LEAF AND SEED DEVELOPMENT

IN PEA (*Pisum sativum* L.)

by

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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.

Jennifer Yaxley

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Abstract

This thesis examines the development of the compound leaf in pea (*Pisum sativum* L.), focusing on regional identities within the leaf. Novel interactions between the pea leaf mutants *insecatus(ins), cochleata(coch), unifoliata(uni), stipules reduced(st), tendril-less(tl)* and *afila(af)* are utilised to investigate the role of regional identity in the determination of leaf form. Secondly, a new viviparous pea mutant, *viviparous(vip)*, is characterised and used to investigate the processes controlling normal seed maturation and the prevention of precocious germination.

Leaf development.

Plants which are homozygous for both the *coch* and *st* mutations show that the *coch* mutation transforms the stipules into organs which act as leaf tissue. The stipules of *coch* mutants respond to other mutant genes as a leaf would, and do not respond to the mutant gene *st*, which alters only stipule tissue. Plants of the genotype *coch coch Tl tl* show reduced *Tl* expression at the base of the leaf. This provides evidence for gradients of gene expression within the pea leaf, which have been proposed in other studies. Examination of the apical meristems of *coch* and *uni* mutants has revealed that the phenotypes of these mutants are visible earlier in the development of the leaf primordia than other previously characterised pea leaf mutants. Plants homozygous for the *coch* mutation show greatly retarded development of the stipule primordia. Also, the apical domes of *uni* mutants are smaller than those of wild-type plants. A difference in apical dome size has not been previously reported for any pea leaf mutant.

A new allele of a poorly characterised mutant, *ins*, has been found and *ins* has been shown to be a dominant mutation with poor penetrance rather than a recessive mutation. Plants homozygous for the *ins* mutation have a range of tendril and notched structures at the tip of some leaflets. This has been found to be due to additional growth at the tip of the leaflet, which occurs late in development. The double mutant *ins tl* has leaflet-like structures, rather than tendril structures, present at the affected leaflet tips. This indicates that the *ins* mutation alters the identity of the leaf tip. The double mutant *ins uni* shows that the simple leaf of the *uni* mutant has a region at its tip which corresponds to the tip of the leaflet in a compound pea leaf.

Previous studies of pea leaf development have focused on the *af* and *tl* mutants, which affect the leaflets and tendrils. This thesis provides information about the development of the stipules at the leaf-base which has not been addressed in detail in previous models of pea leaf development.

Seed development.

The *vip* mutant is the first viviparous mutant to be reported in a legume species. The seeds of the *vip* mutant germinate in the pod near contact point (when the liquid endosperm disappears). They stay green and will die through desiccation if they remain in the pod. The phenotype of the seed is determined by the embryo. Seeds of the *vip* mutant will survive if they are removed from the pod and planted before they dehydrate. However, growth of seedlings homozygous for the *vip* mutation is slow for the first few weeks, and there is increased seedling death, compared with wild-type seedlings. Mature *vip* mutant plants produce one pod per inflorescence rather than two. Two *vip* alleles have been isolated (*vip-1* and *vip-2*). The mutation is inherited as a monogenic recessive, with the gene located at the bottom of linkage-group five. A paucity of *vip* seeds from crosses suggests that *vip* gametes have reduced viability, as there is no increased abortion of *vip* seeds.

Compared with wild-type seeds, seeds of *vip-1* and *vip-2* mutants show some alteration in free-abscisic acid (ABA) levels during development. However, comparison with seeds of the pea ABA-deficient mutant *wilty*, which have very low ABA levels and are not viviparous, suggests that the altered ABA levels in *vip* seeds are not responsible for their precocious germination. Free-ABA levels in the shoots of the *vip* mutants, and their ABA synthesis in response to droughting, are the same as wild-type shoots. Seeds of the *vip* mutants are somewhat insensitive to ABA during their early growth. There is no evidence of overproduction of bioactive gibberellins in *vip* seeds. Messenger RNAs for proteins found during seed maturation, which are reduced in wild-type germinating seeds, continue to accumulate in *vip* seeds even though they are germinating precociously.

Thus, *vip* mutant seeds are deficient in a single factor, which is necessary to prevent precocious germination, but does not reduce expression of maturation genes. This factor does not appear to be homologous to the *Arabidopsis* gene *ABSCISIC ACID INSENSITIVE3 (ABI3)*. The *vip* gene may relate to ABA sensitivity early in seed development, which would indicate that this is the critical time when ABA prevents precocious germination. Alternatively, the ABA insensitivity found may be a secondary effect, and *vip* seeds may be altered in a novel factor that normally prevents precocious germination. Such factors have been proposed to exist by other studies examining the control of germination.

Contents

Abbreviations

ABA	abscisic acid
ABI	*ABSCISIC ACID INSENSITIVE*
ABR	*Abscisic acid responsive*
af	*afila*
apu	*apulvinic*
C	control
cDNA	complementary (copy) DNA
ce	*cerise*
cf	compared with
CNRS	Centre National de la Recherche Scientifique
coch	*cochleata*
cri	*crispa*
CSIRO	Commonwealth Scientific and Industrial Research Organisation
cv.	cultivar
D	droughted
DAO	days after flower opening
dCTP	deoxycytosine triphosphate
df	degrees of freedom
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMS	ethylmethanesulfonate
ESEM	environmental scanning electron microscopy
F	filial generation
FLO	*FLORICAULA*
FUS	*FUSCA*
FW	fresh weight
GA	gibberellin
GA_x	gibberellin x
GC	gas chromatography
gp	*green pods*
HL	Hobart line
HL107	cultivar Torsdag
(H)L303	*vip-1*
(H)L353	*vip-2*
IF	inflorescence
Ins	*Insecatus*
JI	John Innes Institute line
kb	kilobase
KN	*KNOTTED*
l	lateral primordia of *coch* stipule
L	leaflet
LD	long days (photoperiod 18 hours)

