNA was isolated from HL107, HL244 (*pim-1*) and a *pim-2* plant segregating in the F_2 from the backcross to HL107. The DNA was digested with either *Eco*RI, *Eco*RV, *Bam*HI or *Hin*dIII, size fractionated on an agarose gel, blotted onto Zetaprobe and probed with the *peasqua* clone as described in **Chapter II** (and see below).

Production of the peasqua *probe*

The *peasqua* clone was isolated from a wild-type pea shoot apex cDNA library by Margaret Knox (Department of Applied Genetics, JIC) using the *Squamosa* gene from *Antirrhinum* as a probe.

To prevent non-specific cross-hybridisation to other MADS-box containing genes, the *peasqua* cDNA clone was double digested with *Spe*I and *Xho*I to remove both the ca. 450-bp MADS-box fragment and the 2.9-kb pBluescript DNA. The required 850-bp fragment from the *peasqua* cDNA was excised from an agarose minigel, and either electroeluted in dialysis tubing (JIC), or centrifuged through a small ball of plastic wool (Hobart). The probe was cleaned with a phenol: chloroform: IAA

(25: 24: 1) extraction, ethanol precipitated, and redissolved in a minimal volume of TE buffer.

Expression analysis

In situ hybridisation analysis was performed using Digoxygenin (DIG) labelled sense and antisense probes synthesised from the *peasqua* plasmid (digested with *Spe*I to remove the MADS-box) using T3 and T7 RNA polymerases, respectively, and DIG-11-UTP. The RNA probes were hydrolysed in carbonate buffer at 55°C to yield 100-200-bp fragments. Tissue samples were fixed in 4% formaldehyde overnight in a cold room. Fixed samples were dehydrated in ethanol, cleared in Histoclear, and embedded in Paramat extra wax. 8-µm serial sections were attached to poly-L-lysine coated slides and probed with sense and antisense DIG-labelled *peasqua* RNA. The slides were developed using alkaline phosphatase-conjugated anti-DIG antibodies, BCIP and nitroblue tetrazolium and left for 48 h. Fast green was used as a counter-stain prior to final mounting.

Isolation of total RNA and northern analysis were performed as described in **Chapter II**.

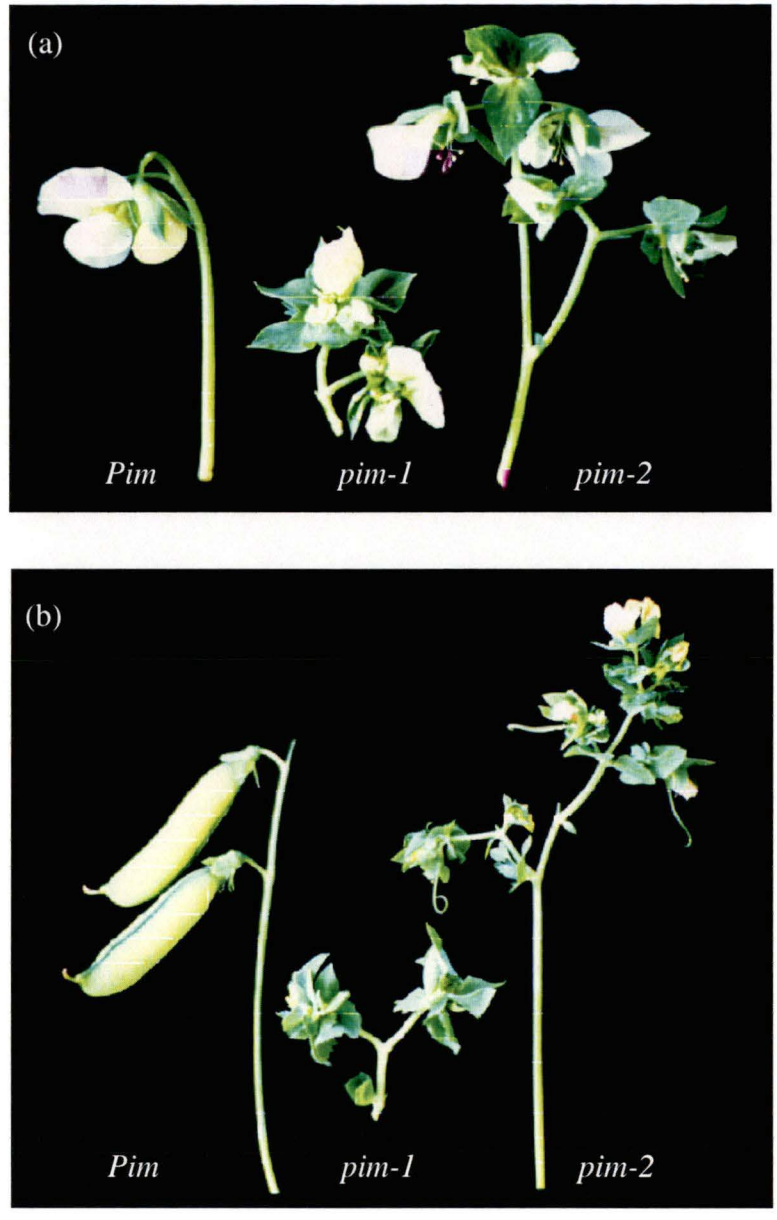


Figure V.1: *Pim* allelic series. (a) Young wild-type and *pim* mutant flowers. (b) Older flowers. The *pim-1* line was dwarf (*le*), which is reflected in the shorter secondary inflorescences.

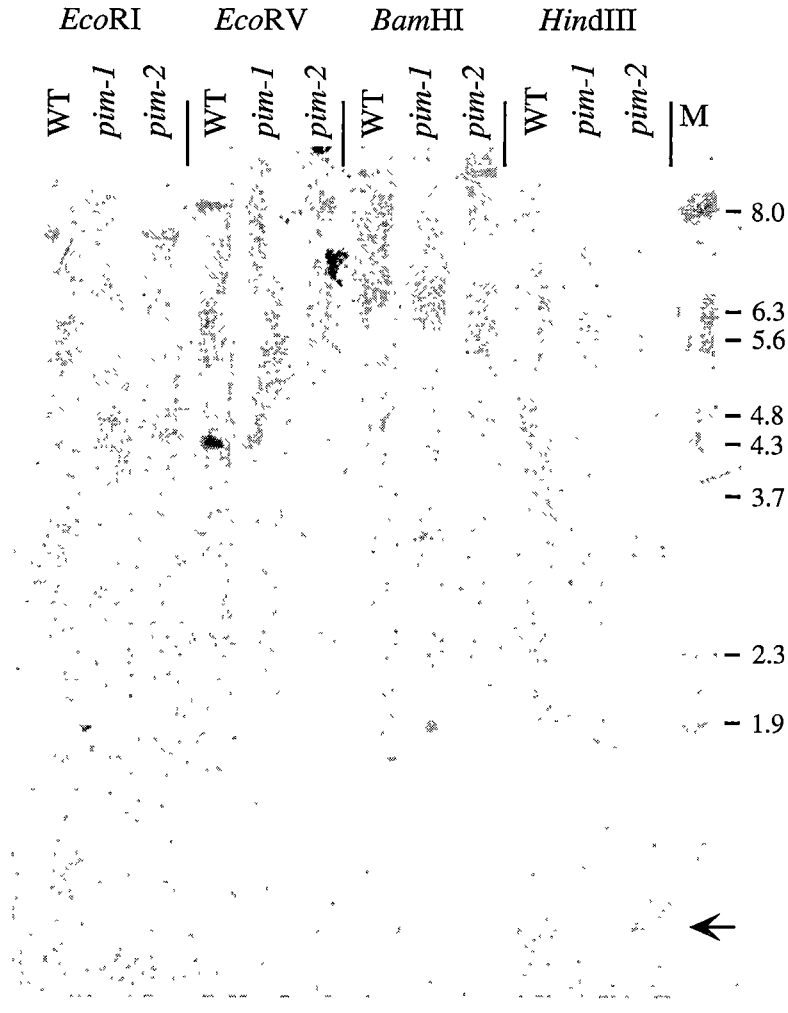


Figure V.2: Southern analysis of the two known *pim* alleles probed with the *peasqua* cDNA. *pim-1* possesses a complete deletion of the *peasqua* gene and no band is present in digests of this mutant. The *pim-2* mutant retains the restriction pattern of its progenitor. WT = wild type (HL107), M = marker lane (Marks' it, AMRESCO), with sizes given in kb. The band produced from the *Hin*dIII digested DNA is marked with an arrow.

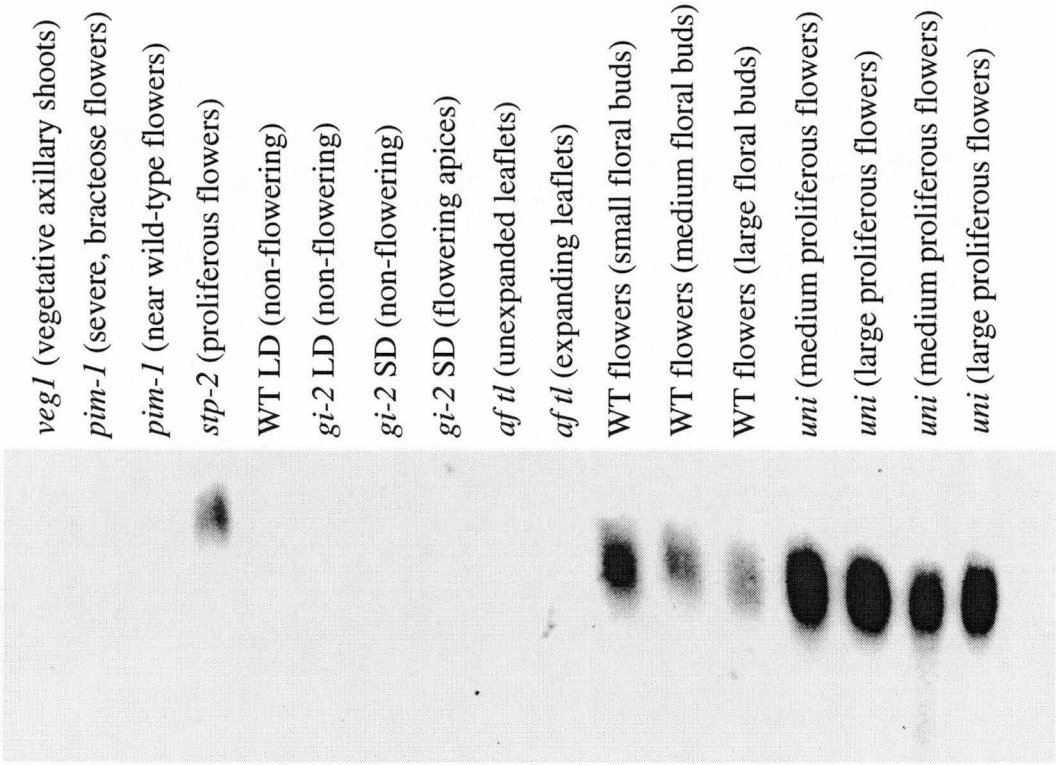


Figure V.3: Expression of *peasqua* in floral mutants of pea. Northern analysis reveals that *peasqua* is only expressed in floral tissue. However, it is not expressed in the flowers of the *pim-1* mutant (*pim-1* is the result of the complete deletion of *peasqua*). Expression in the wild-type (WT, from HL107) flowers decreases with increasing size of the floral bud (i.e. age of the flower). Expression in the proliferous flowers of *uni* exceeds that of small WT flowers. The weak expression of *peasqua* in the flowering apicies from the *gi-2* mutant is not readily apparent in the figure. *pim-1* flowers were divided into those consisting entirely of bract-like structures, or those that were relatively wild type. All other samples were grouped according to the size of the floral buds.

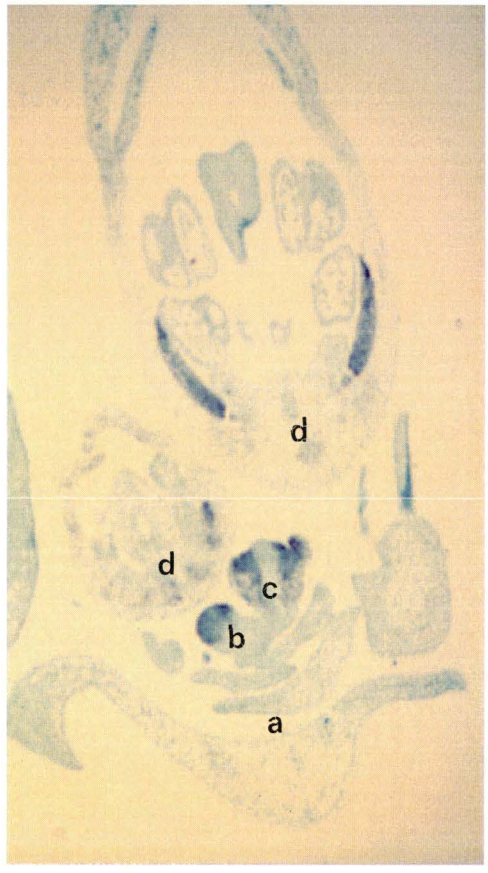


Figure V.4: Analysis of *peasqua* expression in a wild-type flowering pea apex by *in situ* hybridization with DIG-labelled antisense *peasqua* probe (without MADS-box). Four floral buds of different ages can be seen (b - d). *Peasqua* expression was confined to the flower and pedicel, and was not detected in vegetative tissue (a). Expression occurred throughout the youngest floral primordia (b), although expression appeared to be weaker in the centre. Expression was lost in the centre whorl later in flower development (c), but remained strong in the presumptive petal and stamen common primordia, and on the adaxial side of the sepals. Late expression of *peasqua* was restricted to the petals and inner surface of the sepals (d).

Figure V.5: Analysis of *peasqua* expression in a wild-type flower (as described in **Figure V.4**). This oblique longitudional section through the filaments indicates that *peasqua* may also be weakly expressed late in the development of the flower within the filaments of the stamens.

Chapter VI. The vegetative mutants *gi*, *veg1* and *veg2*

Introduction

Unique to the garden pea are three mutants – *gigas* (*gi*), *vegetative1* (*veg1*) and *vegetative2* (*veg2*) – that have what appear to be completely vegetative phenotypes (Reid and Murfet 1984, Murfet and Reid 1993, Taylor and Murfet 1994, Beveridge and Murfet 1996, Reid *et al.* 1996; **Figure VI.1**). Production of flowers and secondary inflorescences is completely prevented by the most severe alleles known at two of these loci (*veg1* and *veg2-1*), and is prevented under specific environmental conditions by both known mutations at the third locus *Gi* (alleles *gi-1* and *gi-2*). Single gene recessive mutants with a completely vegetative phenotype have not been identified in other species, although severe *flo* mutants from *Antirrhinum* (Coen *et al.* 1990), and the *ap1 lfy* double mutant in *Arabidopsis* (Huala and Sussex 1992, Weigel *et al.* 1992, Bowman *et al.* 1993), do not produce flowers, but show a proliferation of the inflorescence.

Grafting studies have revealed that one of the vegetative mutants (*gigas*) is deficient in a graft-transmissible floral stimulus (Taylor and Murfet 1994, Beveridge and Murfet 1996). Grafting mutant *gi* shoots onto a leafy wild-type stock is able to induce flowering under conditions where the mutant normally fails to flower (Taylor and Murfet 1994, Beveridge and Murfet 1996). In contrast, the *veg1* mutant cannot be made to flower either by grafting or by manipulation of the growing conditions (Reid and Murfet 1984).

The involvement of *veg1* in inflorescence development is indicated by its interaction with *determinate* (*det*). In *det* plants the apical meristem of the primary inflorescence ceases growth to form a hairy terminal stub, characteristic of a secondary inflorescence (Singer *et al.* 1990). Thus, *Det* function is required to maintain the indeterminate growth habit of the apical meristem and the racemose inflorescence (panicle) seen in pea. The interaction between the *det* and *veg1* mutations results in the production of a solitary terminal flower in the double mutant (Singer *et al.* 1994), indicating that *veg1* may play a role in the development of the secondary inflorescence. This also indicates that *Veg1* function is not an absolute requirement for floral meristem identification. In contrast, the two mutant alleles at *Veg2* are completely epistatic to *det* (Murfet *et al.* 1995).

Thus, grafting studies and genetic interactions have begun to reveal the distinct nature of the three vegetative mutants. This chapter discusses results that combine a genetic and physiological dissection of the function of these genes. This series of results further define and support the distinct roles of the *Gi*, *Veg1* and *Veg2* genes in the control of flower and inflorescence development in pea. The interactions between these three genes and the *Late flowering* (*Lf*) gene complements this work, and these results are described in **Chapter VII**.

Results

The interaction between gi-2 *and* det

The F_2 from a cross between pure breeding *Det gi-2* and *det Gi* plants clearly segregated into four classes when grown under SD conditions. These four classes corresponded to a 9 wild type: 3 very late (*gi-2*): 3 determinate (*det*): 1 very late determinate (*gi-2 det*) ratio ($\chi^2_{9:3:3:1} = 0.41$; $P > 0.9$). Under a SD photoperiod, *gi-2* plants are usually able to flower, although at a much later node than that of their wild-type siblings. The genotype at *Det* could be determined solely on the inflorescence morphology, without regard to the genotype at *gi*. The close linkage (in coupling phase) between *det* and the wrinkled seed character (*r*) provided additional confirmation of the genotype at the *Det* locus. The F_2 *det gi-2* double mutants flowered at a node similar to that of *Det gi-2* siblings and *Det gi-2* parental controls (**Figure VI.2**), although they possessed the typical *det* mutant inflorescence morphology. The late flowering behaviour was confirmed in the F_3 and F_4 progeny derived from a single *gi-2 det* F_2 plant. This suggests a simple addition (independent expression) of the *det* and *gi-2* phenotypes under SD conditions.

In contrast, when the *det gi-2* F_3 plants were grown under a LD (18 h) photoperiod, they flowered and produced a determinate inflorescence type similar to that of the *det Gi* controls (**Figure VI.3**). Under these conditions, *Det gi-2* plants typically remain completely vegetative (**Figure VI.1** and **VI.3**). This result was confirmed in the F_4 progeny from *det gi-2* F_3 plants grown under both long and short days. Flowering of the *det gi-2* double mutant was significantly later than the control *det Gi* plants under SD (by 17.22 nodes; $t = 5.62$; $P < 0.001$), but did not differ significantly from *det Gi* plants when the two genotypes were grown under LD (0.33 node difference; $t = 0.54$; $P > 0.5$). However, the epistasis of *det* over *gi-2* under the LD conditions was not complete; the *det gi-2* plants often produced completely

vegetative lateral shoots after termination of the main apex, which pointed to their *gi-2* mutant genotype.

Grafting studies: veg2-2

The flowering behaviour of *veg2-2* mutant scions was unaffected by grafting onto wild-type or day-neutral root-stocks (**Figure VI.4**). Nor were *veg2-2* mutant root-stocks able to influence flowering in wild-type scions (**Figure VI.4**). Thus, like *veg1*, the phenotype of *veg2-2* is not influenced by grafting and the function of *Veg2* appears limited to the apex.

The intermediate allele at Veg2*:* veg2-2 *and flower development*

Plants carrying the *veg2-2* allele flower significantly later than their wild-type progenitor (Murfet and Reid 1993, **Figure VI.4**). In addition to the delay in appearance of recognisable flower initials, *veg2-2* mutants also show substantial abnormalities in their floral development (Murfet and Reid 1993, **Figure VI.5a** and **VI.5b**). Effects on the secondary inflorescence are most obvious, and *veg2-2* plants are characterised by their leafy inflorescences showing indeterminate growth. Terminal stubs are never produced (**Figure VI.5**). The flowers of *veg2-2* plants also show a marked tendency towards a leafy shoot morphology. The outer whorls were most often disrupted with the loss of the standard petal and disruption of the sepals in over 80% of first flowers produced (**Figure VI.6**). Chimeric organs, typically sepal / petal or sepal / leaf were also common. However, there was a marked acropetal improvement of the flower phenotype, and later flowers were often completely normal (**Figure VI.5c**). This effect was also seen in *pim* mutant plants (**Chapter V**), and has been described for floral meristem identity mutants from *Arabidopsis* (e.g. Irish and Sussex 1990, Huala and Sussex 1992, Weigel *et al.* 1992, Bowman *et al.* 1993, Schultz and Haughn 1993).

The relationship between pM6 *and* veg1

Collaborative work with Cristina Ferrándiz, then a PhD candidate under the supervision of Dr. José-Pío Beltrán (Valencia, Spain), and Dr. Noel Ellis (JIC, UK) resulted from the mapping of a pea MADS-box containing gene (*pM6*; see Beltrán *et al.* 1996) to the lower end of linkage group V (Ferrándiz 1995). Murfet (unpublished)

had previously mapped the *Veg1* gene to this general region and it was considered important to investigate the relationship between the *pM6* clone and the *veg1* mutation. Initial evidence indicated that a line carrying the *veg1* mutation (HL245, *det veg1*) also possessed a complete deletion of the *pM6* gene (**Figure VI.7**). Analysis of the same Southern with *Rbcs1* (which is also on the lower end of linkage group V) indicated that the deletion did not involve a large region (data not shown). Cosegregation analysis using an F_2 population from the cross between HL245 (*det veg1 Cri*) and HL34 *(Det Veg1 cri*) supported the hypothesis that the *veg1* phenotype was a result of this deletion in *pM6* (**Figure VI.8a**). To further confirm this hypothesis, DNA from a population of the original type line segregating for the *veg1* mutant (HL172) was extracted, digested with *Eco*RI or *Hin*dIII and probed with the *pM6* gene. Again the cosegregation of a deletion in the *pM6* gene and the *veg1* mutant phenotype (**Figure VI.8b**) suggests that the phenotype of *veg1* results from this deletion.