LEC	*LEAFY COTYLEDON*
lh	*length h*
LeT6	*Lycopersicon esculentum T6*
LFY	*LEAFY*
lo	lobe
m	mass
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
NS	not significant
p	probability
P	plastochron
prob	probability
PSKN	*Pisum sativum KNOTTED*
QTL	quantitative trait loci
RCV	recombination value
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	stipule
SD	short days (photoperiod 8 hours)
SDS	sodium dodecyl sulfate
sil	*sinuate leaf*
SIM	selected ion monitoring
SSC	sodium chloride/sodium citrate
st	*stipules reduced*
tKN	*tomato KNOTTED*
tl	*tendril-less*
tl^{pet}	*tendril-less petiolules*
uni	*unifoliata*
uni^{tac}	*unifoliata tendrilled-acacia*
vip	*viviparous* (pea)
vp	*viviparous* (maize)
V	pea linkage group five
v/v	volume for volume
wb	*vix-cerata*
w/v	weight for volume
wil	*wilty*
wlo	*wachlos*
WT	wild-type
z	atomic number

General Introduction

Pea has been a favoured model species for experimental biology for over a hundred years. During this time a great deal of genetic and biochemical knowledge has accrued, especially in the areas of leaf development (Makasheva, 1973; Lyndon, 1977; Hedley and Wang, 1987; Murfet and Reid, 1993) and seed development (Wang and Sponsel 1985; Casey et al., 1993; Wang and Hedley, 1993; Chandler et al., 1994; Vitale and Bollini, 1995; Liu et al., 1996). The study of development in pea has been aided by the large number of mutants available. The use of mutants is a powerful tool in the dissection of complex biological and developmental processes.

Recently, the development of compound leaves has been the focus of increased research interest (Kerstetter and Hake, 1997; Poethig, 1997; Tsiantis and Langdale, 1998; Goliber et al., 1999; Sinha, 1999). This has excited discussion about the fundamental nature of the leaf (Lacroix and Sattler, 1994; Jackson, 1996; Sinha, 1997; Tsukaya, 1998), and about the evolution of leaf form (Baum, 1998). There is a range of well characterised leaf development mutants in pea (Marx, 1987; Murfet and Reid, 1993; Goliber et al., 1999), which makes this an excellent model species in which to study compound leaf development. There is also a wide range of pea seed development mutants (Wang and Sponsel, 1985; Hedley and Wang, 1987; Casey et al., 1993; Liu et al., 1996). However, until now there have been no pea mutants affecting dormancy and control of precocious germination, which is a poorly understood fundamental aspect of plant growth and development (Bewley, 1997; Li and Foley, 1997; Holdsworth et al., 1999).

This thesis will utilise pea mutants altered in leaf and seed development to investigate compound leaf development and the control of precocious seed germination.

CHAPTER ONE

Introduction: Leaf Development in *Pisum*

The wild-type pea leaf

A normal, wild-type pea leaf is compound pinnate, with three distinct parts (Fig. 1.1a). (Makasheva, 1973; Marx, 1987). At the leaf base there is a pair of sessile stipules that partially enclose the proximal side of the stem. The stipules are elliptical in shape and may be slightly pointed at the tips; their sides typically overlap, and their bases are typically somewhat crenulated. Oval leaflets are borne on the rachis in the middle of a normal leaf. They have very short petioles and are found in pairs opposite each other, although occasionally a leaflet may be paired opposite a tendril. The distal portion of the leaf consists of pairs of simple, unbranched, cylindrical tendrils placed opposite each other on the rachis, and a final terminal unbranched tendril. Lateral structures are initiated acropetally on the leaf primordium (Lyndon, 1983; Lyndon, 1977; Meicenheimer et al., 1983)

Leaves are placed alternately on the main stem and display heteroblasty (Wiltshire et al., 1994). Conventionally, the cotyledons are considered to be inserted at node zero, and these are normally found beneath the ground. At nodes one and two there are small scale leaves, with the first true compound leaf at node three. The first compound leaves have only two leaflets with a single short terminal tendril, although sometimes the tendril is absent. At subsequent nodes the number of pairs of leaflets and tendrils increases, up to a maximum of three pairs of leaflets and four pairs of tendrils in some lines. The maximum leaf complexity is reached just prior to the first flowering node.

Terminology

The genetic nomenclature for describing pea leaf mutants currently varies somewhat from the *Arabidopsis* nomenclature. Pea mutants usually have a three letter symbol, and dominant genes start with a capital letter (eg. *Ins*). Recessive genes are symbolised with all lower-case letters (eg. *vip*). The wild-type gene has no special designation, and must be detailed at each particular locus (rather than being designated with all-capital letters as it is in *Arabidopsis*). This pea nomenclature is currently under review, but this thesis will make use of the currently accepted pea genetic nomenclature as described in the journal *Pisum Genetics* (Murfet, 1997).

Unless stated otherwise, the italicised recessive genotypes represent the homozygous condition.

Throughout, leaves at the shoot apex are numbered using the plastochron index (Erickson and Michelini, 1957; Lamoreaux et al., 1978) to describe their state of development. The plastochron 1 (P_1) leaf is the youngest leaf that has been initiated on the flank of the apex, the plastochron 2 leaf (P_2) is the next oldest primordium, and so on. Plastochron zero (P_0) marks the site where the next leaf will be initiated on the flanks of the apical dome.

In developmental biology, mutants that have homeotic or heterochronic features are considered to be of special significance. Homeotic mutants have organs or tissues that appear in an incorrect place. They are considered significant because it is thought that they represent 'master-switch' genes which turn on the genetic pathway leading to the development of that particular organ or tissue where they are expressed. Heterochronic mutants have features that are expressed at the incorrect time. For example, they might maintain juvenile features throughout adulthood, or show precocious onset of adult features. Both heterochronic and homeotic mutants are of evolutionary significance because they illustrate how new features may have been incorporated into an organism's developmental programme. However, in the case of mutants that have not yet been fully characterised, such as the pea leaf mutants, it is not often possible to say whether a mutation is homeotic or heterochronic. For example, it is possible that a mutation which causes an organ to appear in the wrong place may be heterochronic. This is because the change has been caused by an alteration in the timing, rather than the position of gene expression. In this thesis I will refer to mutants in which organs appear in the incorrect place as 'homeotic'. However, this is not meant in the strictest sense as some of the phenotypic aspects of these mutants could also have heterochronic features.

Pea leaf development mutants

There are several well-known mutants affecting the development of the pea leaf (Marx, 1987 #22; Hedley, 1987 #4; Murfet, 1993 #28; Hofer, 1998 #429). The *afila (af)* mutant (Fig. 1.1c) (Goldenberg, 1965; Kujala, 1953) has no leaflets. It consists of branched tendrils in the proximal portion of the leaf and unbranched tendrils in the distal portion of the leaf. The *tendril-less (tl)* mutant (Fig. 1.1b) (Vilmorin and Bateson, 1912) has normal proximal leaflets, but also has leaflets replacing the simple tendrils in the distal portion of the leaf. Both these phenotypes are caused by single gene recessive mutations, and are considered homeotic in nature because certain organs (tendrils or leaflets) appear in places in which they do not normally occur (Marx, 1987). These mutations affect only the form of the leaflets and tendrils. The stipules and flowers are wild-type in appearance. The mutant *stipules reduced (st)* (Fig. 1.1g)(Pellew and Sverdrup, 1923) reduces the stipules to simple triangular or strap-like appendages. It occurs in a single recessive gene and affects only the stipules.

The recessive *unifoliata (uni)* mutant (Fig. 1.1e)(Eriksson, 1929; Lamprecht, 1933) has leaflets and tendrils replaced by a simple laminar leaflet, or in some cases with a trifoliate leaf. The stipules are normal, but the infertile flowers consist of many carpeloid and sepaloid structures. The less severe allele *uni^{tac}* (Fig. 1.1f) has normal proximal leaflets and a terminal leaflet subtended by two tendrils. Flowers of the *uni^{tac}* mutant are also less severely affected; they have some petal and stamen tissue. The altered gene in these mutants has been cloned (Hofer et al., 1997) and is a homologue of the floral meristem identity genes *LEAFY (LFY)* in *Arabidopsis* and *FLORICAULA (FLO)* in *Antirrhinum*. The floral phenotype shown by the *uni* mutant resembles *LFY* (Hofer et al., 1997), but the simple leaves of the *leafy* and *floricaula* mutants have no mutant phenotype. It seems possible that in pea the function of this floral gene has been put to use in the development of the compound leaf.

The *cochleata (coch)* mutation (Wellensiek, 1959) also alters both leaves and flowers. In the leaves only the stipules are altered. In the lower and upper nodes of the plants they are reduced to simple forms. Mostly commonly, they appear strap-like, triangular, spatulate or filamentous. However, in the middle nodes of the *coch* plant the stipules undergo a homeotic change and are replaced by structures that look and behave as compound leaves without stipules (Fig. 1.1h). The flowers are also variable and are only partially fertile. They contain multiple organs, and show abnormal organ fusing (Gottschalk, 1973; Molhova et al., 1988).

In a 1959 paper, Lamprecht described a mutant affecting the leaflet tips, which he named *insecatus (ins)* (Lamprecht, 1959). In this mutant some of the leaflets, mostly the first pair of leaflets in a leaf, had an incised notch in their tips. A tendril-like structure extended into the incision and out beyond the edge of the leaflet (Fig. 1.1j). Somehow the developmental potential of the leaflet tip is changed allowing a tendril to be produced there.

Interactions between pea leaf development mutants

Interactions between some of the pea leaf developmental mutants result in novel leaf phenotypes. The best known is the double mutant *afila tendril-less (af tl)* in which the proximal and distal portions of the leaf contain branching compound tendrils that each terminate in a miniature leaflet (Fig. 1.1d). Thus, the double mutant combines features of each of the single-mutant phenotypes. The *uni* mutant has the general effect of reducing the complexity of leaf form when it is combined with other mutants (Hofer and Ellis, 1996). For example, the double mutant *uni tl* has only a small number of relatively large leaflets, compared with the equivalent leaflets of the *tl* mutant alone.

The mutant *sinuate leaf (sil)*(Marx, 1977) has undulating leaflet margins. Alone its phenotype is not homeotic, but the double mutant *af sil* has deeply incised stipule tips from which tendrils protrude (Fig. 1.1i). In the triple mutant *af tl sil,* the tendrils that protrude from the incised leaflet tips terminate in small leaflets. Thus,

when *sil* is combined with other leaf mutants, homeotic effects are produced. In this way some combinations of leaf mutants provide more information about gene action in pea leaf development than the single mutants alone. This type of information is invaluable for building a model of the genetic control of leaf development in pea.

Models of pea leaf development

Some models of pea leaf development have been proposed which attempt to integrate knowledge from several mutants into a common developmental pathway or plan, but these are still incomplete.

Sachs (1969) performed a classic experiment where he examined regeneration of pea leaf primordia of different sizes, which he produced by cutting the leaf primordium. He found that lateral primordia, which would normally have developed into leaflets, often formed tendrils after the primordia were cut. He then suggested that the difference between leaflet and tendril primordia was a change in primordium size at a critical age.