Discussion

Despite the similarity in mutant phenotype, the *Veg1*, *Veg2* and *Gi* genes play separable and distinct roles in the control of flowering and inflorescence development in the garden pea. However, the phenotypic similarities suggest that the three genes probably cooperate in the same developmental pathway.

Veg2 *is essential for flower and inflorescence identification*

The tendency of both flowers and inflorescences to display a leafy shoot morphology in *veg2-2* mutant plants suggests that the *Veg2* gene plays a significant role in the reduction of a branched leafy shoot into an inflorescence bearing flowers. As the production of secondary inflorescences bearing flowers is the primary characteristic of the floral transition in pea, the *Veg2* gene must play a central role in flowering in pea. Therefore, it is suggested that expression of *Veg2* would be the earliest recognisable signal that the plant is undergoing the transition from vegetative to reproductive growth. This proposed central role of *Veg2* makes the molecular characterisation of this gene extremely important for understanding of the transition to flowering.

The role of *Veg2* in the identification of the secondary inflorescences, including the production of the terminal stub; the epistasis of mutant *veg2* alleles over *det*; and the replacement of the indeterminate primary inflorescence with a terminal stub in *det*

mutants, all suggest that the mutant phenotype of *det* may result from ectopic expression of *Veg2*. Thus, the wild-type *Det* allele may actively prevent the expression of *Veg2* in the apical meristem, maintaining its indeterminacy. This is similar to the proposed function of *Centroradialis* (*Cen*) from *Antirrhinum* and its *Arabidopsis* homologue *TERMINAL FLOWER* (*TFL*) (Bradley *et al.* 1996a, 1997). Activity of these genes is thought to prevent the expression of *Flo* and *Squa* (in *Antirrhinum*) and *LFY* and *AP1* (in *Arabidopsis*) in the apical meristem, also resulting in the maintenance of the indeterminate inflorescence (Shannon and Meeks-Wagner 1991, Alvarez *et al.* 1992, Huijser *et al.* 1992, Okamuro *et al.* 1993, Shannon and Meeks-Wagner 1993, Gustafson-Brown *et al.* 1994, Bradley *et al.* 1996a, 1997). Although the proposed function and mutant phenotype of *Det* is similar in many respects to that of *Cen* and *TFL* (Singer *et al.* 1994), the relationship between *Cen* / *TFL* and *Det* remains to be determined.

Veg1 *and* pM6

Part, or all of the *veg1* mutant phenotype may result from a deletion in a MADS-box gene. The phenotype of only one other MADS-box gene in pea has been identified, *pim* (see **Chapter V**), and this mutation specifically affects the flower. No other MADS-box mutant currently identified has a completely vegetative mutant phenotype, although the mutant phenotype of *AGL2*, the *Arabidopsis* MADS-box gene most closely related to *pM6* (Beltrán *et al.* 1996), is unknown. The apparently specific nature of the *veg1* mutation on the transition to flowering may suggest that only a single gene is involved. However, the size and nature of the deletion is unknown, and it remains possible that the *veg1* phenotype results from the loss of a number of closely linked genes. Identification of weak alleles at *veg1*, and their molecular lesions, will aid in resolving this possibility, as will careful mapping of the deletion present in the *veg1* mutant lines. Finally, transformation of the *veg1* mutant plants with the *pM6* clone should reveal if it is able to restore a completely wild-type phenotype, or if other aspects of flower or inflorescence development remain disrupted.

The role proposed for *Veg1* in inflorescence identification (Singer *et al.* 1994) suggests that *Veg1* represents one of the earliest acting MADS-box genes involved in flowering. It is also possible that the *Veg1* product interacts with other MADS-box proteins (such as Pim) to control specific aspects of flower development, as described for other MADS-box genes (Huang *et al.* 1996). This would depend highly on overlapping expression patterns, but could, in part, explain some of the redundancies present in the *pim* mutant phenotype (**Chapter V**). In addition, the similarities in the

veg1 and *veg2* mutant phenotypes, and the overlapping function of the wild-type alleles in the identification of the secondary inflorescences, may suggest that *Veg1* also interacts with *Veg2*. The effect of the *veg2-2* allele on flower development (**Figure VI.5**) suggests that *Veg2* may also interact with *Pim* in the identification of the floral meristem. Therefore, it is possible that *Veg2* also represents a MADS-box gene, and thus the transition to flowering may involve a 'cascade of MAD-ness'. However, it is certainly premature to second guess the molecular nature of a gene that has only been studied at the morphological level.

Does the floral stimulus interact with Det?

The most intriguing result from this series of investigations is the environmentally dependent interaction between *gi-2* and *det*. Under LD conditions the mutant *det* allele is epistatic to *gi-2* in respect to node of flower initiation on the main axis. In contrast, under SD conditions the phenotype of the *det gi-2* double mutant is a simple addition of the two single mutant phenotypes. Why should the environment have such a profound effect on the phenotype of this double mutant combination? And what underlies the interactions between daylength, the floral stimulus and *det* that results in this effect?

The production of vegetative lateral shoots on *det gi-2* plants grown under LD may provide a clue to the nature of the interaction between the floral stimulus and *Det*, and the difference seen in the double mutant plants grown under LD versus SD conditions. Despite the severe effect *gi-2* has on the production of the floral stimulus, plants carrying the *gi-2* are able to flower under certain conditions (Taylor and Murfet 1994) suggesting that there is either some residual production of stimulus or an alternative source of the floral stimulus. This assumes that the floral stimulus is an absolute requirement for flowering and that the flowering of *gi-2* plants under SD does not result from an alternative inductive pathway. Thus, under LD conditions the limited production of floral stimulus by *gi-2* mutant plants may still be sufficient to trigger flowering in a *det* mutant background, but insufficient to maintain the flowering response. This would imply that the *det* mutant lowers the threshold requirement for the floral stimulus for flowering. However, there is no evidence that *det* affects the node of flower initiation of wild-type (*Gi*) plants (Murfet 1989, 1992).

More floral stimulus is required for flowering under SD conditions. This is indicated by the observation that more wild-type donor tissue is required to promote flowering in *gi-1* mutant scions under SD than LD conditions (Beveridge and Murfet

1996). Thus, if the *det* mutant reduces the total requirement for the floral stimulus, and this reduced threshold permits the transition to flowering in a *gi-2* mutant background under LD conditions, there may be insufficient floral stimulus available under SD conditions to overcome even this lowered threshold. If the *det* mutant does reduce the total requirement for the floral stimulus for flowering, it may be possible to promote earlier flowering in the less severe *gi-1* mutant under both LD and SD conditions by adding the *det* mutant allele.

We may ask what is so special about the *det* mutant background? The phenotype of the *det* mutant resembles that of the *tfl* and *cen* mutants from *Arabidopsis* and *Antirrhinum*, respectively, and it is possible that *Det* represents the pea homologue of *TFL* and *Cen* (Singer *et al.* 1994). However, in addition to the production of a terminal flower, *tfl* mutants also promote earlier flowering (Alvarez *et al.* 1992, Zagotta *et al.* 1993, Bradley *et al.* 1997). Thus the promotion of flowering seen in the *det gi-2* double mutant under LD may reveal a similar promotive effect of *det* on flowering time in pea. That this effect is not apparent in a wild-type (*Gi*) background potentially reveals some of the evolutionary divergence in the control of flowering and inflorescence development between *Arabidopsis* and pea.

Methods

Grafting technique

All grafts were made epicotyl to epicotyl using the cleft-wedge grafting technique as described by Murfet (1971c) on seven to nine-day-old seedlings. Stock plants were sown singly in 14-cm slim line pots. Plants required as scions were germinated in 14-L tote boxes. The grafting procedure consisted of cutting the epicotyl of the scion into a wedge shape, and slitting the epicotyl of stock plants down the middle for approximately 1.5 cm. The grafts were secured with a small plastic ring. To prevent excessive water loss, and to assist in the establishment of the graft, plastic bags were placed over the pots. The bags were turned daily to avoid water congestion and encourage some transpiration in the scion. Once the graft union had become established, the corners of the plastic bags were cut to improve ventilation. The bags were removed when the graft had become well established and the scion had commenced further growth (approximately one week).

After grafting, lateral shoots normally grow from buds in the cotyledonary axils of the stock. These lateral shoots were regularly removed in order to prevent

them competing with the scion for available nutrients. However, to produce a plant that possessed two genetically different shoots, one of these laterals was allowed to grow. This results in what is known as a 'Y' graft, as opposed to an 'I' graft where all cotyledonary laterals were removed. The vigour of the scion and lateral shoot were taken into account during the interpretation of the results.

The floral phenotype of *veg2-2* mutant shoots (Wt16123) was examined in both ungrafted and grafted plants. Grafting appeared to have no significant effect on floral morphology regardless of the genotype (*Veg2* or *veg2-2*) of the rootstock.

Mutant analyses

The interaction between *det* and *gi-2* was examined in the F_2, and in *det gi-2* F_3 and F_4 segregants from cross T10 (Wt16015, *Det gi-2* x HL216, *det Gi*). The F_2 population was grown under 8-h SD conditions. The *det gi-2* F_3 and F_4 plants were derived from a single F_2 segregant and were grown under both LD (18 h) and SD (8 h) conditions.

Cosegregation of the *veg1* mutant and MADS box clone *pM6* was examined in the F_2 and F_3 populations of cross 909 (HL34 x HL245) supplied by Prof. I.C. Murfet and grown under standard JIC conditions. Cosegregation was also examined in segregating populations of the original type line, HL172, grown in Hobart under a LD photoperiod (see **Chapter II**). As the *veg1* mutant is completely sterile, it is maintained as a heterozygote. Thus, HL172 segregates both wild-type (*Veg1*) and mutant (*veg1*) plants. Total genomic DNA, restriction digestion, and Southern blotting was carried out as described in the general methods (**Chapter II**). The Southern displayed in **Figure VI.7** was made at the JIC by S.A. Taylor, and probed by C. Ferrandiz with the *pM6* probe (without MADS-box) at the JIC. The Southerns in **Figure VI.8** were made and probed by S.A. Taylor (**VI.8a** at the JIC and **VI.8b** in Hobart). The *pM6* insert used as a probe was supplied by Cristina Ferrandiz.

Gi *gi-2*

Figure VI.1: the vegetative phenotype of *gi*, *veg1* and *veg2* plants results from the reiteration of leafy shoots in the absence of flowering. This photograph compares wild-type (Wt3519) and *gi-2* (Wt16015) plants grown under an 18-h photoperiod. *gi-2* plants remained vegetative under a LD photoperiod, but eventually flowered under a SD photoperiod. In contrast, *veg1* and *veg2-1* single mutants never flowered in either photoperiod. In all three cases the non-flowering plants have a very similar phenotype

Gi det *gi-2 det* *gi-2 Det*

Figure VI.2: Under an 8-h photoperiod the effects of the *gi-2* and *det* mutations are additive. The *Gi det* control plant has flowered with the production of the terminal secondary inflorescence characteristic of plants carrying the *det* mutation. Neither of the two plants carrying *gi-2* have visible flowers at this time. However, the *gi-2 det* plant eventually produced an inflorescence structure typical of *det* mutant plants, while the *gi-2 Det* plant produced the indeterminate primary inflorescence of the wild type.

Gi det *gi-2 det* *gi-2 Det*

Figure VI.3: The *det* mutation is epistatic to *gi-2* under an 18-h photoperiod. The *Gi det* and the *gi-2 det* plants both flowered and produced the typical *det* mutant inflorescence type. In contrast, the *gi-2 Det* single mutants remained completely vegetative.

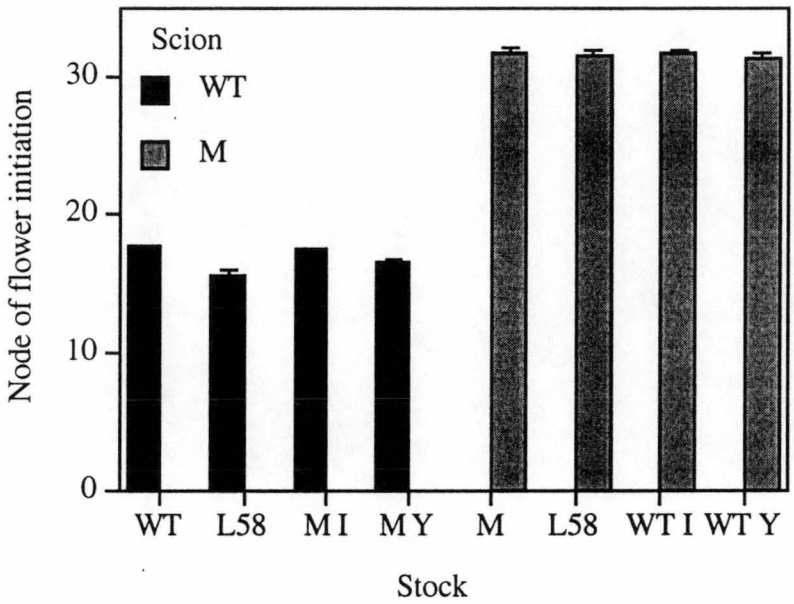


Figure VI.4: The effect of the *veg2-2* mutation is not graft-transmissible. Reciprocal grafting between *Veg2* (WT, Wt4042) and *veg2-2* (M, Wt16123) plants did not influence the flowering behaviour of the scion. Similarly, grafting onto a highly promotive rootstock (HL58; see Murfet 1971c) did not influence flowering behaviour in the *veg2-2* scion. 'I' and 'Y' refer to grafts with (Y) and without (I) a leafy lateral shoot arising from the cotyledonary node of the rootstock. Photoperiod 18 h. Bars indicate SE of the mean. n = 10-12.

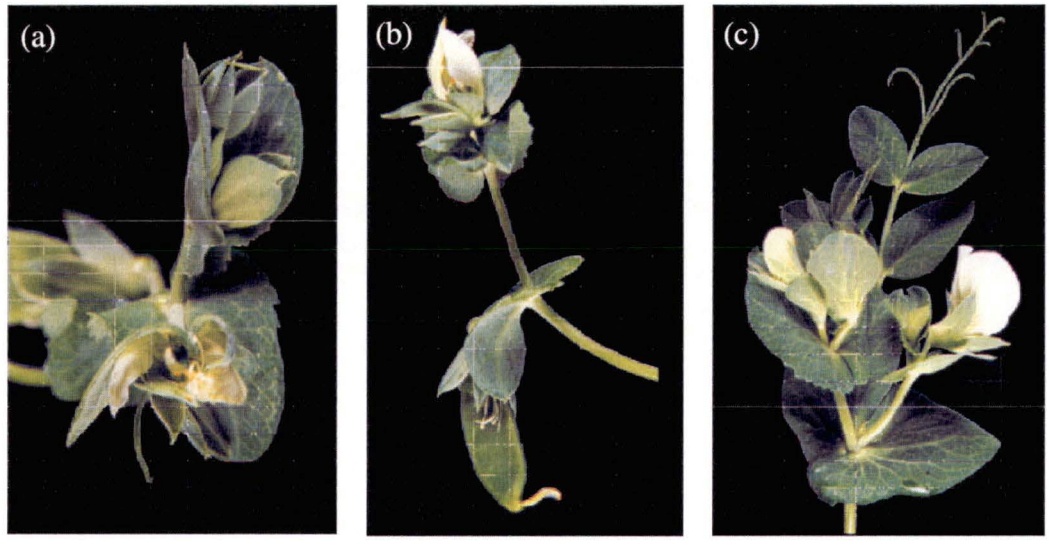


Figure VI.5: Abnormalities in the *veg2-2* mutant flowers. (a) The almost complete conversion of the sepal whorl to leaf-like structures including the production of tendrils, the reduction in petal numbers and loss of stamens are typically found in early flowers on the *veg2-2* mutant. Although later flowers may be more complete (b) or completely normal (c), the abnormalities in the secondary inflorescence remain.

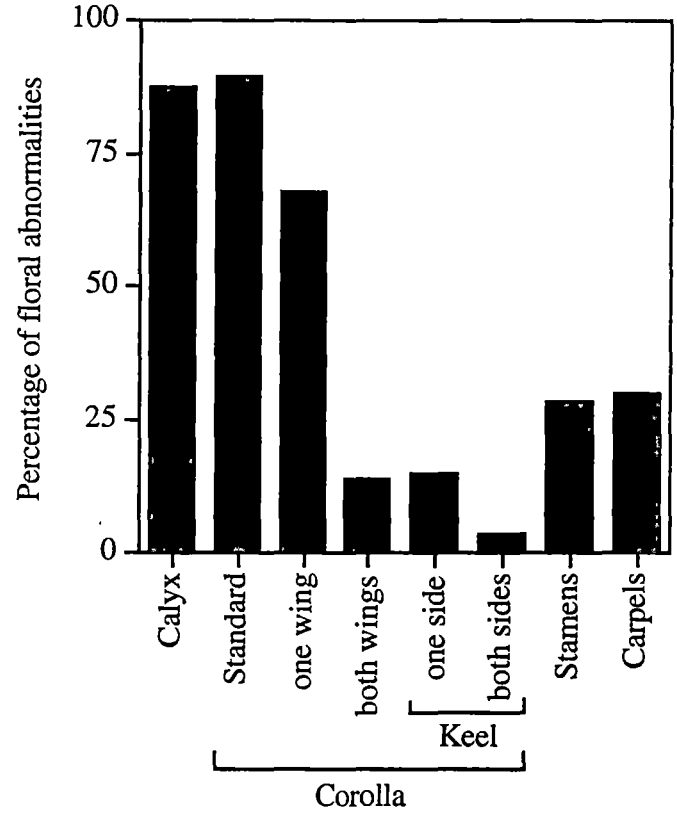


Figure VI.6: Floral abnormalities in the *veg2-2* mutant. The first flowers from grafted and ungrafted *veg2-2* plants were examined, and the proportion of abnormal organs present in each whorl recorded. Abnormalities in the wings or keel of the corolla were described as affecting one or affecting both petals. Most abnormal flowers possessed many characteristics suggesting a transformation into a leafy shoot. n = 56.

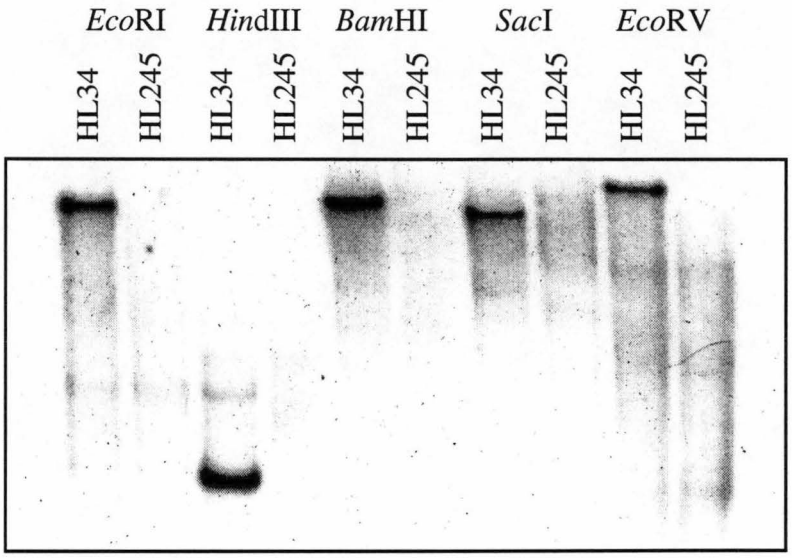


Figure VI.7: RFLP analysis of HL34 (*Veg1 Det*) and HL245 (*veg1 det*) probed with the pea MADS-box cDNA, *pM6*.

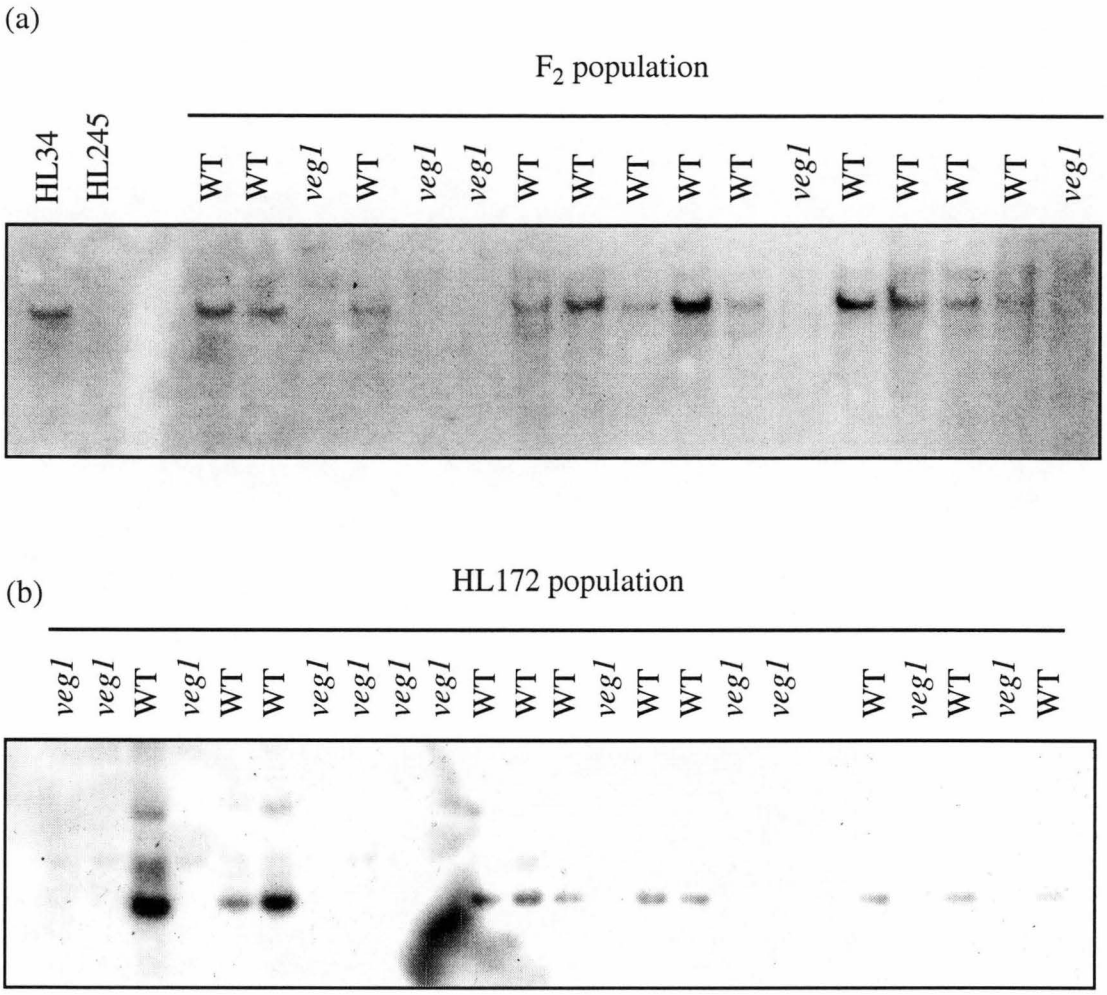


Figure VI.8: Cosegregation of the *veg1* mutant and the deletion in the *pM6* pea MADS-box clone identified in the RFLP analysis (**Figure VI.7**). (a) *Eco*RI digests of total genomic DNA isolated from parental lines and F_2 segregants from the cross between HL34 (*Det Veg1*) and HL245 (*det veg1*) probed with the *pM6* cDNA. (b) *Hin*dIII digests of total genomic DNA isolated from a population of the original mutant line segregating for *Veg1* and *veg1* (HL172) as the *veg1* allele is maintained through heterozygous plants. In each case only a subset of the segregating populations is shown.

Chapter VII: The transition to flowering; *Lf*

Introduction

Mutations at the *Late flowering* (*Lf*) locus can be divided into four classes of alleles: *Lf-d*, *Lf*, *lf*, and *lf-a* (Murfet 1975, Reid *et al.* 1996). These different *Lf* classes are defined by minimum flowering nodes of 15, 11, 8, and 5, respectively (Murfet 1978, 1985). All but one of the many induced mutations at *Lf* fall into one of these four classes (Murfet and Reid 1993, Taylor and Murfet 1993). These classes may represent a stepwise disruption of some critical aspect of the genes' function or structure. Alternatively, they may represent different developmental constraints on the initiation of flowering. For example node 5 may represent the earliest node competent to respond to the flowering signal. *Lf* represents the wild-type class as defined by Murfet and Reid (1993), and many pea cultivars carry an allele in this class. It also seems likely that *Lf* represents the most ancestral state as it appears to be present in many primitive lines (van de Kamp J. and Murfet I.C. unpublished). Despite this, induced mutations from *Lf* to *Lf-d* have not been identified, and the origin of the *Lf-d* allele(s) remains unknown.

The *Lf* gene appears to be the first flowering gene acting in the apex. As such it would presumably interact with the earlier acting genes controlling the floral stimulus and those involved in the response to photoperiod, and it has been suggested that the different *Lf* alleles confer different degrees of sensitivity to the flowering signal (Murfet 1971c, 1975). *Lf* would also interact with later acting genes specifically involved in the production of the inflorescence and flowers. The earlier flowering nature of the recessive *lf* and *lf-a* mutants suggests that the primary role of *Lf* is to prevent or delay flowering, presumably by inhibiting the function of a gene or genes central to the flowering process (Weller *et al.* 1997b). This chapter describes work investigating this inhibitory role of *Lf* by examining the interactions between mutations in *Lf* and mutations in both earlier and later acting genes.

Results

Lf *and inflorescence development: abnormalities in* lf-a *plants*

Although *lf-a* mutant plants are primarily characterised by their very early flowering behaviour (Murfet 1975, Murfet 1985), abnormalities in the secondary

inflorescences produced early in lines carrying *lf-a* have also been noted (I.C. Murfet pers. com.). Abnormalities in the first or second secondary inflorescence of *lf-a* lines HL7 and HL69 were seen in half the plants examined. Typically, the aberrant morphology resulted from the production of a terminal flower, which replaced the terminal stub characteristic of secondary inflorescences (**Figure VII.1**). Abnormalities in the flowers themselves included chimeric sepal / petal structures, an increase in the number of petals, and replacement of the sepals with bract-like structures (**Figure VII.1**). These abnormalities are not seen in *lf* or wild-type plants, and may be specific to the very early flower initiation shown by *lf-a* plants. However, similar abnormalities have been observed in *pim* mutant plants (**Chapter V**), and also in some flowers produced on *det* mutants (Murfet *et al.* 1994). Whether this results from the similar terminal flower phenotype in *det* and *lf-a*, or from reduced *Pim* expression in the early inflorescences of *lf-a* plants, remains to be determined.

Lf *and inflorescence development: interactions with* veg1 *and* veg2

The inflorescence identity mutant *veg2-2* was found to be completely epistatic to the *lf-a* allele present in HL7 (**Figure VII.2**). The F_2 population segregated 36 late flowering: 8 very early initiating (*lf-a*): and 13 very late flowering (*veg2-2*) plants which corresponds to a 9: 3 :4 ratio ($\chi^2 = 1.27$; $P > 0.5$). The very late group possessed proliferating secondary inflorescences and floral abnormalities characteristic of plants with the intermediate *veg2-2* allele (see **Figure VI.6**). The *veg2-2* phenotype segregated from the very early initiating (*lf-a*) F_2 individuals in accordance with the expected 5 (very early): 1 (very late) ratio ($\chi^2 = 0.30$; $P > 0.5$). These *lf-a veg2-2* segregants were indistinguishable from the *Lf veg2-2* controls (**Figure VII.2**) confirming that the *veg2*-2 allele is completely epistatic to *lf-a*. Whether this relationship also exists between *lf-a* and the completely vegetative *veg2-1* allele was not determined, although this possibility seems highly likely.

The *lf-a*-class allele present in HL7 (the *lf-a* type line) was also reported to be hypostatic to *veg1* (Reid and Murfet 1984). Thus, the effect of *lf-a* is not expressed in the non-flowering *veg1* mutant. The presence of the *det* mutant allows *veg1* plants to produce a terminal flower (Singer *et al.* 1994), and this interaction allowed a re-examination of the interaction between *lf-a* and *veg1* in a background where all *veg1* plants were capable of flowering. The cross between HL245 (*Lf veg1 det*) and a plant with the genotype *lf-a Veg1 det* was used to examine the interaction between *lf-a* and *veg1* under genetic conditions where all plants were able to produce flowers. The F_2

population from this cross clearly segregated into four distinct phenotypes (**Figure VII.3**) corresponding to a 9: 3: 3: 1 ratio ($\chi^2 = 1.66$; $P > 0.5$). This indicates that in a *det* background *lf-a* can indeed promote flowering in a plant carrying the *veg1* mutation. The phenotype of the *lf-a det veg1* triple mutant was confirmed by analysis of the F_3 populations derived from the *lf-a* segregants. Plants with a similar phenotype also segregated in the progeny from some of the *Lf veg1* F_2 segregants, suggesting a heterozygous parental genotype of *Lf lf-a*. *veg1 det* double mutant plants never produce a secondary inflorescence, rather the flowers appear to be produced directly from the apical meristem. Thus the node bearing the first secondary inflorescence could not be scored from the *veg1* segregants. The *veg1* mutation delayed the node at which the terminal flower was produced by 5.5 nodes in *Lf* segregants and 3.8 nodes in *lf-a* segregants (**Table VI.1**). If the node of terminal flower is considered the NFI of *veg1 det* plants, then *veg1* delayed flowering by approximately 6 nodes irrespective of the genotype at *Lf*. C-3 occurred at an earlier node in *lf-a* plants compared with *Lf* plants, regardless of the genotype at *Veg1*. Although C-3 occurred later in *lf-a veg1* compared with *lf-a Veg1* plants ($t = 2.31$, $P < 0.05$) a number of the *lf-a Veg1* plants produced their terminal flower, and therefore ceased apical growth, before producing a leaf with three or more leaflets (C-3). This therefore reduced the average node to C-3.

lf-a veg1 segregants were also phenotypically distinct from *Lf veg1* plants, producing lateral branches with long early internode lengths (**Figure VII.3**). The observed differences in lateral length between *lf-a veg1 det* and *Lf veg1 det* plants were similar in many respects to the differences in peduncle length between *Lf Veg1* and *lf-a Veg1* plants and may have resulted from their earlier flowering behaviour. Thus, in summary, *lf-a* is expressed in *det veg1* plants and *veg1* is expressed in *lf-a det* plants resulting in the 9: 3: 3: 1 phenotypic segregation.

An examination of the flowers in populations segregating for *veg1* and *lf-a* also revealed a number of abnormalities in the *det veg1* segregants independent of the segregation at *Lf*. Disruption of keel fusion was noted in many of the flowers produced by *det veg1* mutant plants. However, more severe abnormalities were also seen. These included lack of carpel fusion, ovule to carpel transformations, the production of leafy shoots from within the base of the carpel, and the production of additional flowers from the pedicel (**Figure VI.4**).

These results indicate that *Lf* function is independent of *Veg1*, which contrasts with their relationship described previously by Reid and Murfet (1984) who had used a *Det* background. This work has the advantage of using a *det* background in which the

veg1 plants are able to flower, and therefore the effect of the *Lf* alleles on flowering behaviour could be examined directly. However, the specific effect of *lf-a* on leaflet development and minor effects of *lf-a* on the appearance of *veg1* plants may enable *lf-a veg1* segregants to be distinguished from *Lf veg1* segregants in a *Det* background. To this end, crosses between the white flowered early F_3 segregants with the genotype *a det lf-a veg1* and the red flowered typical late line HL2 (*A Det Lf Veg1*) were made for future analyses. Close linkage between *Lf* and flower colour (*A*) (Murfet 1971b, 1975) will aid in the interpretation of the results from this cross.

Lf *and the hormonal control of flowering*

The *Lf* gene acts as a fulcrum between earlier and late acting genes in the flowering process. It is the earliest known gene acting in the apex, and is proposed to determine the minimum threshold levels of floral stimulus required to trigger flowering (Murfet 1971b, 1975). To directly examine this proposal, the interaction between *lf-a* and *gi-2* (the more severe mutant allele at the *Gi* locus; Taylor and Murfet 1994) was examined. The interaction of *gi-2* and *lf-a* was examined in the F_3 and subsequent generations of the cross between HL7 (*lf-a E Gi*) and Wt16015 (*Lf E gi-2*). None of the F_3 or F_4 decendants from the eight *lf-a* F_2 segregants examined had a late or vegetative *gigas*-like phenotype. Rather all the F_3 and F_4 plants from the *lf-a* F_2 plants shared the common characteristics of the parental *lf-a* line: early, often simultaneous flowering for the first few nodes, and early onset of apical arrest under the 18-h photoperiod conditions. No developmental characteristic was able to distinguish potential *lf-a gi-2* segregants from their *lf-a Gi* siblings. Crosses between very early initiating F_3 segregants and the standard late line HL24 (*Lf e Gi*) identified three F_3 plants carrying the *gi-2* allele. The probability of <u>not</u> obtaining the double mutant from the 59 plants in the combined F_3 and F_4 populations derived from these three individual F_3 plants and their parental F_2 plants is less than 1 in 10^7. This indicates that the *lf-a gi-2* double mutant is indeed early flowering and indistinguishable from the *lf-a Gi* segregants. Thus, *lf-a* is completely epistatic to *gi-2*.

Three plants in the F_2 from the cross between HL7 and Wt16015 initiated flowering at a node comparable with their late-flowering (*Lf Gi*) siblings, but possessed leafy indeterminate inflorescences and underwent vegetative reversion after producing only a few reproductive nodes (e.g. **Figure VII.5b**). The F_3 from these three plants segregated for three distinct phenotypes: very early initiating (VEI; *lf-a*-type), late abnormally flowering (F_2 parental-type), and vegetative (*gi*-type) (**Figure VII.5** and **VII.6**). Analysis of the F_4 generation from the early flowering, and

abnormal late F_3 and F_4 segregants indicated that the three original F_2 plants were likely to have the genotype *Lf lf-a gi-2 gi-2* (**Figure VII.7**). Identical results were also found in the F_5 progeny derived from the early flowering and abnormal late F_4 plants (data not shown). Combined putative segregation of *Lf* in the F_3, F_4 and F_5 generations agreed with a 1 VEI: 3 Late or vegetative ratio ($\chi^2_{(3\ 1)} = 0.002$, $P > 0.9$), but did not agree with a 1: 2: 1 ratio of VEI: Late abnormal: vegetative ($\chi^2_{(1\ 2.1)} = 29.93$ $P << 0.001$). The poor agreement with the 1: 2: 1 ratio results from an excess of vegetative plants, possibly reflecting poor penetrance of the *Lf lf-a* genotypes' flowering behaviour. That is, in some heterozygous plants the *Lf* allele was completely dominant over *lf-a*. Alternatively, the unstable and abnormal flowering which occurred in this genotype (e.g. **Figure VII.5b**) might have been missed in some plants, as this was occasionally represented only by one malformed flower on an essentially vegetative lateral shoot. Evidence of incomplete dominance of *Lf* over recessive *lf* alleles has been reported previously (e.g. Murfet 1971b, 1975). However, the qualitative shift from the vegetative phenotype of the homozygous dominant (*Lf Lf*) plants to the abnormally flowering phenotype of the heterozygous (*Lf lf-a*) plants is unique.

Discussion

The recessive mutant alleles *lf* and *lf-a* cause early flowering, indicating that the wild-type *Lf* gene acts to delay the onset of flowering. The early flowering behaviour of *lf* mutants is dependent on the wild-type function of *Veg2* (**Figure VII.2**), suggesting that the earlier flowering in plants carrying *lf-a* results from earlier expression of *Veg2*. The *veg2-2* mutation prevents the promotion of flowering by *lf-a*. This suggests that *Veg2* acts after *Lf* in the transduction pathway, and that *Lf* may directly or indirectly interfere with *Veg2* activity (**Figure VII.8**).

The putatively null *lf-a*-class allele from HL7 is completely epistatic to the severe *gi-2* mutation. This suggests that *lf-a* completely abolishes the requirement for the floral stimulus for flowering. Because *lf-a* is insensitive to the floral stimulus, and as the proposed function of *Lf* is to prevent flowering, it follows that the stimulus produced or influenced by *Gi* may act to inhibit wild-type *Lf* activity (**Figure VII.8**). It follows that flowering is prevented in the *gigas* mutants as there is insufficient floral stimulus to inhibit *Lf* function. The net result from the interaction between floral stimulus produced in the leaves and the *Lf* gene at the apex is the upregulation of *Veg2* (and therefore flowering). This proposal implies that flowering occurs when the floral

stimulus reaches sufficient levels to block *Lf* inhibition of *Veg2* activity (**Figure VII.8**).

Inherent in this proposal is a mechanism by which flowering is eventually triggered. Reid (1979) has suggested that each leaf becomes more promotory with age, implying that plant growth results in an accumulation of more promotive leaves. This would lead to a steady increase in the amount of the floral stimulus reaching the apex, eventually reaching a sufficient level to shut off the activity of *Lf*, which in turn permits floral initiation.

The minimum flowering node of 5 for plants homozygous for an *lf-a*-class allele may represent the earliest undetermined node available in the embryo. A minimum leaf requirement for flowering was identified in the late cultivar Greenfeast (*Lf*) (Paton 1967, 1968, 1978). This work was supported by the results of Taylor and Murfet (1994) who found minimum leaf requirements for i) a flowering response and ii) a 100% flowering response in *Lf gi-2* scions grafted onto wild-type stocks with leafy lateral shoots bearing various numbers of leaves. Thus, the minimum flowering nodes of 8, 11 and 15 for the *lf*, *Lf* and *Lf-d* classes, respectively, may be the sum of the minimum leaf requirement (determined by the particular *Lf* allele, and the production / export of floral stimulus in / from the leaves) and the determined nodes present in the apex. Factors interfering with the production of the floral stimulus, or its transport to the apex, will delay flowering by increasing the minimum leaf requirement. One of these factors is presumably the presence of the floral inhibitor, as the quoted minimum flowering nodes are found in day-neutral (*sn*) plants (Murfet 1978, 1985), which lack the flower inhibitor (Barber and Paton 1952, Barber 1959, Murfet 1971c, Murfet and Reid 1973).

Veg1 *and the transition to flowering*

These results do not clarify the role of *Veg1* in the transition to flowering. Unlike *Veg2*, *Veg1* is not required for *Lf* function as the *veg1* mutation does not prevent the expression of the *Lf* / *lf-a* difference. However, the differences between the *det veg1* and *det Veg1* plants (e.g. **Figure VII.2**) would indicate that *Veg1* is required for the identification of secondary inflorescences (as proposed by Singer *et al.* 1994). *Veg1* also plays some role in floral development as suggested by the floral abnormalities noted in *det veg1* plants. These abnormalities are distinct from those described in *det Veg1* plants (Murfet *et al.* 1995). This may represent a late function of the *Veg1* gene (**Figure VII.8**), or it could result from the loss of activity of closely

linked genes (see **Chapter VI**). The disruptions in carpel and ovule development, and in floral determinancy, suggest that this may result from the incorrect expression of C-class genes. MADS-box genes are capable of producing homo- and hetero-dimers with differing binding affinity (Huang *et al.* 1996, Davies *et al.* 1996) and it is possible that the proposed dual activity of *Veg1* (which is potentially a MADS-box gene; **Chapter VI**) results from the production of heterodimers with different partner-MADS-box proteins. Selective mutagenesis of the '*Veg1* MADS-box region' (e.g. by using PCR-mutagenesis and transforming the products into mutant *veg1* plants) may be able to separate the early role in inflorescence development from its later role in flower development.

The role of E

If we consider the *Lf* (or *Lf-d*) class to be ancestral in the genus *Pisum*, then the nature and function of the gene *E* in flowering is open to question. *E* has no effect on flowering behaviour in a plant carrying *Lf* or *Lf-d* (Murfet 1985). Its effect on flowering is only revealed in a *lf* or *lf-a* background where it promotes flowering to a lower node; genotypes *lf e* and *lf-a e* flower later than *lf E* and *lf-a E*, respectively (Murfet 1971a, 1971b, Murfet 1985). *E* is thought to suppress the production of the inhibitor in the cotyledons (Murfet 1971a, 1971c). This possible function may indicate that, in the ancestral late flowering background, *E* may have been selected to suppress the pleiotropic effects of the inhibitor on branching and internode length, and/or other aspects of the early growth habit of the ancestral pea. This would permit early growth to be tall and unbranched, and allow the seedling to grow into the light and away from potential competition before inhibitor activity suppressed internode elongation and promoted branching.

The flowering behaviour of the peanut (*Arachis hypogaea*), a distantly related legume, is similar in many respects to the early initiating flowering class of pea (genotype *lf E Sn Dne Ppd*). Both are day-neutral in terms of node of flower initiation (Marx 1968, Murfet 1971a, Summerfield and Roberts 1985, Wallace *et al.* 1993b), but both retain the response to photoperiod and express photoperiodically-controlled effects on vegetative growth and seed development (Marx 1968, Murfet 1971a, Murfet 1985, Wallace *et al.* 1993a, 1993b). Thus, assuming homologous genetic regulation of flowering in this distantly related legume, the peanut homologue of *E* may directly influence flowering behaviour. The flowering behaviour of the ancestral legume is open to question, and node of flowering is influenced by photoperiod in other legumes (such as the sweet pea and the common bean; Murfet and Ross 1985, Wallace *et al.*

1993a, 1993c). Thus, speculation as to the original role of *E* in flowering depends on the flowering behaviour of the prototype legume. This speculation results in a circular argument – if the ancestral legume was late flowering (*Lf* or *Lf-d* type) as seen in the garden pea, sweet pea and bean, then *E* would have little effect on flowering behaviour, and an alternative role for *E* must be sought. Alternatively, if '*lf*' is the ancestral genotype then *E* would have a major influence on flowering. Until *Lf* and *E* homologues in other legumes and in more distantly related species have been identified the role of *E* in flowering and plant development will remain elusive.