This idea was taken up by Young (1983) who formulated a simple algebraic model where the developmental fate of each meristem was determined by its size at a critical stage. Small meristems produced tendrils, intermediate meristems produced leaflets and large meristems produced rachises. The position of the meristem, its previous developmental history, and its interaction with other meristems, were not important. The genes *af* and *tl* were proposed to alter these size thresholds. Moreover, when these two changes in size threshold were combined, the model mimicked the leaf form of the double mutant *af tl*, suggesting that this theory had a real basis. Gould *et al.* (1992) later developed L-system notation to describe the branching patterns of mature leaves of wild-type, *af*, *tl* and *af tl* plants. While they accurately summarised branching behaviour, this notation was descriptive only and could not give any insight into mechanisms. However, the outcomes they outlined were consistent with Young's model (Young, 1983).

The critical factor of meristem 'size' was vague and Young (1983) suggested that it could refer to meristem volume, mass, cell number or concentration of a chemical. Work by Meicenheimer *et al.* (1983) in the same year showed that the meristems of *af*, *tl* and *af tl* mutants were the same as the wild-type until the differentiation of lateral primordia (with some small differences in the timing of primordium initiation). This was confirmed by Gould *et al.* (1986) who also showed specifically that the shoot apical domes of wild-type, *af* and *tl* plants were the same size and shape. A comparative study of wild-type and *af* leaf development by Cote *et al.* (1992) also showed that the early development of the meristem was the same in the two lines, which began to diverge during differentiation of the leaflet and tendril primordia. So it appears that Young's 'size' factor needs to be something else apart from the gross physical character of the early shoot apex.

These studies of pea leaf development, and a further study of growth of different age primordia in culture (Gould et al., 1994), also showed that primordia were not determined at their inception. Differentiation of the pea leaves occurs over four plastochrons, before the primordia are 80μm in length (Sachs, 1969), and development of the primordia proceeds acropetally (Cote et al., 1992; Gould et al., 1986; Meicenheimer et al., 1983; Sachs, 1969). Using observations of meristem growth, Meicenheimer (1983) described the pea leaf mutants in terms of the activity of "marginal meristems" producing flattened laminar structures in various parts of the leaf. The mutant *af* was seen to have no marginal meristems in the proximal domain and so developed with tendrils only. The mutant *tl* had additional marginal meristems in its terminal compartment, converting tendrils to leaflets, while *st* had marginal meristems with a shortened time of action, resulting in reduced stipule size. The activity of these marginal meristems in different areas of the leaf was suggested to be under independent genetic control. The concept of marginal meristems has been partially superseded by clonal analysis experiments showing that leaf lamina growth occurs over most of the leaf surface, for most of the period of development (Dolan and Poething, 1998). Marginal meristems may exist at certain phases, and their importance may vary with species.

In a major review of pea leaf development mutants Marx (1987) pointed out that various mutants appear to operate in different "domains" within the leaf, and that the action of genes might help act to define these domains (Fig. 1.1a). The mutants *coch* and *st* act on the stipules in the basal domain, *af* acts on the leaflets in the middle or proximal domain and *tl* acts on the tendrils in the distal domain. Support for this theory comes from the fact that mutants that operate in different domains tend to act independently and their double mutants are additive; for example, *st* and *tl,* or *coch* and *af.* However, in some cases the double mutant combinations were interactive, such as the *af tl* mutant, which indicates that the genes controlling pattern formation are not entirely independent. In the cases of the *af tl* and *uni* mutants the middle and distal compartments appear to merge into one larger compartment. The phenotype of other mutants, for example uni^{tac} suggested that some compartments, in this case the terminal compartment, could be divided into sub-domains. In thinking in terms of domains Marx was aligning plant development with the compartmentalisation seen in the homeotic mutants of *Drosophila* and other animals (Gehring and Hiromi, 1986; Wolpert, 1996). It is not yet known whether a similar system operates universally in plants. However, such a system does operate in the development of floral organs as described by the ABC model (Coen and Meyerowitz, 1991).

Morphological measurements of leaves of wild-type, *af, tl* and *af tl* mutants revealed that *Af* and *Tl* genes were expressed in the context of pre-existing proximal and distal regions in developing pea leaves (Lu et al., 1996). The assumption was made that the ground-state for determining the effects of *Af* and *Tl* genes is the loss-of-function *af tl* double mutant (in keeping with the ABC model of floral development). Lu *et.al.* (1996) showed that *af tl* double mutant leaves already had distinct proximal and distal regions. So it appears that *Af* and *Tl* genes do not in

themselves specify these regions, as suggested by Marx (1987). However, the Marx proposal that pinna morphology is related to region is supported, whereas Young's assumption (Young, 1983) that position was not important in pinna determination is contradicted. Lu *et al.* (1996) found a strong relationship between pinna identity and relative pinna position, with proximal positions bearing large, complex pinnae and distal positions bearing smaller, simpler pinnae. Pinnae in intermediate regions developed into mixed pairs of complex-simple pinnae. Pinna position appears to be tightly controlled, but not by the genes *Af* and *Tl*.

Lu *et al.* (1996) proposed a heterochronic model for the action of *Af* and *Tl*. They suggested that *af tl* double mutant pinnae first underwent branch formation and then, at the end of development, changed to lamina formation to produce the very small leaflets that appear on the distal end of tendrils in this genotype. The proposed action of *Af* from this *af tl* ground state was to induce precocious lamina formation. The action of *Tl* was to halt leaf development before the final lamina expansion stage and to cause premature termination of pinnae branching. This action in timing lamina formation and axis termination explains wild-type, *af*, *tl* and *af tl* mutant phenotypes. It also gives an explanation for the flattened tendrils/narrow leaflets seen in *Tl tl* heterozygotes, an issue that was not addressed by previous authors. In addition, (Lu et al., 1996) also suggested that a gradient model for specification of organ identity, such as occurs in animals, might be more consistent with the existence of mixed pinna pairs and a relative positional boundary in the pea leaf. It was postulated that the action of *Tl* produced a tendril-inducing or branch-inhibiting morphogen at the leaf tip, whereas *Af* was suggested to produce a lamina-inducing morphogen at the leaf base (Lu et al., 1996).