The *gi-2* mutation has a profound effect on flowering in an *Lf* background (e.g. Taylor and Murfet 1994) suggesting that the floral stimulus is central to the transition to flowering in pea. *lf-a* is completely epistatic to *gi-2*; the *lf-a E Gi* and *lf-a E gi-2* genotypes are phenotypically indistinguishable indicating that *Lf* and *Gi* are acting in the same pathway controlling the transition to flowering (**Figure VI.8**). In contrast, *lf-a e Gi* plants flower significantly later than *lf-a E Gi* plants (Murfet 1985). This implies two things. First, the *E* gene is able to function normally in a stimulus-deficient (*gi-2*) background, and therefore *E* cannot act is the same pathway as *Gi*. Secondly, the interaction between *E* and *Lf* is profoundly different to the interaction between *Gi* and *Lf*. If the type *lf-a* allele from HL7 is a complete null and removes the requirement for the floral stimulus for flowering, how does the loss of *E* activity delay flowering in *lf-a e Gi* plants? *lf-a E/e sn* plants typically flower at an earlier node than *lf-a E Sn* plants (Murfet 1975, Murfet 1978, Murfet 1985, Taylor and Murfet 1993), probably as a result of the production of inhibitor in the cotyledons. Replacing *E* with *e* in a photoperiodic background would increase the level of inhibitor supplied from the cotyledons (by relieving the inhibition of its production; Murfet 1971c). Therefore the later flowering of *lf-a e Gi* plants may be directly related to an increase in inhibitor levels present in the germinating seedling (Murfet 1985). Examining the effect of introducing the *e* and *sn* (or *ppd / dne*) mutations to the *lf-a gi-2* background may provide some insights into the interactions between *Lf*, *E*, *Gi* and the photoperiod gene system. Clearly the role of *E* in the transition to flowering in both early (*lf*) and very early (*lf-a*) initiating plants, and its relationship to both *Gi* and the photoperiod gene system of pea, deserves further investigation.

Possible molecular nature of Lf

Although mutations in *Cen* from *Antirrhinum* result in the production of a terminal flower (similar in many respects to the phenotype of the *det* mutant of pea) Bradley *et al.* (1996a) propose that the ancestral role of *Cen* may have been to delay the

onset of flowering. This role is identical to that suggested for *Lf*. In addition to their effects on inflorescence development mutations in the *Arabidopsis* homologue of *Cen*, *TFL* (Bradley *et al.* 1997), also promote early flowering (Shannon and Meeks-Wagner 1991, Zagotta *et al.* 1993, Bradley *et al.* 1997). Mutations at *Lf* or *Det* produce flowers in places normally occupied by a vegetative shoot, either at earlier nodes, which results in earlier flowering, or at the apex, resulting in determinancy. These mutant phenotypes may result from the ectopic expression of *Veg2*. In addition, both *lf-a* and *det* are epistatic to mutations in *gi-2* (**Chapter VI** and above). Thus, although the *lf* and *det* mutant phenotypes are distinct, their cause may be related. This hypothesis is further supported by the abnormalities seen in early inflorescences on plants carrying an *lf-a*-class allele (**Figure VI.1**). These abnormalities often resemble the transformation of the apical meristem of the secondary inflorescence into a terminal flower, akin to the mutant phenotype of *det*, *cen* and *tfl*. Therefore *Lf* may represent a functionally divergent paralogue of *TFL* and *Cen* from *Arabidopsis* and *Antirrhinum*. The proposition that *Lf* may represent a homologue of *Cen* requires a great leap of faith. However, it provides a particularly interesting avenue for future research.

Methods

Genetic interactions

As it is possible that the same early flowering (*lf* or *lf-a*) phenotype may result from different mutant lesions (with potentially differing physiological effects), I have attempted to use *Lf* alleles derived from the same source to enable a direct comparison of the interactions examined. The *lf-a*-class allele used in these studies and those in **Chapter III** is sourced from HL7 (*lf-a E Sn Gi*; Vilmorin's 'Acacia'), which is the type line for *lf-a* (Murfet 1985). HL69 (*lf-a E sn Gi*), HL71 (*lf-a e Sn Gi*) and HL117 (*lf-a E Sn Gi*) are derived from HL7 (Murfet pers. com.).

The effect of *lf-a* on inflorescence development was examined in 12 HL7 and 6 HL69 plants grown under an 18-h photoperiod. The abnormalities observed were independent of the presence of *sn* in HL69. The interaction between *lf-a* and *veg2-2* was examined in the F_2 from a cross (T29) between HL7 (*lf-a Det Veg2*) and Wt16123 (*Lf Det veg2-2*). The *lf-a Veg1 det* plant used in the analysis of *lf-a det veg1* interaction was an F_4 segregant from a cross (C794) between HL7 and HL216 (*Lf det Veg1*). Cross T41 was made between the *lf-a Veg1 det* segregant and HL245 (*Lf det veg1*). The interaction between *lf-a* and *gi-2* was examined in the F_3, F_4 and F_5 populations derived from a cross (C795) between HL7 (*lf-a E Gi*) and Wt16015 (*Lf E*

gi-2). The F_2 population from cross 795 and the F_4 segregant from cross 794 were supplied by Prof I.C. Murfet . All plants were grown in typical Hobart conditions as described in **Chapter II**.

Table VII.1: Developmental characteristics of the *Lf / lf-a* and *Veg1 / veg1* F_3 segregants in a *det* mutant background (cross T41). Data is shown as mean ±SE. C-3, node of change to three or more leaflets per leaf; NFI, node of flower initiation. Photoperiod 18 h.

Genotype	C-3	NFI	Node of terminal flower	n
Lf Veg1	13.88 ±0.55	17.50 ±0.5	17.75 ±0.59	8
Lf veg1	13.92 ±0.46	–[a]	23.28 ±0.56	16
lf-a Veg1	10.82 ±0.12[b]	9.00 ±0.17	11.26 ±0.15	38
lf-a veg1	11.47 ±0.34	–[a]	15.05 ±0.47	18

a: *veg1 det* plants do not produce secondary inflorescences. Therefore NFI (node bearing the first secondary inflorescence) could not be scored off the *veg1* segregants but the node of terminal flower may also be considered the node of flower initiation in these plants

b: Some *lf-a Veg1* plants failed to reach C-3 as a result of the limited apical growth

Figure VII.1: Secondary inflorescences from *lf-a* plants.
The terminal stub characteristic of the secondary inflorescence of pea, arrowed in (a), is often replaced by a terminal flower in secondary inflorescences produced early on *lf-a* plants [shown as a progression from (b) to (d)]. Development of these terminal flowers is disturbed and abnormalities often occur. The inflorescence in (e) possessed two flowers fused at their pedicels, while the arrowed flower in (f) contained four standard petals instead of one. The most common abnormality is the production of bract-like sepals, as seen in (b), (c), (d) and (g). The production of these terminal flowers in *lf-a* mutant plants supports the proposition that *Lf* is a homologue of *Cen* and *TFL*. All the inflorescences shown are from HL7 plants grown under an 18-h photoperiod.

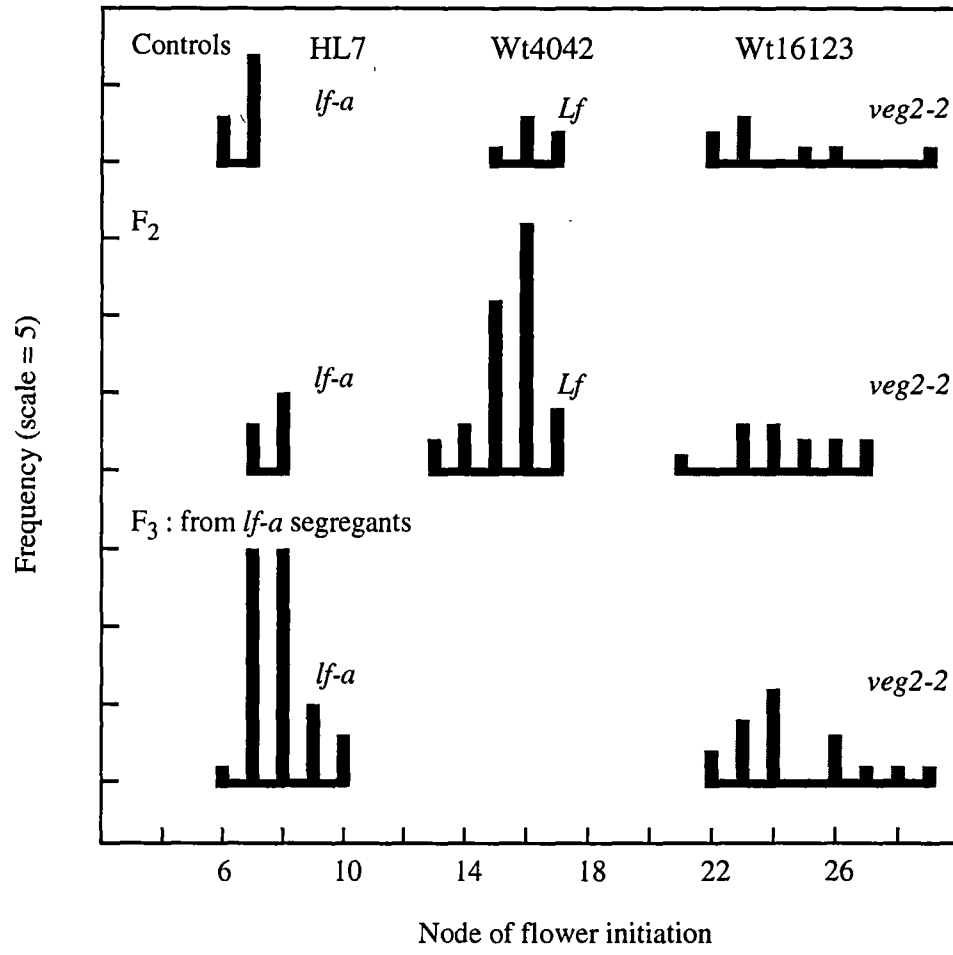


Figure VII.2: The interaction between *lf-a* and *veg2-2*. The F_2 population from the cross between HL7 (*lf-a Veg2*) and Wt16123 (*Lf veg2-2*) produced three distinct classes based solely on node of flower initiation, which corresponded to the three control genotypes (HL7, very early initiating; Wt4042, late flowering wild type; and Wt16123, very late flowering). The observed numbers fit a 3: 9: 4 ratio ($\chi^2 = 0.30$, $P > 0.5$) Approximately two thirds of the F_3 populations derived from the very early initiating (*lf-a*) class segregated two phenotypes, very early and very late. The very late flowering class possessed all the characteristics of the original *veg2-2* mutant, which indicates that *veg2-2* is completely epistatic to *lf-a*.

Lf Veg1 *Lf veg1* *lf-a Veg1* *lf-a veg1*

Figure VII.3: Four phenotypic classes segregated from the cross between an *lf-a det Veg1* plant and HL245, *Lf det veg1*. The four plants shown (left to right) represent the genotypes *Lf det Veg1*, *Lf det veg1*, *lf-a det Veg1*, and *lf-a det veg1*. The *Lf / lf-a* difference was similar in both *Veg1* and *veg1* backgrounds (see also **Table VII.1**). Photoperiod 18 h.

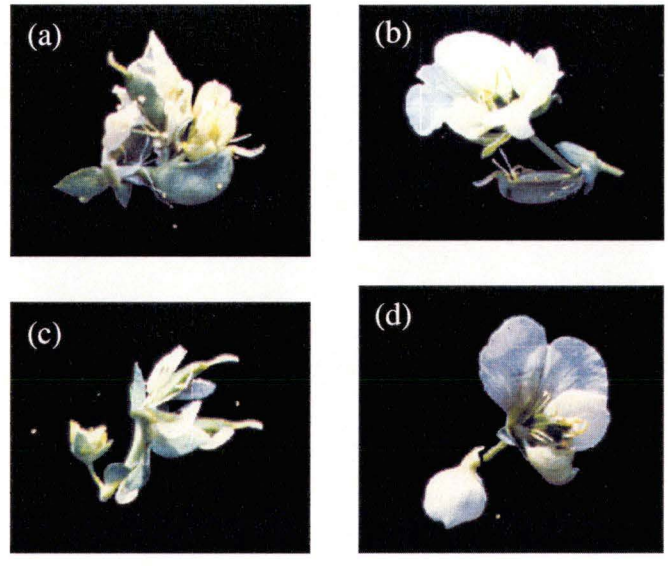


Figure VII.4: Floral abnormalities seen in *veg1 det* double mutant plants. These included homeotic shifts from ovules to carpels (a and b), the production of extra whorls of organs (c and d), ectopic flowers arising from within the central carpel (a and b) and also from the pedicel of the flower (c). Chimeric organs were also produced, including petal / sepal and sepal / carpel mosaics. Although many flowers possessed abnormalities, occasional flowers with an almost completely wild-type appearance were produced.

(a) (b) (c) (d)

Figure VII.5a: Segregation of *Lf / lf-a* in a *gi-2* mutant background. Four plants representing the three classes: vegetative (a, putatively *Lf Lf*), late abnormal (b and c, *Lf lf-a* heterozygotes), and very early initiating (d, *lf-a lf-a*), which segregated from the late abnormal F_2, F_3 and F_4 plants in cross 795. Photoperiod 18 h.

Figure VII.5b: The abnormalities in the late abnormal class (genotype *Lf lf-a gi-2 gi-2*) included vegetative reversion (upper section of plant), and the proliferation of the secondary inflorescences (arrowed). Photoperiod 18 h.

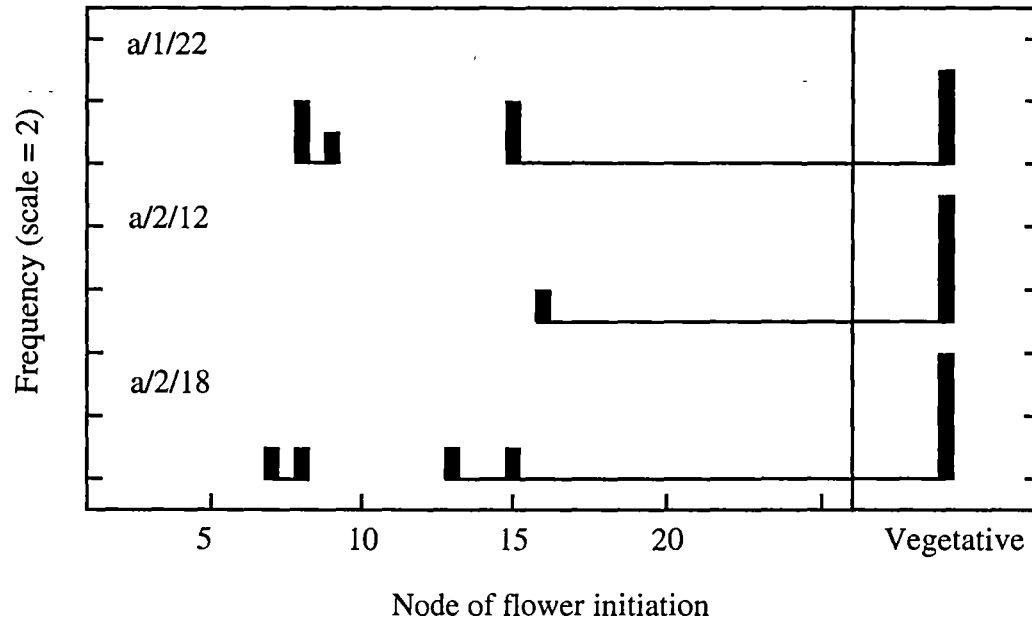


Figure VII.6 F_3 progeny analysis of three late abnormal flowering F_2 plants from the cross 795 between HL7 (*lf-a Gi*) and Wt16015 (*Lf gi-2*). Although the F_2 plants flowered at a node comparable with *Lf Gi* controls, they possessed leafy inflorescences and underwent vegetative reversion. The segregation of very early (*lf-a*-type) and vegetative (*gi-2*-type) plants in the F_3 suggests an F_2 parental genotype of *Lf lf-a gi-2 gi-2*. The late flowering F_3 segregants also possessed the leafy inflorescences and vegetative reversion like their F_2 parents (see **Figure VII.5b**). The bars connect sibling of genotypes *lf-a lf-a* and *Lf/-*; the numbers on the left identify individual progenies. Photoperiod 18 h.

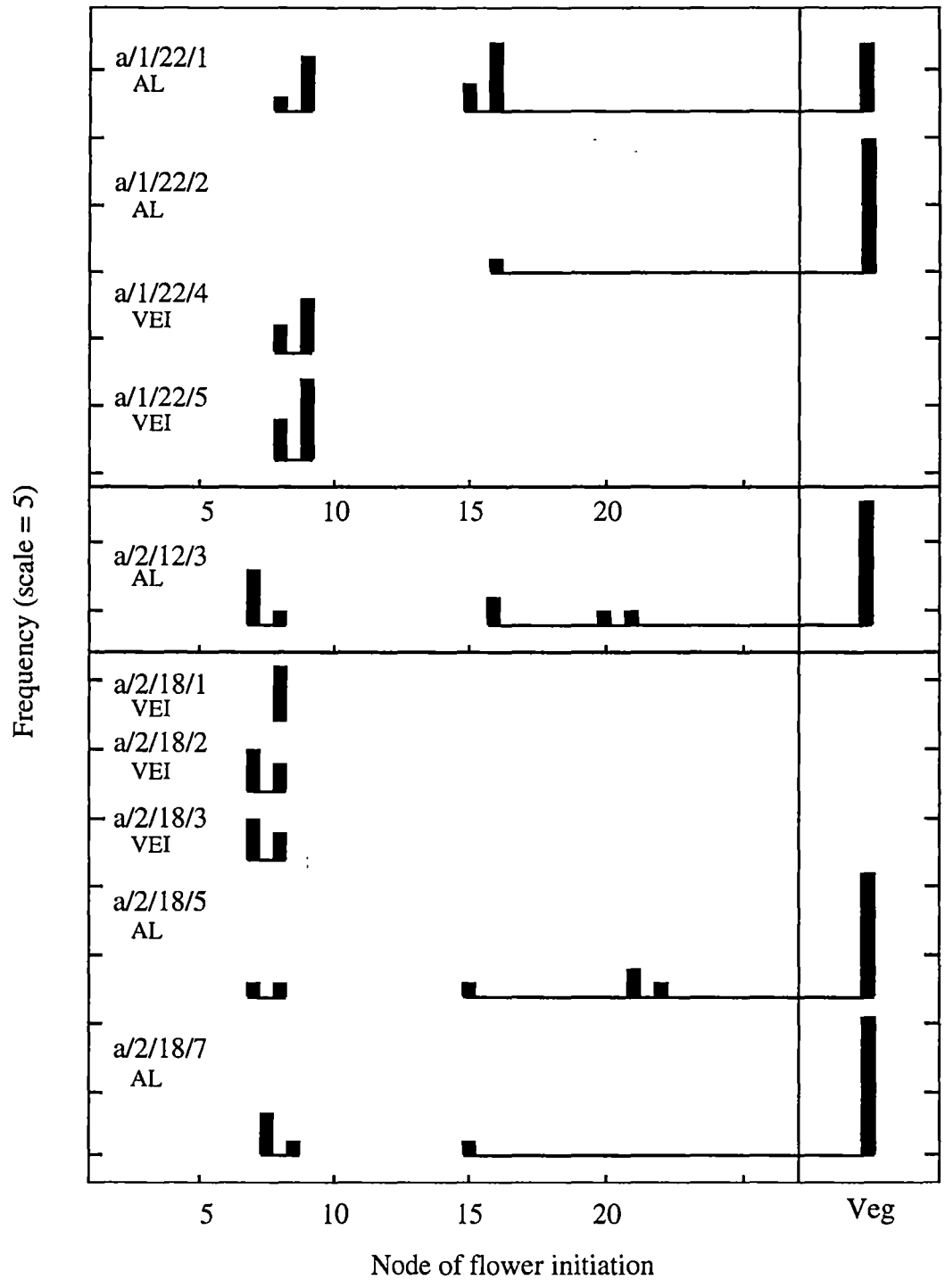


Figure VII.7: Progeny analysis of the very early initiating and late abnormal flowering F_3 plants from the cross between *lf-a Gi* x *Lf gi-2* shown in **Figure VII.6**. F_4 segregation analysis confirmed the results of the F_3 analysis. The very early initiating (VEI) F_3 segregants only produced very early initiating F_4 progeny, while the abnormal late (AL) F_3 segregants produced vegetative (Veg), very early initiating and abnormal late flowering plants. Again, the late flowering segregants in these progenies also possessed the leafy inflorescences and vegetative reversion of their F_2 and F_3 parents (see **Figure VII.5b**). The bars connect siblings of genotypes *lf-a lf-a* and *Lf/-*, the numbers on the left identify individual progenies. Photoperiod 18 h.

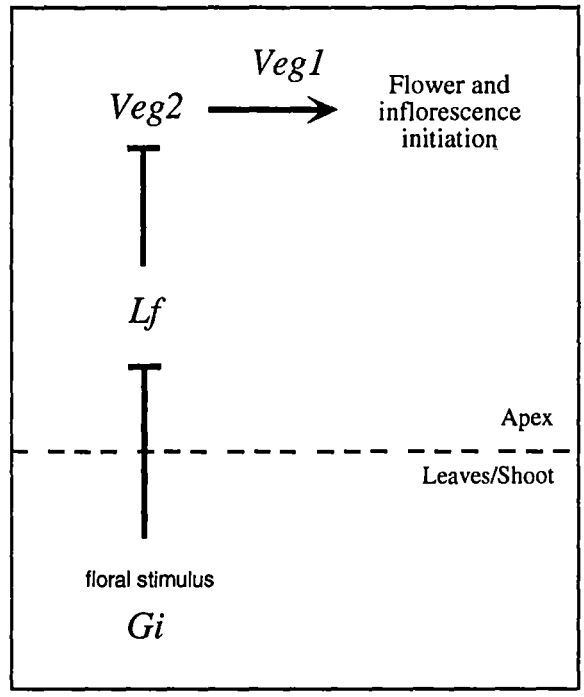


Figure VII.8: Proposed interaction between *Lf*, *Gi* and *Veg2*. In this proposal, the floral stimulus produced under the control of *Gi* is transported to the apex where it inhibits the activity of *Lf*. *Lf* functions in the apex to inhibit the activity of *Veg2*. The activity of *Veg2*, in combination with that of *Veg1*, results in the identification of the axillary secondary inflorescences, and of the flowers. The regulation of *Veg1* is independent of both *Veg2* and *Lf* activities. Positive regulation (⟶). Negative regulation (⊣).

Chapter VIII. The transition to flowering; *Aero*

Introduction

The *aeromaculata* phenotype results from the separation of epidermal and subepidermal layers of the pea leaflets and stipules leading to greyish mottling on the leaves (Blixt 1962, Hoch *et al.* 1980, Marx 1986a, **Figure VIII.1**). The expression of this phenotype is influenced by a number of separate genes: for example the dominant allele at the *Argenteum* locus (*Arg*) results in complete silvering of the leaves, whereas *Flecking* (*Fl*) confers the silver flecking commonly seen on the leaflets and stipules of most pea cultivars. Recessive mutations at *Arg* produce the typical wild-type leaf colouring, whereas the *fl* allele removes the remaining silver flecking resulting in a completely green-leaved phenotype. In contrast, recessive mutations at *Aero* or *Obovatus* result in the promotion of the *aeromaculata* phenotype to *supaeromaculata* (Lamprecht 1958a, Blixt 1962, Marx 1986a). However, unlike *obovatus* mutations, *aero* mutations do not alter the structure of the leaflets or flowers and they remain fully fertile (Blixt 1962, 1972). Mutations in *Aero* occur frequently (Blixt 1962, Marx 1986a, Sidorova and Uzhintseva 1995) and are easily scoreable; thus *aero* is a useful Mendelian trait.

Sidorova and Uzhintseva (1995) identified nine *supaeromaculata* type mutations of differing severity, all at the *Aero* locus. Two of these mutations reduced plant productivity, reducing the number of seeds produced per plant. In addition, a second effect of specific *aero* alleles on total plant height was also noted (Sidorova and Uzhintseva 1995). These two characteristics did not seem to be related as one mutant line produced fewer seeds, although it reached a final height that exceeded that of the initial line. Similarly, the original *aero* type line described by Marx, although significantly shorter in stature, had a seed yield almost identical to that of its initial line (Sidorova and Uzhintseva 1995). Blixt (1962) also noted that plants carrying an *aero* mutation are 'somewhat different' in appearance, although exactly what this means was not explained. The relationship between these additional differences and the *aero* phenotype was not examined.

This chapter presents further work examining the MIII/122 (WL5880) mutant derived from cv. Virtus. This mutant carries a *supaeromaculata* mutation (Marx 1986a, **Figure VIII.1**), but it also shows other phenotypic differences such as earlier flowering and changes in leaflet number (Taylor 1993). Indeed MIII/122 was originally brought under investigation in Hobart as an early flowering mutant. This

chapter forms an initial examination of the genetic relationship between the *aero* phenotype present in MIII/122 and the effects of the mutation on various aspects of plant development.

Results

MIII/122 carries a mutation at aero

Although Marx (1986a) included MIII/122 (WL5880) in his initial examination of the *aero* phenotype, there is no mention of allelism tests between this line and the *aero* type line. To determine whether the *supaeromaculata* phenotype seen in the MIII/122 mutant does result from a mutation in the *Aero* gene, reciprocal crosses were made between MIII/122 and JI2767 (also known as WL5837 and K11), the type line for *aero*. All F_1 and F_2 plants possessed the *supaeromaculata* phenotype indicating that MIII/122 does indeed carry an allele of *Aero*. For simplicity, the type allele found in WL5837 will be referred to as *aero1-1* and the MIII/122 allele as *aero1-10*. The other alleles described by Sidorova and Uzhintseva (1995) are numbered *aero1-2* to *aero1-9*.

The M1a/324 supaeromaculata *mutant is not an allele of* aero

The M1a/324 mutant line (ex HL107) from which the *stp-2* mutant allele was isolated (**Chapter III**) also segregated for a *supaeromaculata* mutation, although the phenotype was weaker than that seen in *aero1-1* and *aero1-10* plants (**Figure VIII.1**). Allelism tests between line MIII/122 (*aero1-10*) and a *supaeromaculata* segregant from line M1a/324 indicated that the two mutants are not allelic as the F_1 plants were wild-type. The new mutant was symbolised *aero2-1*. Segregation of wild-type and *aero* plants in the F_2 did not fit the expected 9 : 7 ratio (43 wild type: 17 *aero*; $\chi^2 = 5.79$, $0.02 > P > 0.01$). However, *aero2-1* was found to segregate as a recessive characteristic in accordance with a 3:1 ratio in a separate cross (30 wild type: 11 *aero*; $\chi^2 = 0.07$, $P > 0.7$). Thus M1a/324 identifies a second *supaeromaculata* gene, *Aero2*. Additional *supaeromaculata* mutations are known (Marx 1986a), but their relationship to JI2767, MIII/122 and M1a/324 is currently unresolved.

Aero1 shows strong linkage with the group IB marker *i* (green cotyledons) (Marx 1986a). A small population segregating for both *aero2* and *i* in repulsion indicated free recombination between *aero2* and *i* (20 *Aero2 I*: 10 *Aero2 i*: 10 *aero2 I*: 1

aero2 i; joint seg. $\chi^2 = 2.41$, $0.2 > P > 0.1$). The map location of *aero2* has not been found.

*The phenotype of MIII/122 (*aero1-10*) revisited*

An examination of a number of traits under LD and SD revealed multiple differences between the MIII/122 mutant and its wild-type progenitor cv. Virtus (**Figure VIII.2** and **VIII.3**). In addition to the fewer seeds and shorter plant height seen in some *aero* type mutants (Sidorova and Uzhintseva 1995), MIII/122 plants also showed earlier leaflet change, flowering, and apical arrest (fewer total nodes) than the wild-type controls (**Figure VIII.2**). In addition, the response to daylength was reduced. The MIII/122 plants also showed a smaller difference in the trait flower / leaf relativity (FLR) between LD and SD than the initial line (**Figure VIII.2**). FLR estimates the tendency towards reproductive growth; negative FLR's indicate that flower development is lagging behind leaf development and the flowers are opening below the apical bud (Murfet 1982). Wild-type lines typically show lower FLR values under SD than LD conditions as SD favour vegetative growth (Murfet 1985). Thus, the effect of short photoperiods on partitioning of resources towards vegetative growth (indicated by the low FLR values in Virtus plants under SD conditions; **Figure VIII.2**) is reduced in MIII/122 plants, indicating a tendency towards day-neutrality.

These several differences in developmental characteristics appeared to cosegregate with the *aero1-10* in F_2 populations derived from the cross between Virtus and MIII/122. While *aero* plants were always clearly recognisable from the *supaeromaculata* trait, no other single developmental characteristic was sufficient alone to distinguish between the *aero* and wild-type classes.

A principal component analysis (PCA) was used to maximise the separation of MIII/122 (*aero1-10*) and cv. Virtus (*Aero1*) plants grown under SD conditions into two classes on the basis of all developmental traits measured (**Figure VIII.4a**). Principal component 1 comprised 63% of the variability of the data. Equally influencing this component were stem length (L_{1-9}), nodes of change in leaflet number (C-*x*), node of flower initiation (NFI), node of flower development (NFD), total nodes produced on the main stem (TN), and number of seeds. Principal component 2 was based primarily on FLR, and comprised a further 12% of the variation. This PCA was also able to distinguish between the majority of *aero1-10* and *Aero1* F_2 segregants

from the cross between cv. Virtus and MIII/122 based solely on their developmental characteristics (**Figure VIII.4b**).

Although this analysis separated the majority of wild-type and *aero1-10* mutant segregants into two distinct groups, five plants (four *aero1-10* and one wild type) fell between the two major groupings (**Figure VIII.4b**). F_3 populations from these five plants were examined to resolve the cause of their intermediate nature. The single wild-type plant in this group proved to be heterozygous for *Aero1 / aero1-10.* The single *aero* F_3 segregant from this wild-type plant flowered and underwent leaflet changes much earlier than its wild-type siblings (**Figure VIII.4c**), indicating that the differences in developmental behaviour between plants carrying *Aero1* and *aero1-10* are maintained in the intermediate background. This would suggest that this aberrant wild-type plant carried additional background differences not associated with *aero1*. The four intermediate *aero1-10* F_2 segregants flowered and underwent leaflet changes atypically late, although their values were still earlier than typical *Aero1* segregants. The progeny of these four *aero1-10* segregants were either identical to, or much earlier than, their parent, which also suggests the presence of other background differences influencing their development. MIII/122 and the Virtus line used in these analyses are not completely isogenic as indicated by the segregation of an early initiating class (EI class; see Murfet 1971a) in their F_2 that is not apparent in the pure breeding lines (see **Figure VIII.4b**).

It appears that the *aero1-10* mutation is associated with a major shift in plant growth towards earlier development and senescence, in essence an acceleration of wild-type plant development. All changes in developmental characteristics occur earlier than seen in wild-type. However, the sequence of the changes remains identical to that seen in wild-type plants (**Figure VIII.3**). A significant question arising from this work concerns the nature of the relationship between the *aero* phenotype and the accelerated development of the plants. There appears to be no obvious relationship between these two characteristics, although similar effects (on seed yield and total plant height) have been noted in other *aero* mutants (Sidorova and Uzhintseva 1995). Thus, a more detailed investigation was necessary to test whether or not the *supaeromaculata* phenotype and the acceleration of plant development result from a mutation in a single gene. Therefore the effect of the original *aero* mutation, *aero1-1*, on developmental characteristics was examined.

The phenotype of JI2767 (aero1-1)

The *aero1-1* allele present in JI2767 has a more severe effect on leaf flecking than *aero1-10* found in MIII/122 (**Figure VIII.1**). The *aero1-1* allele also proved to have a more severe effect on other aspects of plant development. Plants carrying *aero1-1* flowered, senesced and underwent changes in leaflet number much earlier than their wild-type initial line (**Figure VIII.5**). In addition, plants carrying *aero1-1* reached a maximal leaf development of eight leaflets (C-8) more frequently than plants carrying *aero1-10*, although this may reflect differences in backgrounds (*aero1-1* is ex Torsdag and *aero1-10* ex Virtus); 43.7% of *aero1-1* segregants reached C-8 compared with only 5.5% of *aero1-10* segregants in backcrosses to their respective wild-type progenitors. Leaves bearing seven or eight leaflets were rarely seen in wild-type plants, but were common in both *aero* mutants examined (**Figure VIII.6**). The pleiotropic effects apparent in *aero1-1* also cosegregated with the *aero* phenotype in the F_2 from a cross between JI2767 and its progenitor cv. Torsdag (HL107) (**Figure VIII.7**). These results are in agreement with those of Sidorova and Uzhintseva (1995) on yield and stature and they support the proposition that the *aero1* mutations are associated with a tendency towards earlier development and arrest of growth.

Does Arg *also alter developmental traits?*

Although the dominant *Arg* mutation results in a complete silvering of the leaves rather than leaf-flecking, Marx (1986a) includes *Arg* as a *aeromaculata* mutant. A comparison of *Arg* (silver) and *arg* (wild-type) plants grown under a 10-h SD photoperiod failed to identify any significant differences in node of leaflet change (C-3; $t = 0.25$, $P > 0.7$), node of flower initiation ($t = 1.46$, $P > 0.1$), and total nodes ($t = 1.20$, $P > 0.2$). This indicates that the promotion of heteroblasty seen in *aero* mutants is specific to *aero1* (and possibly also *aero2*) and is not a consequence of the air spaces under the leaf epidermis (the *aeromaculata* phenotype).

The interaction of aero1 *with major flowering genes*

Both the *aero1-1* and *aero1-10* mutants accelerate numerous aspects of plant development; leaf changes, flowering and apical arrest all occur earlier in the *aero1* mutants than their wild-type progenitors. Similar effects have been described for both *lf* and the day-neutral mutants (*sn*, *dne* and *ppd*) in pea (Barber 1959, Murfet 1985, Wiltshire *et al.* 1994). Therefore crosses were made to examine the relationship

between the heterochronic effects of *aero*, and those identified in *lf*, *sn*, *dne* and *ppd* plants.

A PCA was unable to completely separate *Aero1* from *aero1-10* plants in the F_2 of the cross between MIII/122 (*a aero1-10 Lf*) and HL60 (*A Aero lf*) (**Figure VIII.8a**). Despite this, *aero* segregants possessed a marked tendency for earlier transition of all developmental characters measured: node of leaflet changes, node of flower initiation and development, total nodes, and seed yield. These characters contribute equally to PC1 which comprises 54.9% of the variability of the data, and is the axis on which most of the *aero1-10* segregants are differentiated from their *Aero1* siblings (**Figure VIII.8a**). Variations in flower / leaf relativity, node of flower development and flowering time produced a further 19.3% of the variability (PC2). Two of the *Aero* segregants in this population produced leaves bearing seven leaflets. Although uncommon, this does occur in wild-type plants, and does not substantially alter the hypothesis that *aero* affects the rate of plant development. Alternatively these may represent rare recombinant events. Although the base gene for anthocyanin production (*A*) was segregating in this cross, and is known to be linked to *Lf* (Murfet 1971c, 1975) clear separation of *Lf* and *lf* individuals based on flower colour was not possible as the two traits were segregating in repulsion. However, there does appear to be a trend for plants carrying recessive *a* (from MIII/122, which carries *Lf*) to flower at a later time and node (**Figure VIII.8**). This is consistent with these plants also carrying *Lf*, and suggests that the effects of the *lf* and *aero1-10* mutations are additive.

The interaction between *Lf* and *Aero1* was re-examined in the F_2 of a cross between HL31 (*Lf-d A Aero1*) and JI2767 (*Lf a aero1-1*). Although a PCA was able to distinguish between *Aero1* and *aero1-1* siblings (**Figure VIII.9**), the difference between the putative *Lf-d* (red flowered individuals) and *Lf* (white flowered individuals) was unclear (**Figure VIII.9**). However, again there was a trend towards lower PC1 values for the putative *Lf* individuals suggesting earlier flower initiation and apical arrest (compare **Figures VIII.8** and **VIII.9**). In both crosses, the interaction between *Lf* and *Aero1* is unclear, which may reflect overlap of the mutant phenotypes.

The *ppd-2* and *aero1-10* segregants in the F_2 population from the cross between MIII/122 (*Ppd aero1-10*) and M2/830 (*ppd-2 Aero1*) were grouped together using a PCA analysis on developmental characteristics (**Figure VIII.10a**). These formed a distinct group from the late photoperiodic (*Ppd Aero1*) plants. However, *ppd-2* plants could normally be distinguished from *aero* segregants not only because of

the normal level of leaf flecking, but because they rarely achieved a leaflet number of four or greater before undergoing apical arrest. The C-5 and C-6 characters were included in the initial PCA analysis and as a result most of the *ppd-2* segregants were excluded. To counteract this the PCA was repeated without including C-5 and C-6 as defining characters (**Figure VIII.10b**). This retains the discrete separation of *aero1-10* and *ppd-2* segregants from late photoperiodic plants, and confirms the similarity between the effects of a mutation (*ppd-2*) conferring day-neutrality and that of the *aero1-10* mutation on plant development.

Discussion

Aero1 would appear to be a further major gene influencing both leaf and flower development. Although the *supaeromaculata* phenotype appears to be confined to the leaves, both the MIII/122 (*aero1-10*) and the JI2767 (*aero1-1*) mutant lines also underwent leaflet changes, flowered and senesced at earlier nodes than their respective progenitors (**Figures VIII.2** and **VIII.5**). In addition, the MIII/122 line produced fewer seeds, took fewer days to flower and was shorter than its progenitor (**Figure VIII.2**). Some of these characteristics have also been found in other *aero1* mutants (Sidorova and Uzhintseva 1995). Principal component analyses of populations segregating for *aero1-1* or *aero1-10* suggest that these pleiotropic characteristics are associated with the *supaeromaculata* phenotype (**Figures VIII.4** and **VIII.7**), despite the independence of the *aero1-1* and *aero1-10* mutational events. The nature of the relationship between the *supaeromaculata* phenotype and the promotion of plant development is unclear as the two traits appear to have no obvious connection. However, the presence of at least two mutant alleles displaying a similar range of pleiotropic effects would suggest that this represents an additional aspect of the *Aero1* genes' function, and that it is not the result of a second, closely linked mutation.

The new mutation, *aero2*, also seemed to promote a number of developmental characters, and *aero2* plants often had a maximum leaflet number of seven or eight. This raises the possibility that other, non-allelic *supaeromaculata* mutations may also influence other aspects of plant development. Unfortunately a pure breeding line of *aero2* was not developed within the time frame available, precluding a detailed examination of this mutant. However, the numerous *aero*-type mutations identified in various mutagenesis programmes (e.g. see Blixt 1962, Marx 1986a, Sidorova and Uzhintseva 1995) may prove useful in confirming this proposal.

The pleiotropic phenotype of the *aero1-1* and *aero1-10* mutants is significant as it suggests a promotion of total plant development (**Figure VIII.3**); in effect the plant rushes through its developmental programme to the detriment of seed yield. Thus, the mutant should be considered heterochronic, and the action of *Aero1* is to delay the aging process.

Aero1 *heads a hierarchy influencing heteroblasty, flowering and* supaeromaculata

The pleiotropic effects of the *aero1* mutations indicate that the *Aero1* gene controls a number of developmental processes. Mutations such as *Arg*, which promotes the *aeromaculata* phenotype without corresponding effects on plant development, and the day-neutral (e.g. *ppd-2*) and *lf* mutations, which promote earlier flowering without the corresponding effects on leaf flecking, may identify genes influenced by *Aero1*. Therefore, it should also be possible to identify genes specifically, or primarily, affecting leaflet changes without the corresponding effects on flowering, or leaf-flecking. Further characterisation of *Aero2* should reveal if it also affects flowering and leaf development in a similar manner to *Aero1*, or represents a gene controlling a subset of these processes.

aero1 *mutations may accelerate the decline in inhibitor levels*

The eventual flowering of wild-type pea plants under non-inductive conditions is thought to result from a gradual decline in the level of floral inhibitor as the plant ages (Murfet 1971c, 1985, Reid 1979). Reducing the inhibitor levels even further (as in the day-neutral mutants) promotes earlier flowering and earlier apical arrest (Murfet 1971a, 1985, King and Murfet 1985, Arumingtyas and Murfet 1994, Wiltshire *et al.* 1994, Taylor and Murfet 1996). Early flowering and apical arrest is also seen in the *aero1* mutants (e.g. **Figures VIII.2** and **VIII.3**), and *aero1* plants could be described as approaching day-neutrality. This similarity is also exemplified by the clustering *ppd-2* and *aero1-10* segregants together by the principal component analysis (**Figure VIII.10**). Therefore it is possible that the phenotype of the *aero1* mutants results (in part) from a reduction in the level of floral inhibitor, or more likely, a more rapid decline in the floral inhibitor levels with time. This effect is directly opposite to that of the *Hr* allele, which is proposed to prevent the decline in inhibitor production with age (Murfet 1973, Reid 1979). Therefore, addition of *Hr* to an *aero1* mutant background may partially relieve the *aero1* mutant phenotype. Physiological studies will also help to resolve the effects of *aero1* on inhibitor levels.

Do the aero1 *mutations accelerate the decline in COPS activity?*

The phenotype of the *aero1* mutants is more simply explained as an acceleration of the entire developmental programme of the plant. As the development of the garden pea is associated with a decline in the levels of inhibitor then this too is accelerated. However, the effects of *aero1* mutants do not appear to be specific to the inhibitor, but rather may result from a disruption in the ability of the plant to monitor or respond to developmental time.

Changes in character states during a plant's development have been attributed to the activity of a factor termed COPS (controller of phase shifting; Schultz and Haughn 1993, Haughn *et al.* 1995) and it has been suggested that the decline in inhibitor levels with time forms a component of the COPS activity in pea (Weller *et al.* 1997b). The general effects of *aero1* mutations on plant development suggest that *Aero1* may directly or indirectly influence the activity of COPS, possibly as part of a clock mechanism. If *Aero1* activity provides (or influences) some (or all) of the COPS activity in pea, then the potential effects of *aero1* on inhibitor levels may indicate that inhibitor does not form a component of COPS. Rather, the decline in inhibitor levels may also be a response to altered COPS activity.

The pleiotropic effects of the *aero1* mutations are difficult to reconcile with the *supaeromaculata* phenotype. One possibility is that the increased flecking in *aero* mutants also represents an acceleration of a developmental process occurring in wild-type plants, as wild-type leaves do show some leaf flecking (e.g. **Figure VIII.1a**). Thus, the initiation and development of the air spaces between epidermal and subjacent cell layers may occur more rapidly in *aero* mutants in comparison to their wild-type siblings, in much the same way that the increase in leaf complexity, or the transition to flowering is accelerated. This might suggest that in a background incapable of producing leaf flecks, such as in plants carrying *fl*, *aero* would be unable to influence leaf-flecking, although the acceleration of other developmental traits by *aero* would be unaffected. This may explain the intriguing comment of Blixt (in Marx 1986a) that *aero* mutants 'show recessive inheritance, indeed recessive even to *fl*'. The investigation of the pleiotropic effects of *aero* opens a new and exceedingly fertile area of future investigation in the garden pea.

Materials and methods

Allelism tests

Allelism between MIII/122 and *aero* was examined in a cross between MIII/122 and JI2767, the type line for *aero*. Allelism between the *aero*-like mutant in M1a/324 was examined in F_1 and F_2 populations derived from a cross between MIII/122 and a plant expressing the *aero* phenotype in the original M1a/324 mutant population.