In a series of papers Villani and DeMason (1997, 1999a, 1999b, 2000) described detailed morphological measurements and histology of *af*, *tl* and *af tl* leaves and their primordia. Their work supports the Lu *et al.* hypothesis (1996) that differences in leaf form are due to differences in timing of branching and lamina formation. Lamina initiation occurs 1.5 to 2.0 plastochrons later in *af tl* primordia than in wild-type primordia (Villani and DeMason, 1997). In *af*, pinna development in the proximal region is delayed compared with the wild-type (Villani and Demason, 1999), which supports the proposal that *Af* causes precocious lamina formation. In addition, the broad-tendril/narrow-leaflet heterozygous pinnae of *Af Af Tl tl* plants are more leaf-like than those of *Af af Tl tl* plants, demonstrating the role of each *Af* gene in lamina formation (Villani and DeMason, 1999). The pinnae of *af tl* plants are more complex that those of *af* plants in all positions and tendril differentiation in *af* occurs before higher order branching is complete in *af tl* (Villani and Demason, 1999). This supports the Lu *et al.* proposal (1996) that *Tl* causes premature termination of branching. Also supporting this is the fact that *af af Tl tl* plants have a branching complexity intermediate between those of *af* and *af tl* (Villani and DeMason, 1999). These *af af Tl tl* plants also have their distal tendril tips slightly expanded (Villani and DeMason, 1999), which seems to reflect the reduced ability of a single *Tl* gene to suppress lamina formation. The ability of *Tl* to suppress lamina formation is also

suggested by the fact that distal leaflets of *Af af tl tl* are initiated earlier than the broad-tendril/narrow-leaflet of *Af af Tl tl* (Villani and DeMason, 2000).

The actions of the *Af* gene are largely, but not exclusively, seen in the proximal part of the leaf (Villani and Demason, 1999a+b), whereas the actions of the *Tl* gene are largely seen in the distal part of the leaf (Villani and DeMason, 1999b). Yet these papers by Villani and DeMason (1997, 1999a, 1999b, 2000) also show that the genes *af* and *tl* have complex pleiotropic effects which alter leaf development in all domains at multiple levels of organisation, and also alter shoot development and flower development (Villani and DeMason, 2000). Mutant *af* and *af tl* plants have their vegetative development accelerated (have more complex leaves at earlier nodes) and their flowering delayed, relative to the wild-type and *tl* (Villani and Demason, 1997, 1999a, 1999b, 2000). They showed that the genes *Af* and *Tl* help to regulate elongation, and hence position along the leaf axis (Villani and DeMason, 1997). They also showed that the pinnae of *af* and *af tl* leaves in older leaves are initiated bidirectionally, rather than purely acropetally. The pinnae second from the leaf base are initiated first, followed by the most proximal pinnae and then the pinnae third from the leaf base are initiated (Villani and Demason, 1997; Villani and DeMason, 1999a). The *Af* gene seems to promote development of pinna one. In addition, lamina initiation in *af tl* primordia proceeds basipetally from the terminal to the distal areas of the leaf as a whole and within each compound pinna itself (Villani and DeMason, 1997). In *af,* tendril differentiation starts at the terminal pinna and pinna two (the first pinna to develop) at the same time (Villani and Demason, 1999a; Villani and DeMason, 2000). This is in contrast to wild-type plants in which lamina initiation is acropetal. So, *Af* has a role in helping to specify acropetal leaf development. Secondary branching of *af* and *af tl* pinnae still occurs acropetally (Villani and Demason, 1999b). Villani and DeMason (1999b) suggest that the pleiotropic effect of *Af* and *Tl* indicate that they are transcription factors or signalling molecules with multiple target genes.

The small leaflets at the end of *af tl* compound tendrils were shown to be histologically similar to leaves. They had the same types and numbers of cell layers and cell shapes (but with smaller and fewer cells) (Villani and DeMason, 1997). The broad-tendril/narrow-leaflets of *Tl tl* heterozygotes were shown to be similar to leaflets, rather than tendrils. They have the same cell layers as leaves, but with less intercellular space and the same cell types (palisade and spongy mesophyll), although these are less well defined (Villani and DeMason, 1999). It seems that once lamina development has been initiated, then leaf-like histology commences. The heterozygous *Tl tl* leaves seem to be arrested at a late stage of blade expansion; whereas the small *af tl* leaflets are more typical of fully developed leaflets, with the same degree of intracellular space as wild-type leaflets and distinct palisade and spongy mesophyll layers (Villani and DeMason, 1999). Villani *et al.* (2000) also noted that the initial development seen in all pinnae was the development of an adaxial groove. This groove is then accentuated during leaflet development, but lost during tendril development. They propose from this evidence that the ancestral form of pinnae in pea is the leaflet, with the tendril as a secondarily derived structure.

The most comprehensive model of pea leaf development to date is based on the interactions of *Tl, Af* and *Coch* with *Uni* (Hofer and Ellis, 1998). None of these genes is required for lamina formation, as the triple mutant *uni af tl* is trifoliate. By comparing the phenotype of all double and triple mutant combinations of these genes Hofer and Ellis (1998) developed a model that is based on expression of *Uni* in the centre of the leaf primordium where it acts to maintain competency for the production of lateral structures. Hagemann and Gleissberg (1996) have coined the term marginal blastozone for such a leaf region, to avoid confusion with terms such as indeterminacy, which refer to shoot characteristics. The action of the genes *Coch, Af* and *Tl* is then to suppress *Uni* in the lateral portions of the leaf primordium and allow determinate lateral structures to form (Fig. 1.2). This model is largely supported by further work (Gourlay et al., 2000) which shows the expression of the *Uni* gene in the developing mutant and wild-type leaves. *Uni* is expressed in the leaf marginal blastozone when lateral structures are being produced, and is down-regulated on determination. Increased *Uni* expression leads to increased blastozone activity in the leaf (Gourlay et al., 2000).