Mutant phenotypes and genetic interactions

Pure breeding plants of line MIII/122 and its progenitor cv. Virtus (HL235) were grown under 8-h (SD) and 18-h (LD) photoperiods as described in **Chapter II**. Various developmental characteristics were scored off these plants and compared using Students' t-test. Cosegregation of the *aero* phenotype and various developmental characteristics was examined in F_2 populations from crosses between MIII/122 and its progenitor Virtus (cross 832). These plants were grown under SD conditions and the results analysed using a principal component analysis (Stat View™, Abacus Concepts, Inc. 1988). Similarly the phenotype of JI2767 (*aero1-1*) was examined under SD in comparison with its progenitor HL107 (cv. Torsdag). The F_2 from the cross between JI2767 and HL107 was also examined under SD conditions.

The interactions between *aero1-10* and *lf* were examined in crosses between the pure breeding lines MIII/122 (*a Lf aero1-10*) and HL60 (*A lf Aero1*; cross 836) under LD, and *aero1-1* and *Lf* in the cross between JI2767 (*a Lf aero1-1*) and HL31 (*A Lf-d Aero1*) under SD. The interaction between *aero1-10* and *ppd-2* was examined in the F_2 of the cross between MIII/122 (*Ppd aero1-10*) and M2/830 (*ppd-2 Aero1*; cross 819) grown under SD.

The effect of *Arg* on whole plant development was examined in a population from the cross between M2/830 (*arg ppd-2*) and HL224 (*Arg Ppd*) grown under a 10-h photoperiod. Day-neutral (*ppd-2*) segregants were not included in this analysis.

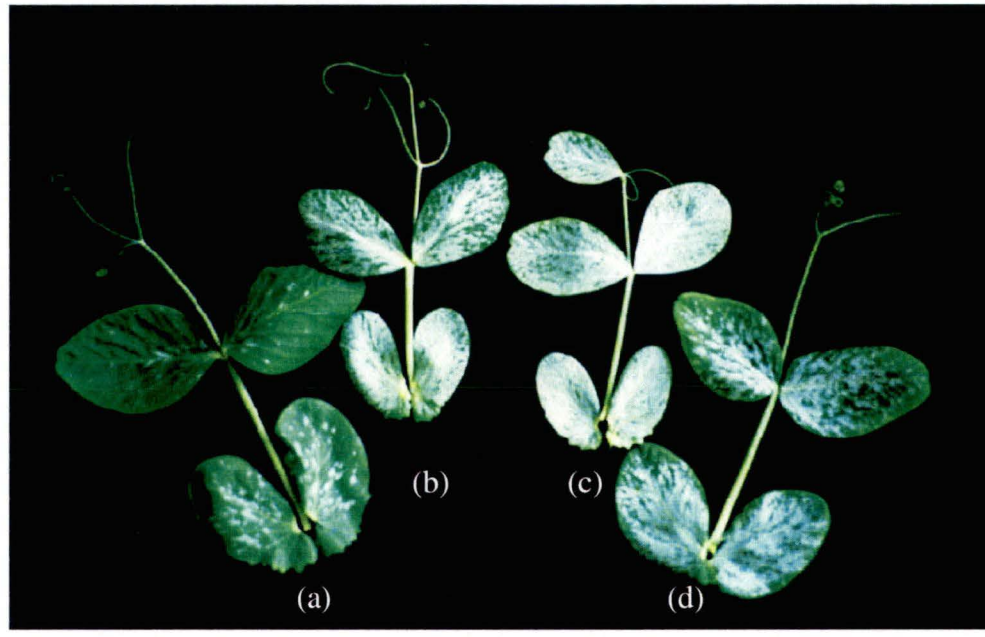


Figure VIII.1: Leaves from equivalent nodes of (a) wild type (*Aero1 Aero2*), (b) *aero1-10* (MIII/122), (c) *aero1-1* (JI2767) and (d) *aero2* (from M1a/324 population). The *supaeromaculata* mutations increase the flecking present on the leaflets and stipules.

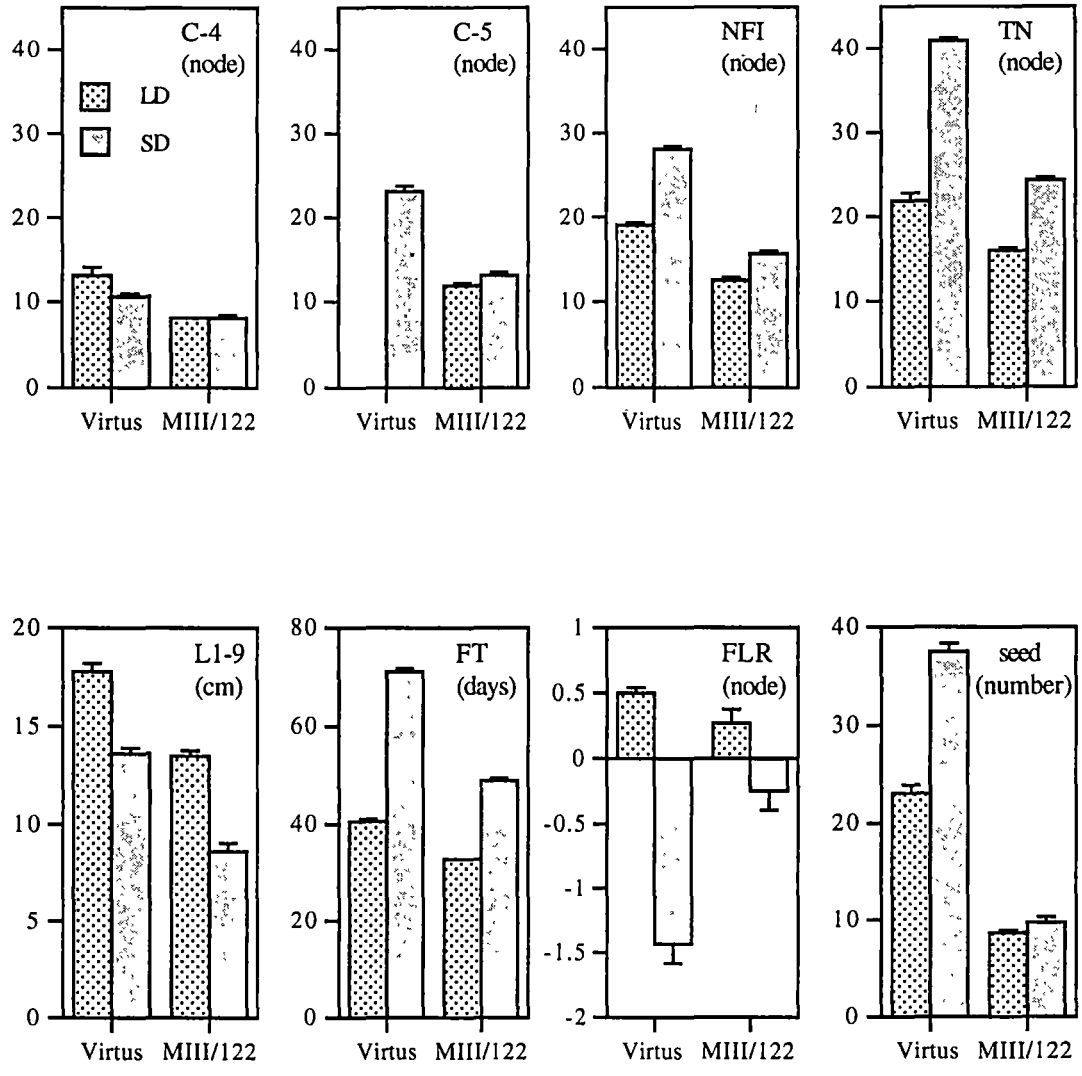


Figure VIII.2: Developmental characteristics of the initial line Virtus and the MIII/122 mutant line under 18-h (LD) and 8-h (SD) photoperiods. All developmental characteristics examined occur at an earlier node in the MIII/122 mutant compared with its initial line Virtus (also see **Figure VIII.3**). MIII/122 plants are also shorter, and undergo apical arrest after producing fewer seeds. In addition, the difference between FLR values in LD versus SD conditions is reduced in MIII/122 plants, further suggesting a tendency towards day-neutrality. Virtus plants did not reach C-5 under the LD photoperiod. The values are means (± SE) of six Virtus and 15 MIII/122 plants under each photoperiod.

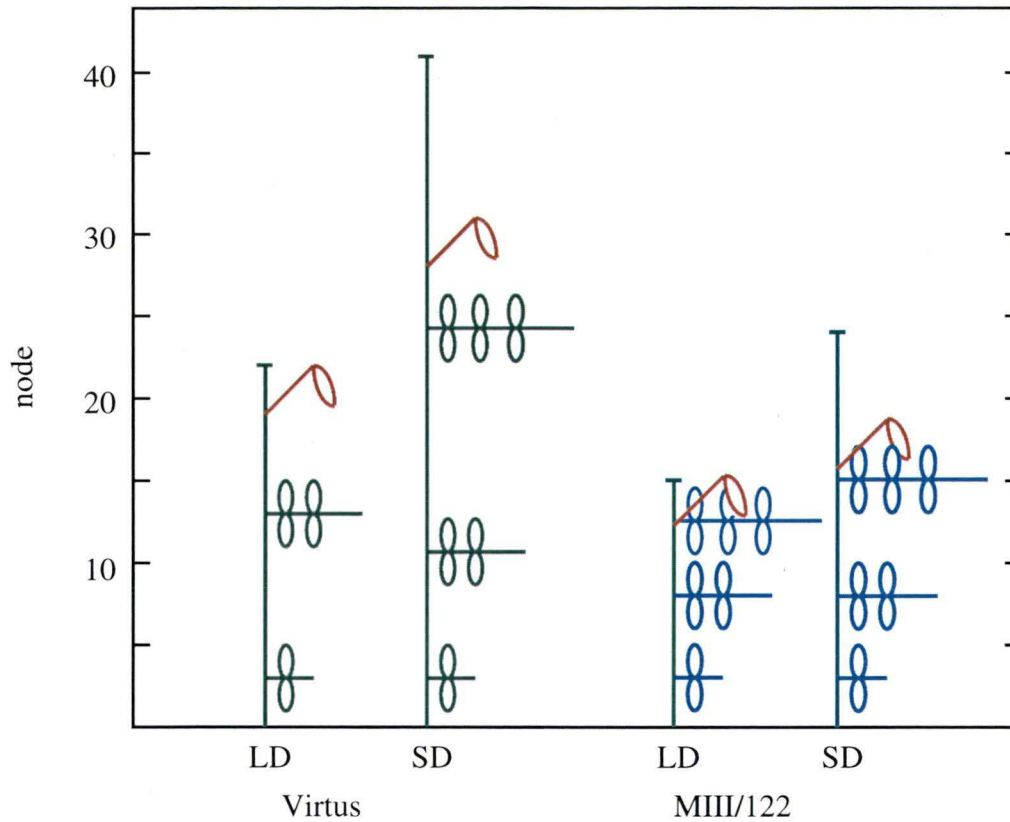


Figure VIII.3: Schematic representation of Virtus and MIII/122 plants, as a summary of the results from **FigureVIII.2**. The MIII/122 mutant line shows an acceleration of all aspects of plant development. This acceleration is seen under both an 18-h (LD) and an 8-h (SD) photoperiod. However, the developmental changes (e.g. C-4, NFI) occur in the same order in mutant and wild-type lines. Developmental characters: C-4 ; C-6 ; NFI ; TN is represented by the cross bar at the apex of the plant.

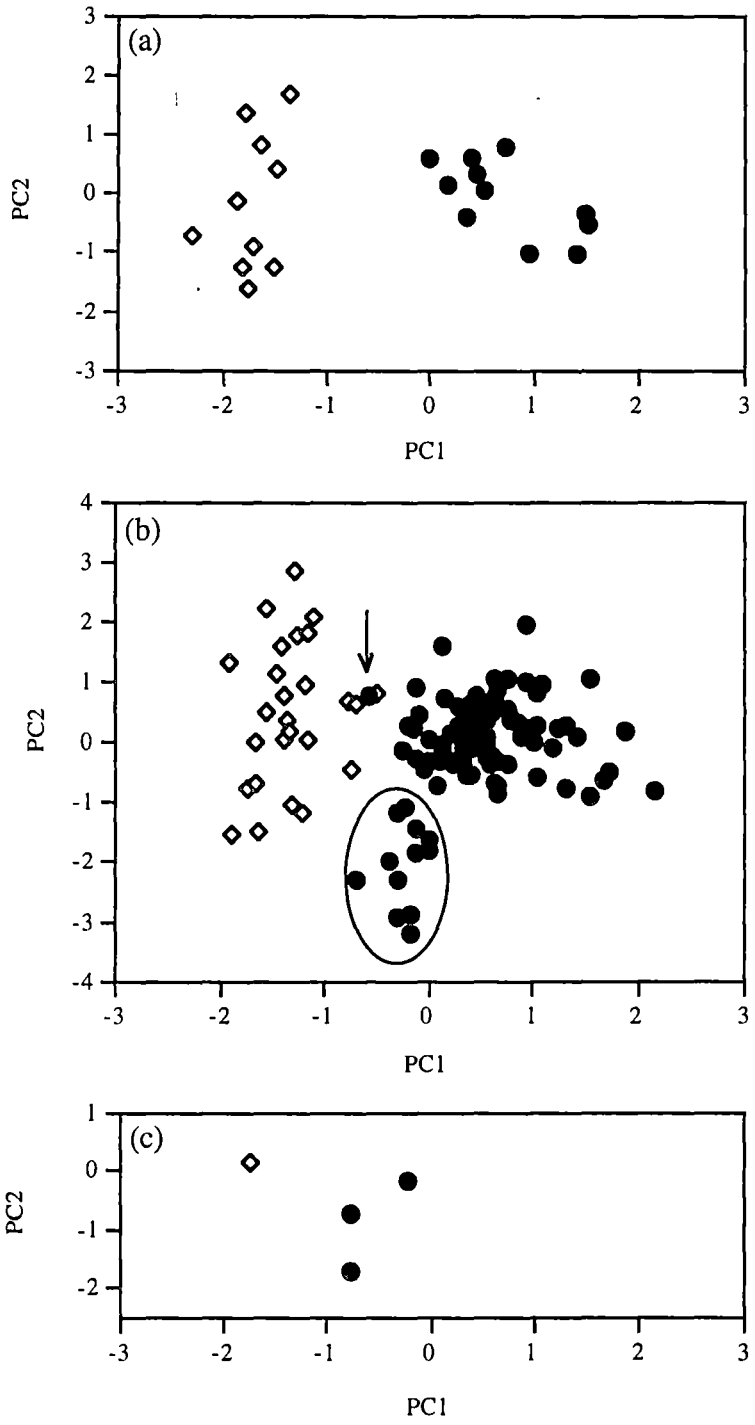


Figure VIII.4: Principal component analysis from the parents, F_2 and F_3 of the cross between MIII/122 (*aero1-10*) and Virtus (*Aero1*). (a) Parental controls. (b) F_2 population; the early initiating (EI class; see Murfet 1971a) individuals are grouped in a circle. (c) F_3 from a heterozygous wild-type (*Aero1/ aero1-10*) plant with intermediate characteristics (arrowed individual in b). PC1 comprises 61% of the variability in the data. Plants carrying *Aero1* are represented by closed circles, homozygous *aero1-10* plants by open diamonds. Photoperiod 8 h.

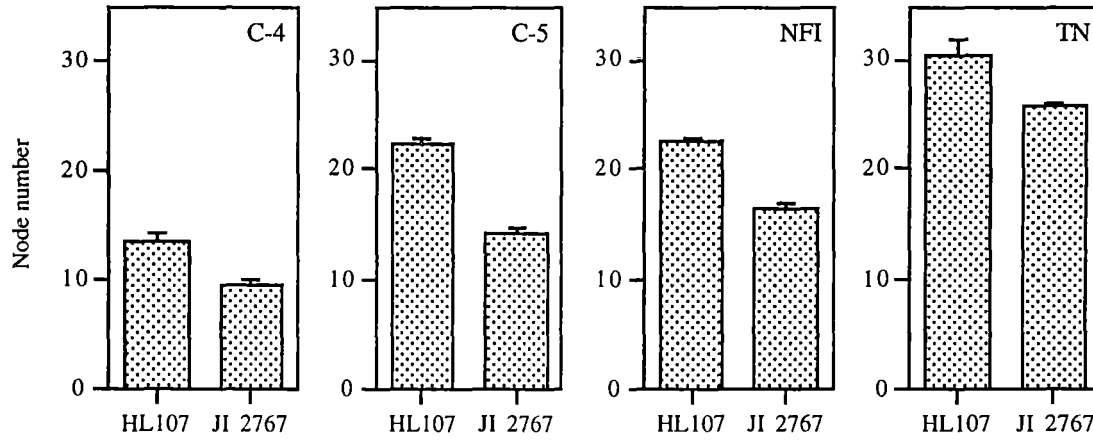


Figure VIII.5: Developmental characteristics of initial line Torsdag (HL107, *Aero1*) and mutant line JI2767 (*aero1-1*). Like MIII/122, the JI2767 mutant also displays an acceleration of all developmental characteristics. Photoperiod 8 h. Data are shown as mean ± SE.

aero1-1 *Aero1*

Figure VIII.6: In addition to the acceleration of leaflet change associated with the *aero1-10* mutant phenotype (e.g. **Figure VIII.3**), both *aero1* and *aero2* mutants also often reached the 'super adult' leaf form bearing eight leaflets per leaf (C-8). This figure illustrates a C-8 *aero1-1* leaf compared with the most complex leaf type (C-6) found on most wild-type plants in this study.

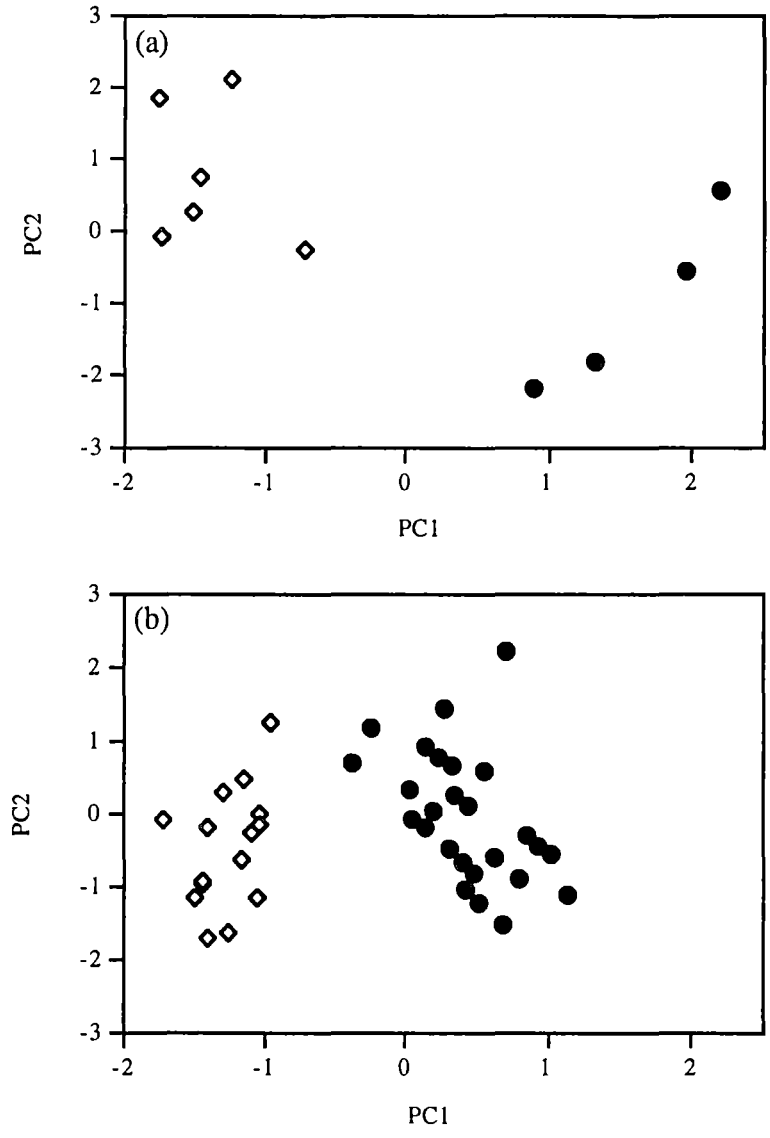


Figure VIII.7: Principal component analysis for the parents and F_2 of the cross between JI2767 (*aero1-1*) and its initial line HL107 (*Aero1*). (a) Parental controls. (b) F_2 population. PC1 comprises 89% of the variability in the data. Separation of *aero1-1* (open diamonds) and *Aero1* (closed circles) segregants was based solely on leaflet changes and NFI.

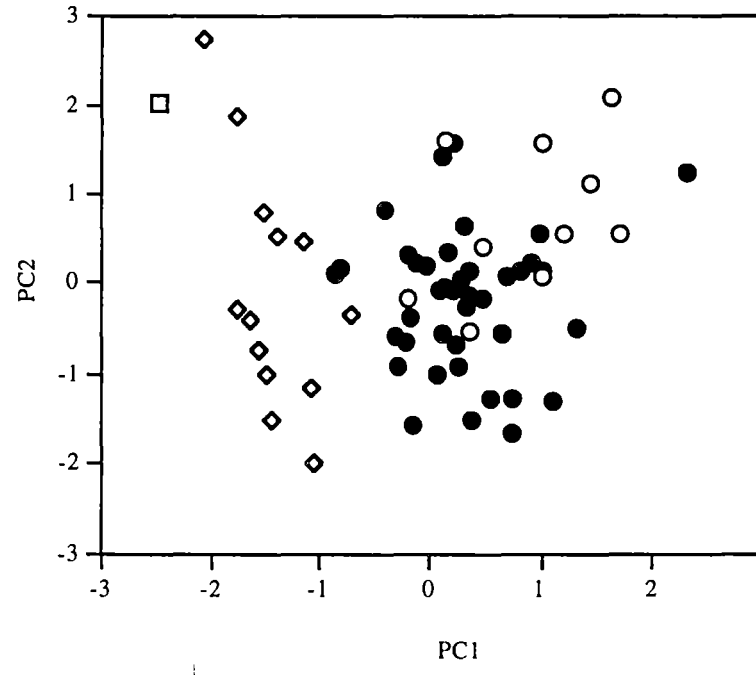


Figure VIII.8: Principal component analysis of the F_2 between HL60 (*A lf Aero1*) and MIII/122 (*a Lf aero1-10*). Closed circles, *A Aero1*; open circles, *a Aero1*; open diamonds, *A aero1-10*; open square, *a aero1-10*. Separation of *Aero1* and *aero1-10* is not discrete. The anthocyanin locus (*A*) and late flowering locus (*Lf*) are linked with an average distance of about 10 cM (Murfet 1971b, 1975). However, as *A* and *Lf* are in repulsion, segregation of *A* does not aid appreciably in the resolution of the interaction between *Aero* and *Lf*.

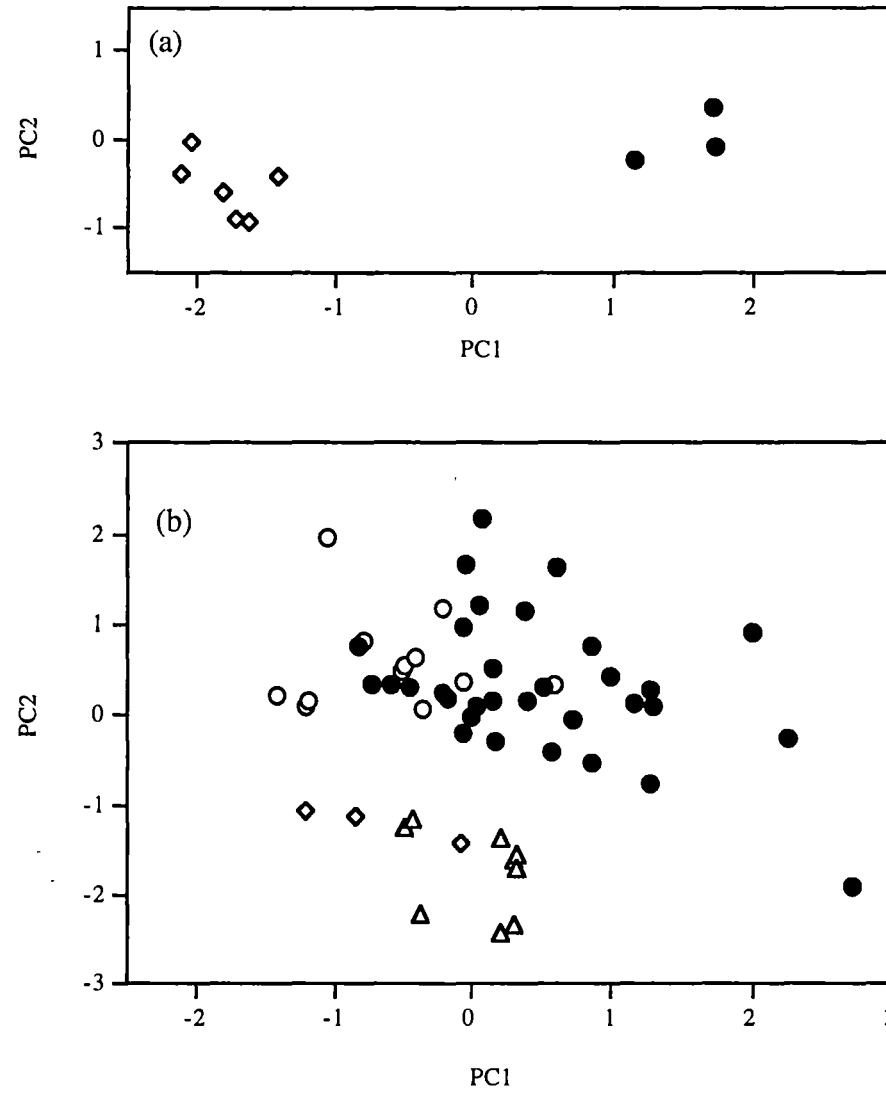


Figure VIII.9: Principal component analysis of the parental lines (a) and F_2 (b) from the cross between HL31 (*A Lf-d Aero1*) and JI2767 (*a Lf aero1-1*). Closed circles, *A Aero1*; open circles, *a Aero1*; open triangles, *A aero1-1*; open diamonds, *a aero1-1*. Separation of *Aero1* and *aero1-1* is based primarily on PC2, which comprises only 17% of the variability of the data. In this cross *A* and *Lf-d* are in coupling. However, the developmental characteristics of the white (*a*) flowered plants (putatively *Lf*) overlap those of the red (*A*) flowered (putatively *Lf-d*) individuals.

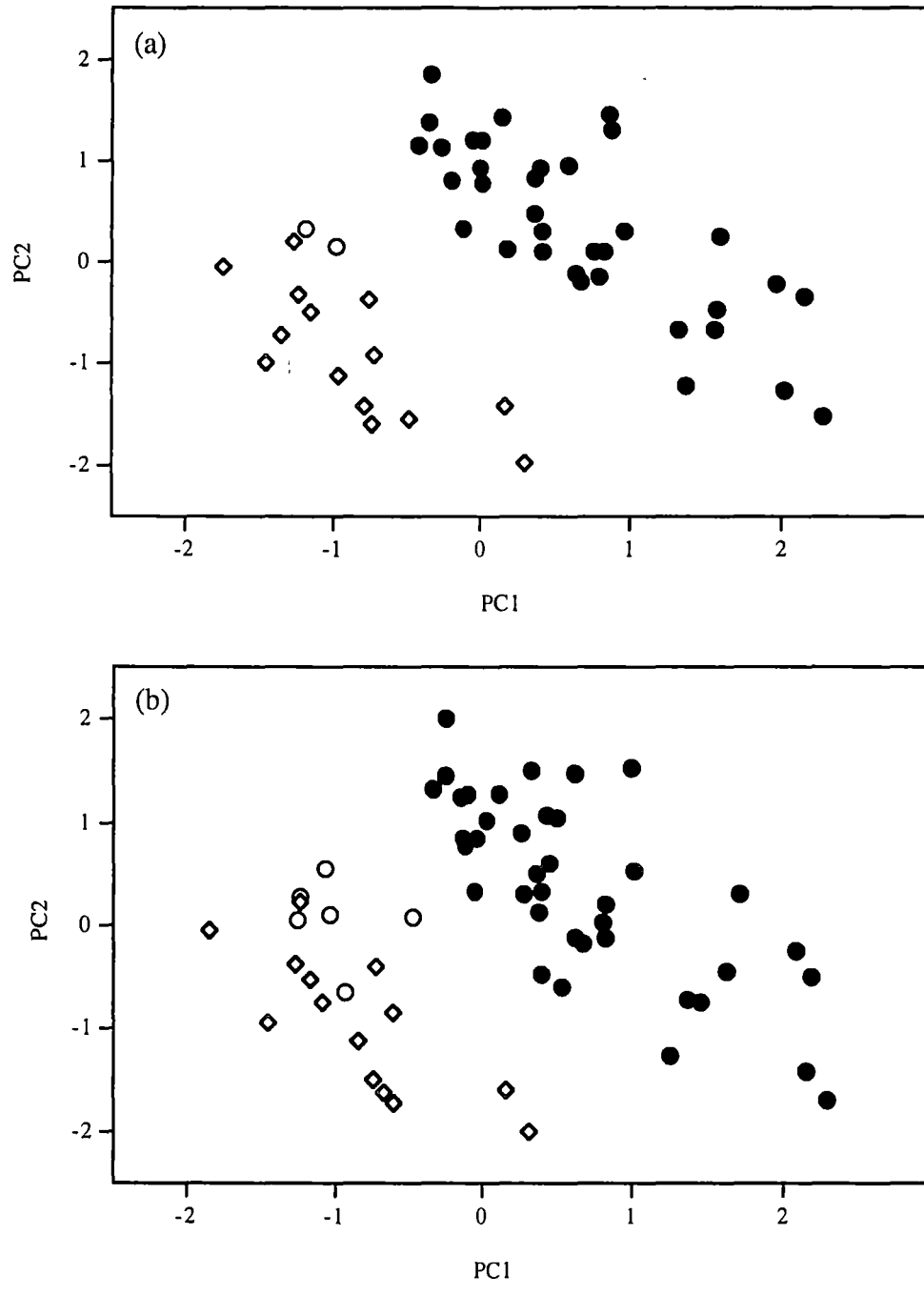


Figure VIII.10: Principal component analysis of the F_2 between M2/830 (*ppd-2 Aero1*) and MIII/122 (*Ppd aero1-10*). Closed circles, *Ppd Aero1*; open circles, *ppd-2 Aero1*; open diamonds, *aero1-10*. The principal component analysis was able to separate *Ppd Aero1* segregants from *ppd-2* and *aero1-10* mutant segregants. However, separation of *ppd-2 Aero1*, *Ppd aero1-10* and *ppd-2 aero1-10* segregants was not achieved. (a) Dataset including C-5 and C-6; (b) dataset excluding C-5 and C-6. Inclusion of the C-5 and C-6 characteristics removes many of the *ppd-2* segregants as they did not attain the C-5 or C-6 stage.

Chapter IX. General discussion

This study has used both genetic and molecular analyses to dissect flower and inflorescence development in the garden pea. The functions and interactions among nine genes involved in flowering were examined - four of these genes in detail. These studies have produced a more detailed overview and understanding of the genes and genetic interactions involved in the flowering process in the garden pea.

Two of the genes examined, *Stp* and *Pim*, are required for the specification of the flower. However, the identification of pleiotropic effects of mutations in *Stp* have indicated that the *Stp* gene is also involved in the development of leaves and inflorescences, and probably acts ubiquitously during plant development. This activity occurs in combination with the meristem identity gene *Uni*. Molecular analysis indicates that *Stp* is the pea homologue of *Fim* from *Antirrhinum* and *UFO* from *Arabidopsis*. Collaborative studies identified *Pim* as the pea homologue of *Squa* and *AP1* from *Antirrhinum* and *Arabidopsis*, respectively.

Abnormalities in the flowers from *veg2-2* mutants and the *veg1 det* double mutant plants suggested that *Veg1* and *Veg2* are also required during flower development, in addition to their previously identified roles in inflorescence identification (Murfet and Reid 1993, Singer *et al.* 1994, Reid *et al.* 1996). Thus, *Veg1* and *Veg2* may interact with, or regulate, the function of *Pim* during the development of the flower. Epistatic relationships between mutant alleles at *Gi* (which influences the production of a floral stimulus) and *Lf*, and between mutant alleles at *Lf* and *Veg2*, have suggested that the floral stimulus may promote flowering by inhibiting a block imposed by *Lf* on *Veg2* activity. A similar interaction may also occur between the floral stimulus and *Det* and *Veg2*. These interactions, and the inflorescence and flower phenotypes described for the *veg2-2* mutant allele, suggest that the *Veg2* gene is pivotal in the transition from vegetative to reproductive growth in pea.

Although the most diagnostic trait of *aero* mutants is their increased leaf flecking, detailed analyses of two *aero* mutants have identified *Aero* as a unique, previously overlooked developmental gene with broad effects on plant development. The *aero* mutant phenotype is associated with an acceleration of several key aspects of plant development, suggesting *Aero* may be involved in the timing of developmental processes.

An integrated model for the control of flowering in pea

The analysis of the genetic control underlying flower and inflorescence identification carried out in these studies (**Chapters V, VI** and **VII**), the detailed analysis and identification of the roles *Stp* and *Uni* in plant development and flowering (**Chapter III**, Hofer *et al.* 1997), and previous physiological and genetic studies of flowering in pea (reviewed in Murfet 1985, 1989, Murfet and Reid 1993, Reid *et al.* 1996, Weller *et al.* 1997b) have led to the development of a detailed model for the genetic control of flowering in pea (**Figure IX.1**). This model is composed of three separate pathways. The first involves the *Stp* and *Uni* genes, which act non-specifically to control meristem growth and development. The second pathway is involved in the response to photoperiod and also acts non-specifically (see also Taylor and Murfet 1994, Reid *et al.* 1996, Weller *et al.* 1997b). The third pathway is specific to flowering and involves genes influencing the production of the floral stimulus (*Gi*), the perception of this stimulus at the apex (*Lf*), and the identification of the secondary inflorescences and flowers (*Veg2*, *Veg1* and *Pim*). *Det* acts in this pathway to maintain the indeterminancy of the primary inflorescence. The *Veg2* gene appears to be central to flowering, and the flowering behaviour of pea may be highly dependent on where and when *Veg2* is active. *Aero* has yet to be placed within any of these three pathways, although current analyses suggest that it may be involved in the response to photoperiod. Alternatively, *Aero* may define a fourth pathway, possibly a clock mechanism, underlying many aspects of plant development.

Some of the ideas derived from these results have been integrated into previous models for flowering in pea (Figure 1 of Reid *et al.* 1996, and Figure 4 of Weller *et al.* 1997b). The model presented in **Figure IX.1** differs from that of Reid *et al.* (1996) in that the functions of *Uni* and *Stp* have been separated from the central pathway controlling flower development. Although *Uni* and *Stp* represent the pea homologues of floral meristem identity genes from *Arabidopsis* and *Antirrhinum* (Hofer *et al.* 1997, **Chapter IV**), analysis of their mutant phenotypes fails to support a flower specific role for these genes in pea (Marx 1987, Hofer *et al.* 1997, **Chapter III**). The model presented (**Figure IX.1**) also differs from that of Reid *et al.* (1996), and extends that of Weller *et al.* (1997b), in the details of the genetic interactions underlying inflorescence development. A deficiency in the model presented lies in the lack of integration of those genes known to be involved in flower development in pea (e.g. see Reid *et al.* 1996). However, the evolutionary conservation of the control of flower development (see **Chapter I**) suggests that the ABC model of flower development, based on results from *Arabidopsis* and *Antirrhinum*, may be directly applicable to pea.

The homology between *Pim*, *AP1* and *Squa* provides a link between flower development in pea, and that of *Arabidopsis* and *Antirrhinum*.

The central pathway for flowering involves a floral stimulus

The phenotype produced by the two *veg2* mutant alleles suggests that the *Veg2* gene is central to the development of the secondary inflorescence and flowers, in essence regulating the transition from vegetative to reproductive growth. This is apparent in the wholly vegetative phenotype of *veg2-1* plants and in the floral and inflorescence abnormalities in *veg2-2* plants (see Murfet and Reid 1993, **Chapter VI**). *Gi* is also essential for the transition to flowering by producing or controlling the production of a floral stimulus in the leaves and shoots (and possibly roots) that triggers reproduction (Taylor and Murfet 1994, Beveridge and Murfet 1996). The transport of the floral stimulus to the apex may allow environmental effects to impinge upon the flowering process (Taylor and Murfet 1994, Weller *et al.* 1997b, below). The abnormal inflorescence development seen in *Lf lf-a gi-2 gi-2* plants (**Figure VII.3**) mimics the phenotype of the *veg2-2* mutant, suggesting a direct link between the functions of *Gi* and *Veg2*. The *Lf* gene appears to provide this link, responding to the level of the floral stimulus produced by *Gi*, and controlling the expression of *Veg2* in the apex (**Figure IX.1** and **IX.2**).

This scenario is complicated by the interaction between *Gi* and *Det* (**Chapter VI**), which suggests that *Det* may also be capable of responding to the level of stimulus present in the plant (**Figure IX.2**). This supports the proposed homology between *Lf* and *Det*. Their apparently identical response to the floral stimulus and proposed function in repressing *Veg2* activity have presumably been inherited from their assumed common ancestral gene. However, their activities have become specialised and are now spatially distinct – *Det* functioning primarily in the apical meristem, while *Lf* presumably acts in the axillary meristems (**Figure IX.2**). However, some redundant activity of *Det* in the axillary meristems would be expected from its interaction with *Gi*. The spatial specialisation of *Lf* and *Det* probably results in their distinct mutant phenotypes.

Overlapping activities of Pim, Veg1 *and* Veg2

Consistent with results found for genes involved in flower development (e.g. Bowman *et al.* 1993, Haughn *et al.* 1995), the functions of *Pim*, *Veg1* and *Veg2*

overlap. *Pim*, like its *Arabidopsis* homologue *AP1*, also acts as a floral homeotic gene, although this activity appears to be highly redundant in pea (**Chapter V**). *Veg1* may interact with *Pim* during floral meristem identification and flower development as both genes contain MADS boxes. However, *Veg1* is also essential for the identification of the secondary inflorescence in pea (Singer *et al.* 1994, **Chapter VI**). *Veg2* is also required for the identification of the secondary inflorescence and flower, but is also necessary for the transition from vegetative to reproductive growth and therefore presumably the identification of the primary inflorescence (**Chapter VI**). These interactions are illustrated in **Figure IX.3**. Thus, the activities of *Pim*, *Veg1* and *Veg2* also overlap the morphological separation of the primary and secondary inflorescences and the flower. These divisions may therefore be inherently artificial, as is the division between floral meristem identification and flower development (Haughn *et al.* 1995).

The interaction between the 'floral inhibitor' and floral stimulus may not be unique to pea

Activity of the photoperiod response genes in pea may influence the distribution of the floral stimulus within the plant (Taylor and Murfet 1994, Weller *et al.* 1997b). Under non-inductive conditions, the activity of the photoperiod gene system influences the partitioning of assimilates within the plant, directing them basipetally (Reid and Murfet 1984, Murfet 1985, Kelly and Davies 1988, Beveridge *et al.* 1992). This distribution of assimilates may interfere with the accumulation of the floral stimulus in the apex, thereby delaying flower initiation (Taylor and Murfet 1994). The control of assimilate partitioning by photoperiod has been identified in a number of diverse species (e.g. see Wallace *et al.* 1993b), which include both monocots and dicots. This suggests that homologues of the genes known to influence assimilate partitioning in pea (*Sn*, *Dne*, *Ppd* and *Hr*) may have been present in the earliest angiosperms (although the possibility that the same phenotype results from the activity of unrelated gene systems cannot be discounted). Moreover, physiological evidence suggests the presence of mobile floral stimulatory substances in an equally diverse group of species (e.g. Lang 1965, O'Neill 1992).

The possibility of *Gi* homologues in other species is also supported by the characterisation of *Indeterminate* (*Id*) from maize (Singleton 1946, Colasanti and Sundaresan 1996, 1997). *id* mutant plants are late flowering, often with proliferous inflorescences, or they may never flower (Singleton 1946, Colasanti and Sundaresan 1997). This flowering behaviour appears to result from a deficiency in a non-cell-

autonomous floral stimulus (Colasanti and Sundaresan 1997). The flowering behaviour and physiological properties of the *id* mutants mirrors the phenotype and properties of the *gi* mutants (Taylor and Murfet 1994, Beveridge and Murfet 1996, **Chapter VI**). Therefore *Id* may be homologous to *Gi*. Alternatively, the *Id* and *Gi* homologues may act together to control the production of the floral stimulus.

Thus, it is possible that the physiological interaction between the 'floral inhibitor' and the floral stimulus may influence responses to daylength and the transition to flowering in diverse groups of plants. This possibility awaits the molecular characterisation of genes involved in the transition to flowering from a number of diverse plant groups.

Do the models for flowering in pea and Arabidopsis *coincide?*

The pursuit of mutant homologues from different species can yield paradigm shifting results. For example, the identification of *Uni* and *Stp* as pea homologues of *Flo / LFY* and *Fim / UFO* have revealed alternative functions of the *Flo* and *Fim* homologues that questions their apparently floral specific role in *Arabidopsis* and *Antirrhinum* (Hofer *et al.* 1997, **Chapter IV**). In contrast, the characterisation of mutant homologues can support and strengthen models proposed in different species (e.g. the flower specific role of *Pim*, *Squa* and *AP1*; **Chapter V**). These two examples are significant as they reveal how generally applicable the theories developed from a single model species may be. The models presented for the transition to flowering are no exception.

The only other species where detailed genetic models for the control of flowering have been published is the small cruciferous weed *Arabidopsis* (e.g. Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995, Peeters and Koornneef 1996). These models are based primarily on the genetic characterisation and environmental responses of early- and late-flowering mutants identified in *Arabidopsis* (e.g. Koornneef *et al.* 1991, Zagotta *et al.* 1992, Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995, Hicks *et al.* 1996, Coupland 1997, Koornneef and Peeters 1997). These characterisations have identified three promotive and two repressive pathways for flowering in *Arabidopsis*: a short day repressive pathway, a long day promotive pathway, a constitutive repressive pathway, a constitutive promotive pathway, and a short day promotive pathway that is mediated by gibberellin (Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995). Plants carrying mutations in either of the two constitutive pathways retain the ability to respond to changes in photoperiod. These pathways are

represented by the late flowering mutants *fca*, *fve*, *fy*, *flc*, *fri*, *fpa* and *ld* (constitutive promotion) and the early flowering mutants *elf1*, *elf2* and *tfl* (constitutive repression) (Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995). Mutants in the long day promotive pathway flower late under a LD photoperiod, but no later than wild-type plants when grown under SD conditions (late, day neutral). This pathway is represented by the mutants *co*, *gi*, and *fha*. The *ft*, *fwa*, *fd* and *fe* mutants are also included in the long day promotive pathway (Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995, Peeters and Koornneef 1996), although these mutants show a substantial delay in flowering under SD conditions (Koornneef *et al.* 1991). The short day repressive pathway is identified by mutants with an early-flowering, day-neutral phenotype (e.g. *elf3*, *hy1*, *hy2*, and *cop1*) (Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995). The fifth pathway is represented by the highly pleiotropic mutants *ga2*, *gai*, *spy*, *aba* and *abi1* (Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995).