Uni expression is required during P_2 to maintain the developing wild-type marginal blastozone, as the *uni* mutant has reduced organogenic potential and the whole *uni* mutant primordium begins to differentiate at P_2 (Gourlay et al., 2000). In the basal portion of the meristem *Coch* suppresses *Uni* in the lateral portions to allow determinate stipules to be produced. In the *coch* mutant, expression of *Uni* in some stipule primordia allows them to become compound structures. The *coch uni* mutant has simple stipules (Gourlay et al., 2000). In the proximal portion of the leaf *Af* suppresses *Uni* in the lateral part of the primordia after P_2, to allow determinate leaflets to be produced. In the *af* mutant, *Uni* expression in these first lateral structures during P_3 allows the formation of secondary blastozones and produces compound branching rachides. Compound structures are not seen at the proximal position of *uni af* or *uni af tl* mutants (Gourlay et al., 2000). *Af* also suppresses primary marginal blastozone activity during P_4. The wild-type compound leaf ceases growing and the distal portion differentiates during P_4, whereas in the *af* mutant growth and lateral organ initiation at the marginal blastozone continues until P_5. In the distal compartment *Tl* acts during P_5 primordium development to suppress both *Uni* and *Af.* The suppression of *Uni* produces a determinate leaf with no further lateral organ initiation, and the suppression of *Af* allows tendrils rather than leaflets to develop. In the *tl* mutant *Af* expression can occur in the distal portion of the leaf, leading to the formation of leaflets there. In the double mutant *af tl*, development of the marginal blastozone continues on until P_6 and P_7 and tertiary blastozones are formed, allowing the *af tl* leaf to have more complex branching than the *af* mutant. Thus, *Tl* also suppresses tertiary blastozone formation in *af* leaves during and after P_5. The addition of *Uni* to the *Af* and *Tl* model of Marx (1987) provides a better explanation of the branching complexity seen in the *af* and *af tl* mutants. Definitive proof for this model awaits the isolation of *Af, Tl* and *Coch* genes.

The Hofer and Ellis (1998) model also proposes that the distal portion of the pea leaf be further subdivided to form two compartments (Fig. 1.3). The phenotype

of the mutant *uni*^{tac} suggested the same idea to Marx (1987). The final fourth compartment would consist of the most distal three tendrils, whereas other, more proximal, paired lateral tendrils would exist in the third compartment. Hofer and Ellis (1998) cite evidence of the mutant *tl*^{pet}, which has narrow leaflets on long petiolules in region three and ovate leaflets in region four, as evidence that these two regions are genetically different. They also propose that the distal leaflet tip differentiates before the penultimate lateral pair of structures, but Villani and DeMason (1999) dispute this. This four domain model of leaf patterning proposed by Hofer and Ellis (1998) also differs from the work of Lu *et al.* (1996) in that proximal, middle and distal domains are set up by gene interactions rather than being pre-existing in the leaf primordium.

In addition to *Uni* there are other, as yet unidentified genes, responsible for the determination of lateral structures in pea leaves. Leaves of the *uni* mutant can be trifoliate and can have a short rachis, so another unknown gene, labelled 'X' by Hofer and Ellis (1998), is also responsible for maintaining some marginal blastozone activity in the pea leaf primordium. There is also the contradictory evidence that *Uni* expression is found in the differentiated small leaflets of the *af tl* double mutant. So, *Uni* expression of itself is not sufficient to ensure marginal blastozone activity, and there must be additional genes that control the differentiation and determination of these small leaflets.

The determination of mutant *uni* leaf primordia is described by Hofer and Ellis (1998) as basipetal, so they suggest that *Uni* has a role in the acropetal development of the wild-type pea leaf. As previously described, Villani and DeMason (1997) suggest that *Afila* is linked to acropetal development in leaf primordia because the *af* and *af tl* mutants initiate their lateral organs bidirectionally. Both genes would appear to be involved, with *Uni* being related to the developmental potential of the leaf and the maintenance of growth at the distal tip of the primordia, and *Af* being related to the determination of the first lateral structures from the primordia.

The action of *Uni* in flowers and leaves appears to have opposite effects on the determinacy of these structures. In flowers the *Uni* gene and its homologues *FLORICAULA* and *LEAFY* (*Antirrhinum* and *Arabidopsis* respectively) act in part to help maintain the determinacy of flowers; the *uni* mutant has a mass of re-iterative sepaloid and carpeloid tissues forming its flowers. However, in pea compound leaves, the *Uni* gene acts to maintain a marginal blastozone ('indeterminacy') and the *uni* mutant has simplified leaves with reduced developmental potential. The homologue of *Uni* in tomato, *falsiflora*, also has a somewhat simplified mutant leaf, with fewer small leaflets than wild-type leaves (Molinero-Rosales et al., 1999). Other *FLO/LFY* homologues from plants with simple leaves do not have a mutant leaf phenotype, although some of these genes are expressed in leaf primordia (Hofer et al., 1997),. It seems that some floral meristem genes may have been recruited to help control compound leaf development and interact with unique components within the compound leaf to have their effect. Alternatively, *Uni* may be a primitive

gene that functioned in the photosynthetic organs of early plants, from which compound leaves and flowers have been derived.