Although a direct comparison of mutant phenotypes (especially one based on the evolutionarily plastic responses to environment; Murfet 1977) may be misleading, four of these five phenotypic classes are also present in pea. The *gi-1* and *veg2-2* mutants possess a late flowering phenotype, but retain the ability to respond to photoperiod (constitutive promotion) (Murfet and Reid 1993, Taylor and Murfet 1994, Beveridge and Murfet 1996, **Chapter VI**). The *lf* mutants flower early, but also retain the ability to respond to photoperiod (constitutive repression). However, the response to photoperiod in *lf* mutants occurs through a delay in flowering time not node of flower initiation (Murfet 1971b, 1975, 1985). Despite this, the 'constitutive promotion' and 'constitutive repression' of flowering describes the proposed functions of *Gi* and *Veg2*, and of *Lf*, respectively (**Chapters VI** and **VII**, **Figure IX.1**). The short day repressive pathway would encompass the early day-neutral mutants of pea, *sn*, *dne* and *ppd* (Barber 1959, Murfet 1971a, 1971b, King and Murfet 1985, Arumingtyas and Murfet 1994), while the long day promotive pathway is exemplified by the phytochrome A (phyA) deficient mutant *fun1* (Weller *et al.* 1997a). However, it appears that the gibberellin-mediated promotive pathway is not present, or is not active, in pea. Gibberellin deficiency does not profoundly alter the responses to daylength in pea (Murfet and Reid 1987).

It is interesting to note that genes proposed to identify the two photoperiodically controlled pathways in pea act in a single pathway according to the scheme presented in **Figure IX.1** (and see Weller *et al.* 1997a). Similarly, those genes proposed to identify the constitutive repressive and constitutive promotive pathways of pea also act within the one pathway (**Figure IX.1**). The first controls the responses to photoperiod (*Fun1*, *Sn*, *Dne*, *Ppd* and *Hr*), and may represent the pea equivalent of the

SD repressive / LD promotive pathways of *Arabidopsis*. The second is considered the central (constitutive) flowering pathway (*Gi*, *Lf*, *Veg2* and *Veg1*). Negative interactions are proposed to occur between the 'early' and 'late' flowering representatives from a single pathway (**Figure IX.1**). It is possible that similar relationships occur between the late and early flowering mutants in *Arabidopsis* (Weller *et al.* 1997b). For example, Simon and Coupland (1996) found that the early flowering mutant *esd4*, which flowers with two vegetative leaves under LD but four under SD conditions, was epistatic to the late photoperiodic mutant *fve*. In contrast, the *co esd4* double mutant had a flowering time intermediate between the *co* and *esd4* parents under both LD and SD conditions (Simon and Coupland 1996).

Regulation of the floral meristem identity genes

The model present in **Figure IX.1** provides a mechanism by which the floral meristem identity gene *Pim* may be regulated – through the activities of *Veg1* and *Veg2*. Similarly, *AP1* (and *CAL*) expression is dependent on the activity of the late flowering genes *FWA* and *FT*, as double *lfy fwa* or *lfy ft* mutants fail to express *AP1* activity (Ruiz-García *et al.* 1997). Although *FWA* and *FT* are placed into the LD repressive pathway for flowering in *Arabidopsis* (Martínez-Zapater *et al.* 1994, Haughn *et al.* 1995), flowering in *fwa* and *ft* (and *fe*) is delayed under SD conditions (Koornneef *et al.* 1991). Thus, the phenotypes of *fwa* and *ft* mutants may match those of *veg1* and *veg2*. However, it seems unlikely that *FWA* or *FT* represents the *Arabidopsis* homologue of *Veg1* at least. The pea MADS-box gene *pM6*, which is completely deleted in *veg1* mutant plants (**Chapter VI**), is most closely related to *AGL2* from *Arabidopsis* (Beltrán *et al.* 1996), and the map position of *AGL2* (on chromosome 5) does not coincide with that of *ft* or *fwa* (Ma *et al.* 1991, Peeters and Koorneef 1996). However, it is in the same general region as the loci of the late flowering mutants *fy* and *flc*.

In contrast to *Pim*, factors which may regulate the activity of *Uni* and *Stp* have not been identified in pea. The *Uni* and *Stp* mutations affect nodes which are laid down in the embryo (**Figure III.9**), and it is assumed that both *Uni* and *Stp* are expressed during all stages of plant development. Consistent with this, *LFY* from *Arabidopsis* is expressed from three days post germination (Blázquez *et al.* 1997, Bradley *et al.* 1997). Under SD conditions *LFY* expression increases gradually. The rate of increase in expression is accelerated by LD photoperiods and continuous gibberellin application (Blázquez *et al.* 1997). *LFY* expression also rapidly peaks after transfer from non-inductive (SD) to inductive conditions (Blázquez *et al.* 1997,

Hempel *et al.* 1997). The difference in *LFY* expression in LD versus SD conditions may be related to *CO* expression, as increasing *CO* expression rapidly results in *LFY* upregulation (Simon *et al.* 1996). Whether the expression patterns of *Uni* are the same is unknown, but the fact that gibberellins do not promote flowering in pea (Murfet and Reid 1987, Beveridge and Murfet 1996) may result from the inability of *Uni* to respond to changes in gibberellin levels. Similarly, there is no evidence for a difference in *Uni* expression in LD versus SD conditions - the flower phenotypes of *Stp* and *Uni* mutant plants are unaffected by photoperiod (**Chapter III**). This may reflect an inability of the environment to influence *Uni* expression in pea, possibly through disruption of the function of a *CO* homologue.

Differences in *Stp* and *UFO* expression patterns are revealed by the overexpression of *UFO* in *Arabidopsis*. Although *Stp*, like *Uni*, must be expressed from an early stage in plant development, *UFO* expression appears to be limited to the apex and floral initials (Ingram *et al.* 1995, Lee *et al.* 1997). Overexpression of *UFO* results in a unique leaf phenotype that is dependent on wild-type *LFY* function in the leaves (Lee *et al.* 1997). This suggests that the potential effects of *LFY* and *UFO* on leaf development in *Arabidopsis* is limited by the lack of *UFO* expression in the leaves. Thus, expansion of *Stp* expression into the leaves may have been a prerequisite for the evolution of the compound leaf in pea.

Problems

The identification of possible homologies between genes in pea and *Arabiodopsis* is hindered by the different classifications of the mutant phenotypes. Mutants in pea are more often described on the basis of their physiological effect. Thus, rather than being described as late photoperiodic, *gi-1* and *gi-2* are described as deficient in the floral stimulus. The vegetative flowering behaviour of *veg1* and *veg2-1* mutants has no counterpart in *Arabidopsis*, although the vegetative phenotype may be considered 'super late' such that flowering does not occur before plant death. Alternatively, the *veg1* and *veg2* mutant phenotypes may be considered homeotic, with leafy shoots replacing secondary inflorescences (Wiltshire *et al.* 1994). The classification of the *Veg1* and *Veg2* genes as flowering time or homeotic influences the choice of potential homologues. Mutants in *Lf* may be considered day-neutral in terms of node of flower initiation (equivalent to leaf number in *Arabidopsis*). However, non-inductive conditions can delay flowering time through the delayed development and/or abortion of early flower initials (Marx 1968, Murfet 1971a, 1985), and a delay in node of flower initiation is only expressed in the absence of *E* activity (Murfet

1971b, 1985). This indicates that *Lf* is not involved in the response to photoperiod in pea. In *Arabidopsis*, the *esd4* mutant is considered photoperiodically insensitive (Hicks *et al.* 1996), yet Simon and Coupland (1996) describe the *esd4* mutant as flowering with four leaves in SD and only two in LD. The differences in the descriptions of the *esd4* mutant phenotype point to potential difficulties in comparing mutants from pea and *Arabidopsis* based on their phenotypic descriptions alone. Direct comparisons between the pea and *Arabidopsis* wild-type and mutant plants grown together under various environmental conditions may overcome this problem. However, this could prove time consuming considering the number of mutants that would have to be considered.

The more intensive mutant screening in *Arabidopsis* has led to the isolation of many more flowering time mutants than are seen in pea. Many of these currently do not have a pea counterpart – although there are seven late day-neutral *Arabidopsis* mutants (*co* and *gi* type), only one has been identified in pea, *fun1*. *fun1* mutants are deficient in phyA, and therefore *Fun1* may be homologous to *PHYA* from *Arabidopsis* (Weller *et al.* 1997a, 1997b). Where are the other late day-neutral mutants in pea? Despite the less intensive mutant screens, three genes with an early day-neutral mutant phenotype have been identified in pea (Murfet 1971a, 1971b, King and Murfet 1985, Arumingtyas and Murfet 1994). These three genes also have an identical physiological function - each is involved in the production of the floral inhibitor (Barber 1959, Murfet 1971c, Murfet and Reid 1973, King and Murfet 1985, Taylor and Murfet 1996). However, many of the early day-neutral mutants in *Arabidopsis* also show an increase in hypocotyl elongation, suggesting that they are involved in responses to light (Martínez-Zapater *et al.* 1994, Hicks *et al.* 1996). Thus, they do not appear to be likely candidates for *Sn*, *Dne* or *Ppd* homologues. The characterisation of more recently isolated early flowering photoperiodically insensitive mutants from *Arabidopsis* (e.g. Hicks *et al.* 1996) may identify potential homologues of the day-neutral mutants from pea.

Although more intensive mutant screening in pea may resolve the differences in the suites of mutants isolated from *Arabidopsis* and pea, other, more fundamental problems exist. Overexpression of *CO* is able to overcome the *fca* mutant phenotype (Coupland 1997), possibly by upregulating *LFY* expression (Simon *et al.* 1996). However, overexpression of genes involved in the photoperiod response in pea (i.e. the equivalent of a *CO* homologue) would not be expected to overcome the block to flowering caused by the *gi* or *veg2* mutations (which putatively belong to the same pathway as *FCA*). This result suggests that there is a problem with the list of possible homologies given above, or that the flowering pathways of *Arabidopsis* and pea have

diverged such that the photoperiod response and the central pathways of pea may no longer intimately interact. It is these types of differences and difficulties that make the pursuit of the homologues of *Arabidopsis* flowering time genes in pea an exciting proposition.

The evolution of flowering

Any consideration of the evolution of the transition to flowering is severely hampered by the paucity of evidence of environmental responsiveness for reproduction in non-angiosperm groups. Fossil evidence and comparative anatomical data support the evolution of the flower as a modified determinate shoot. However, the physiological process of flowering is not preserved in fossils. Physiological studies of flowering have also tended to emphasise the differences in flowering response among different plants, rather than emphasising any similarities (e.g. Rees 1986, Bernier 1988). In addition, the effects of the environment on flowering are only known from a limited subgroup of flowering plants, although many of these plants do show environmental responses (Rees 1986). Despite this, those genes controlling flowering time in pea and *Arabidopsis* are presumed to be derived from a common ancestor. Whether the flowering pathways present in pea and *Arabidopsis* were also present in their common ancestor is currently unknown.

Of the genes known to control flowering time in *Arabidopsis*, only *LD*, *CO*, *TFL* and *FCA* have been cloned (Lee *et al.* 1994, Putterill *et al.* 1995, Bradley *et al.* 1997, Macknight *et al.* 1997). Current molecular evidence suggests that homologues of *CO* and *FCA* are present in a wide range of angiosperms, including both monocots and dicots (Wilson and Dean 1996, Simon and Coupland 1996). This suggests that genes now involved in the transition to flowering in *Arabidopsis* were inherited from the common ancestor of the monocot and dicot groups. However, this does not mean that these gene homologues shared homologous functions. This possibility awaits further analyses.

It seems likely that the original angiosperm was capable of controlling its flowering behaviour in response to environmental conditions. Indeed, this ability may have set apart the angiosperm (or anthophyte) lineage from other seed plants. However, the diversity of flowering responses seen in extant angiosperms suggests that this original control has been highly modified during the diversification of the angiosperms. Intuitively, as the angiosperms diversified and expanded their

geographic range their flowering behaviour had to be modified to suit the prevailing environmental conditions (Murfet 1977). How much, or little, of the genetic control of the original flowering behaviour remains, awaits the molecular characterisation of genes controlling flowering time and the identification of their homologues from diverse angiosperm groups.

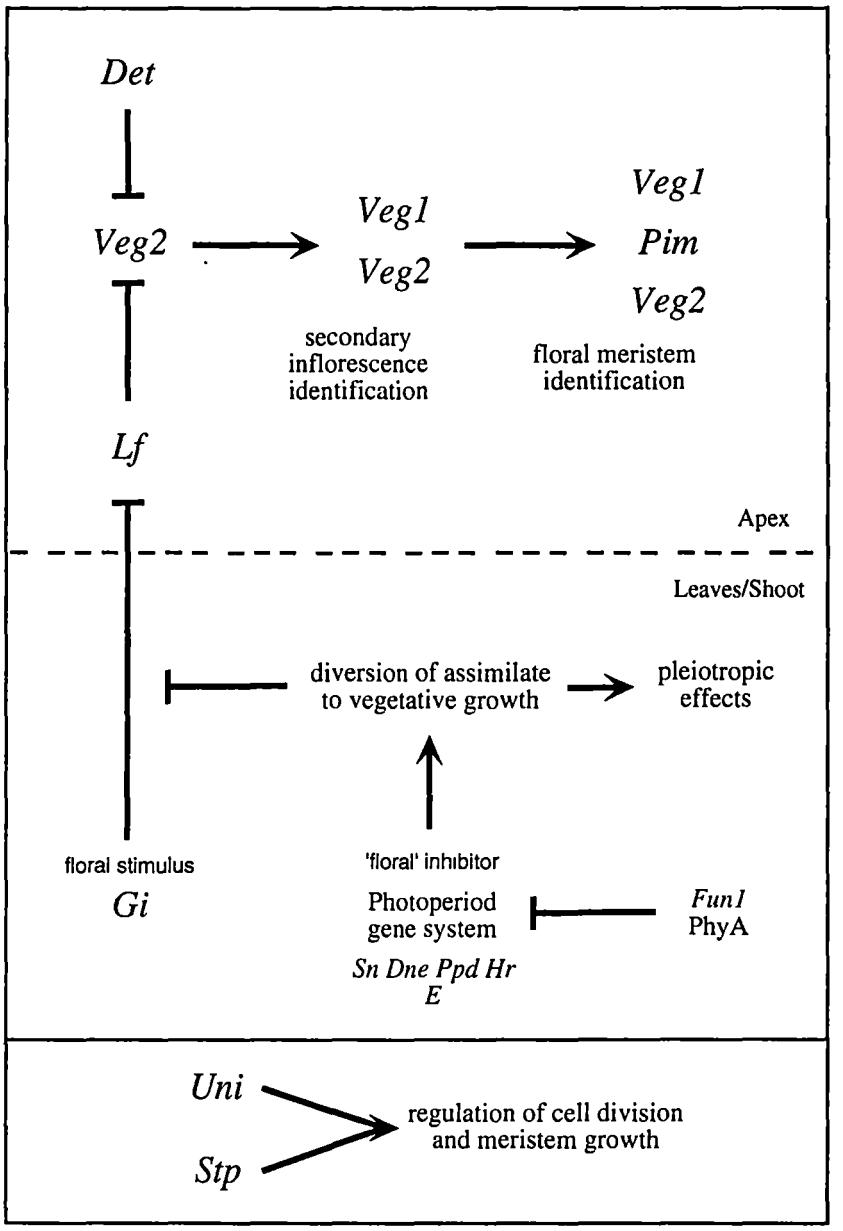


Figure IX.1: The genetic control of flower and inflorescence initiation in pea. This pathway is based on the genetic interactions between individual genes (e.g. *Gi* and *Lf*, *Lf* and *Veg2*) and on the phenotypes and proposed modes of action of individual genes (e.g. *Pim*, *Sn*, *Hr*). The pathway is split into two non-specific pathways (one controlling response to photoperiod and one influencing the development of the meristems), and a central pathway controlling the identification of the floral meristem. This diagram is based on results from **Chapters III, V, VI** and **VII**, Taylor and Murfet (1994) and Weller *et al.* (1997a). Genes directly examined in this thesis are in the larger font size. Positive regulation or influence (⟶). Negative regulation (⊣).

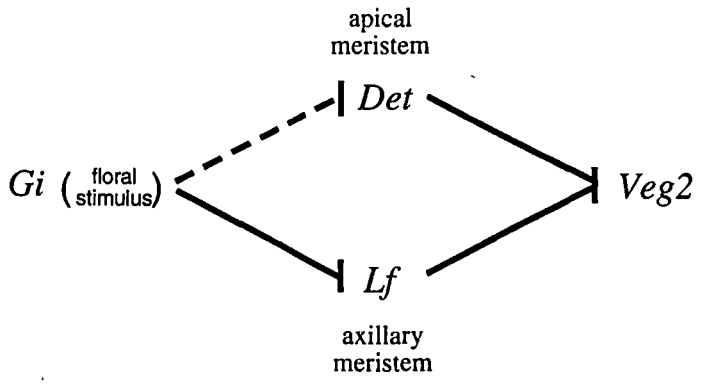


Figure IX.2: Alternative interactions between *Gi* (the floral stimulus), *Lf*, *Det* and *Veg2*. In this model, *Det* and *Lf* represent homologous activities, and the floral stimulus inhibits the activity of both *Det* and *Lf*. Mutations at *Det* and *Lf* are epistatic to loss of floral stimulus production (in the *gi-2* mutant) as the suppression of *Veg2* expression is relieved. However, the activities of *Det* and *Lf* have become specialised. Thus, *Det* is not active in the axillary meristems, whilst *Lf* is no longer active in the apex of the primary inflorescence. The dashed line between *Gi* and *Det* indicates the reduced effect the floral stimulus exerts on *Det* function.

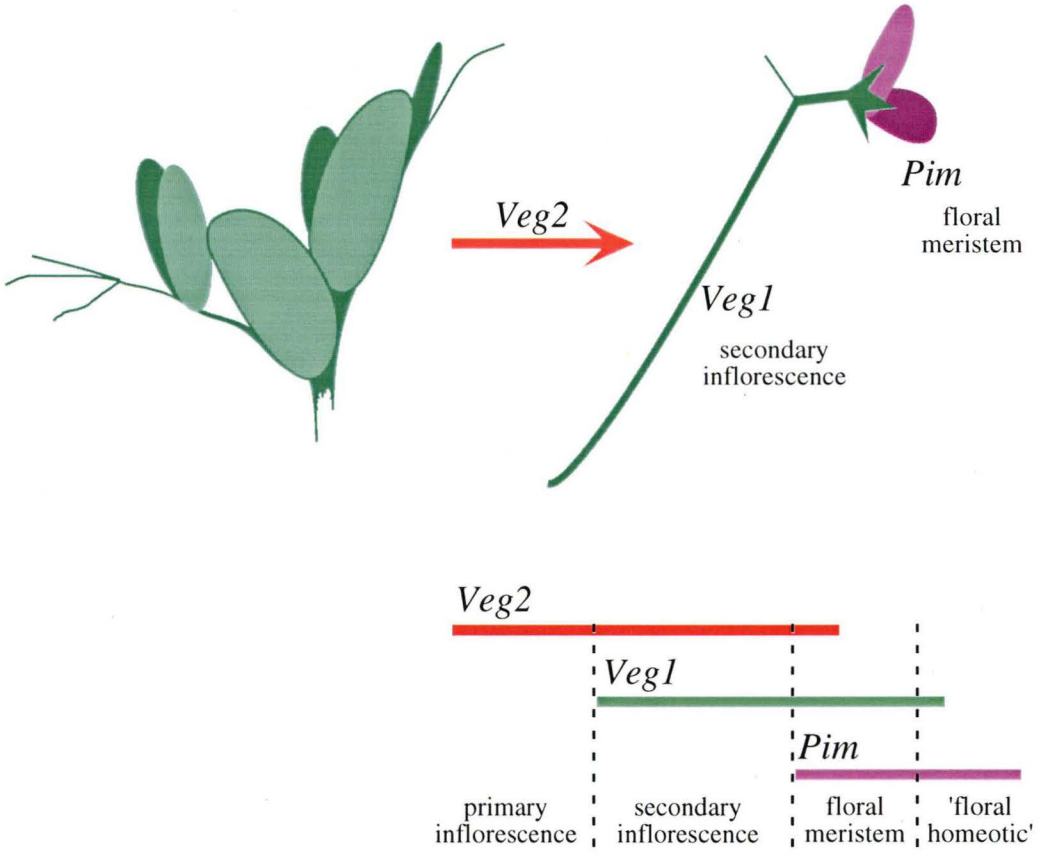


Figure IX.3: An illustration of the overlapping roles *Veg1*, *Veg2* and *Pim* play in the identification of the secondary inflorescences and flowers. In this schematic model, *Veg2* acts to differentiate between reproductive and non-reproductive growth, and therefore its mutant phenotype may result from the loss of activity of flower specific genes such as *Veg1* and *Pim*. *Veg1* acts to identify the secondary inflorescence, but also acts later during flower development. *Pim* is required for floral meristem identification, but also functions as a Class A floral homeotic gene during the identification of the sepal and petal whorls.

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