Compound leaf development

The homology of compound leaves is currently disputed. The partial-shoot theory postulates that pinnate leaves can be interpreted as partially indeterminate lateral shoots (Arber, 1950; Lacroix and Sattler, 1994; Rustihauser, 1995; Sattler and Rustihauser, 1992), intermediate between an indeterminate shoot and a determinate lateral structure. The shoot-like nature of compound leaves is supported by microsurgical experiments on fern leaves (Poethig, 1997; Steeves and Sussex, 1989). In several fern species isolated leaf primordia develop as shoots rather than leaves when grown in culture. There are also compound-leaved species in which leaves are initiated acropetally and inserted transversely onto the rachis, which is similar to leaf initiation at a shoot apex (Fisher and Rustihauser, 1990; Lacroix and Sattler, 1994; Sattler and Rustihauser, 1992; Steingraber and Fisher, 1986). Alternatively there is the majority view that simple and compound leaves are homologous and that a shoot consists of stems and leaves which are fundamentally different (Eames, 1961; Hagemann and Gleissberg, 1996).

Both tomato and pea have unipinnate leaves, but in contrast to peas, tomatoes have unipinnate leaves that are lobed and are developed basipetally (Sinha, 1999). Persuasive evidence for compound leaves as partially indeterminate shoot-like structures was found when it was discovered that in tomato the homeobox genes *tKN1* and *LeT6* are expressed in leaf primordia (Chen et al., 1997; Hareven et al., 1996; Janssen et al., 1998). The genes *tKN1* and *LeT6* are class 1 'knox' (*kn*-like homeobox) genes, which play a role in meristem maintenance and the maintenance of indeterminacy (Hake, 1992; Laufs et al., 1998; Long et al., 1996; Smith et al., 1992; Smith and Hake, 1994). The homologues of *tKN1* in plants with simple leaves; *KNOTTED1* in maize and *KNAT1* in *Arabidopsis*, are expressed in the shoot apical and axillary meristems and are specifically not expressed in the sites of incipient leaf primordia (Jackson et al., 1994; Lincoln et al., 1994). When tomato leaves were transformed with constitutively expressed *KN1* they formed supercompound highly ramified leaves (Hareven et al., 1996). Proliferated leaves with additional pinnae are also seen in the *Mouse ears* tomato mutant which over-expresses *LeT6* (Chen et al., 1997). However, *KN1* expression alone is not sufficient to induce compound structure in tomato leaves, since the tomato *Lanceolate* mutant, which has simple leaves, did not produce compound leaves when *KN1* was constitutively expressed within its leaves (Hareven et al., 1996).

It is not yet known if any class 1 knox genes are expressed in pea leaf primordia. The expression of the one known pea class 1 *KNOX* homologue, *PSKN1*, is similar to that seen in maize and other simple-leaved plants. Its expression is confined to the shoot apex and developing vasculature in the main stem and is down-regulated in leaf primordia and never seen in leaf primordia or older leaves (Gourlay

et al., 2000). In compound-leaved Brassicaceae, leaf initiation sites show a down-regulation of class 1 *KNOX* gene expression, but this is turned back on in leaves at later plastochrons prior to leaflet initiation (Sinha, 1999). Thus, it seems that 'compoundness' may be regulated differently in different compound-leaved species.

An alternative morphogenic program produces compound leaves in palms and *Monstera* (Aracae). In palms both palmate and pinnate leaves firstly produce a series of pleated folds in the leaf primordium by differential growth. The corrugations increase in depth by growth of the intercostal section between the ridges. These folds within the lateral blade surface are then physically cleaved by cell death. The delimited leaflets become separated due to rachis expansion. The marginal regions commonly remain unpleated and are shed as the leaf expands (Kaplan *et al.*, 1982). *Monstera* produces compound leaves via a similar mechanism of localized regions of tissue necrosis in the young leaf primordium (Kaplan, 1984).

It is generally assumed that the ancestral leaf form of angiosperms was simple, and that compound leaves have developed independently in several widely scattered families, some of which may represent a reversion to ancestral cycad-like compound structures (Goliber et al., 1999; Lacroix and Sattler, 1994; Sinha, 1999). Given their different origins it is not surprising that compound leaf form may be regulated differently in different species. It is unclear whether the morphogenic mechanisms seen in pea and tomato are equivalent. There may have been a global alteration in the regulation of *KNOX* genes in tomato (Janssen et al., 1998; Sinha, 1999). In addition, the tomato *LFY/FLO* homologue, while it is expressed in leaf primordia, is not expressed in the central domain of the leaf meristem as *Uni* is in pea (Sinha, 1999). The tomato compound leaf may share characteristics of both shoot and leaf identities. In addition to the evidence of *KN1* expression in tomato leaf primordia, tomato leaves have other stem-like features. When apical and axillary meristems are pinched off, tomato leaflet pseudoaxils will produce axillary shoots (Goliber et al., 1999). The pea compound leaf has been suggested to be a flower-like determinate shoot with the expression of *Uni* in leaves showing a commonality between the compound leaf and inflorescence (Hofer et al., 1997).

Both simple and compound leaves can occur in different species of the same genus, or even within the one shoot (Merrill, 1986). This means there must be a relatively small number of genes that can control the difference between a simple and a compound leaf structure (Goliber et al., 1999; Sinha, 1999). However, no treatments have yet been found which will cause the formation of a compound leaf from a simple leaf, so it is likely that a 'master regulator' of leaf dissection remains undiscovered.

Aims and significance

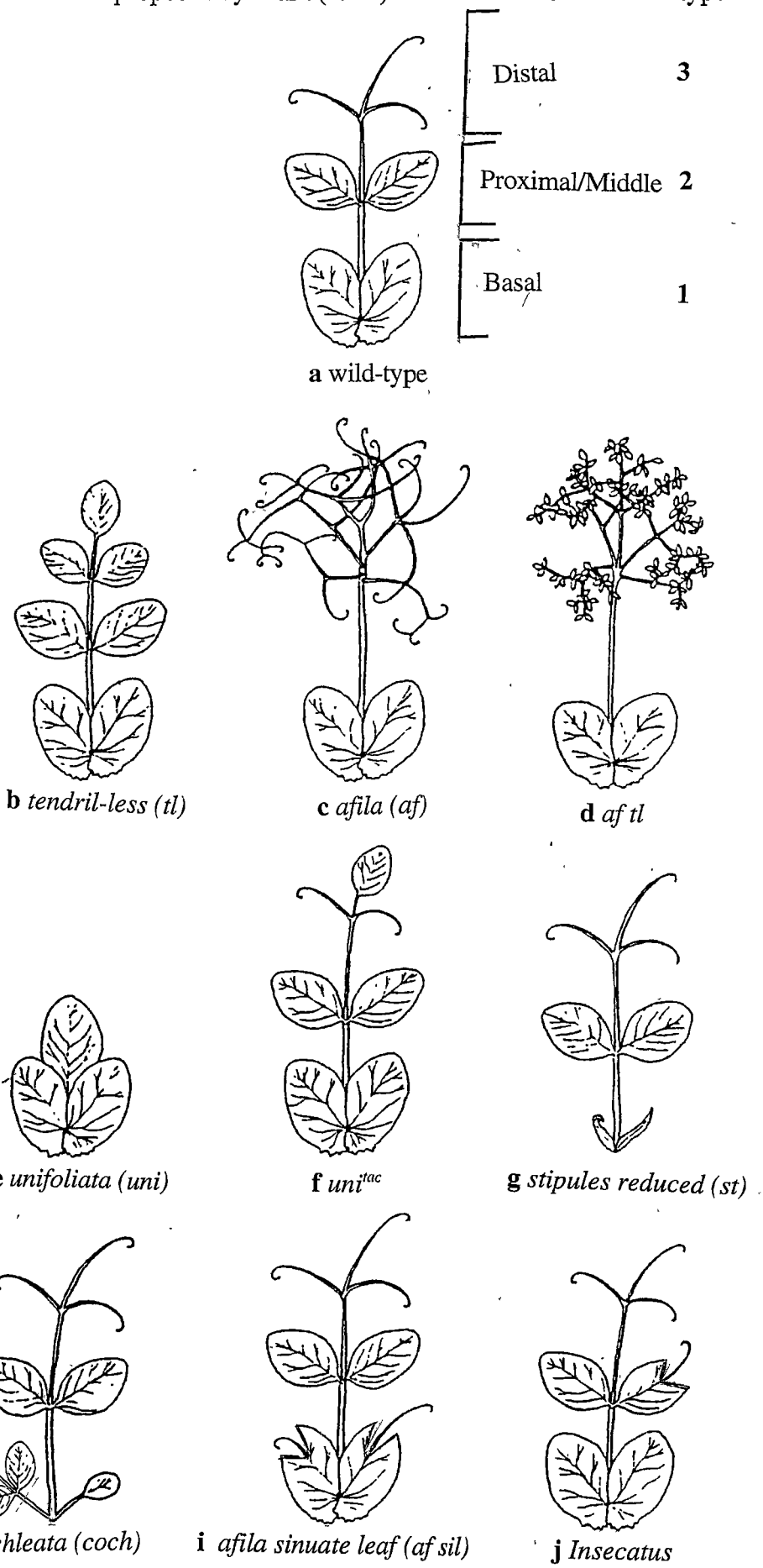
This thesis will describe the phenotype of the *coch* mutant in more detail, as it has not been studied extensively. Interactions of *coch* with other pea leaf development genes will also be examined. This will provide more information about the 'basal compartment' of the compound pea leaf, which is less understood and has been examined less closely than the middle and distal compartments. Like *Uni*, the *coch* mutant also affects both leaves and flowers and may be a further example of the adaptation of floral genes to compound leaf development in pea. A new finding of reduced meristem size in the *uni* mutant and its possible relationship to the mutant's simple leaf form will also be described.

The poorly described leaf development mutant, *insecatus,* will also be examined in more detail, and its interactions with other pea leaf development genes determined. The phenotype of this mutant suggests that it may have increased developmental potential at leaflet tips, similar to the increased developmental potential seen at stipule tips in the *sil af* double mutant.

New knowledge about leaf development is most likely to come from the study of leaf mutants, as these provide the most direct and unambiguous link we have to the normal process. For a full understanding of the process of leaf development a multidisciplinary approach is necessary, involving morphological descriptions, microscopy, classical genetics, tissue culture and other treatments in addition to cloning these genes and describing their action at the molecular level. Leaf development is less well understood than flower development, yet is an equally important basic developmental process. In fact the leaf is the developmental 'ground state' and so is an excellent model system for investigating the genetic control of development. The compound wild-type pea leaf is already segmented and specialised, and a range of mutants altering its development is available, so it is an excellent tool for exploring leaf pattern formation, the evolution of compound leaves and the current debate regarding the overlapping nature of shoots and compound leaves.

In addition peas are an important crop species, and there is commercial significance in understanding their control of leaf development, as it would then be possible to manipulate leaf development to suit particular markets, agronomic practices and processing or distribution requirements (Huyghe, 1998). For instance, many European pea cultivars now express the *afila* phenotype; these are called 'semileafless' peas. The *afila* mutant gene greatly increases the number of tendrils on the pea plant, which provides considerable interlocking of adjacent plants and improves standing ability and resistance to lodging (Hedley and Wang, 1987). Circulation of air in the canopy is also improved, helping to prevent disease and allowing pods to dry more quickly. The more open canopy also allows greater penetration of light for photosynthesis (Cote et al., 1992). Peas of the *afila* type do not alter leaf angle (which reduces incident photosynthetically active radiation) during water deficit, as they maintain rigidity through their tendrils (Ridao et al., 1996). They also have improved standing ability in rainy conditions (Uzan and Ackigoz, 1998).

Figure 1.1 A comparison of the wild-type pea leaf with a range of single and double mutants affecting leaf development (after Murfet and Reid, 1993). The 'domains' of gene action proposed by Marx (1987) are illustrated on the wild-type leaf.



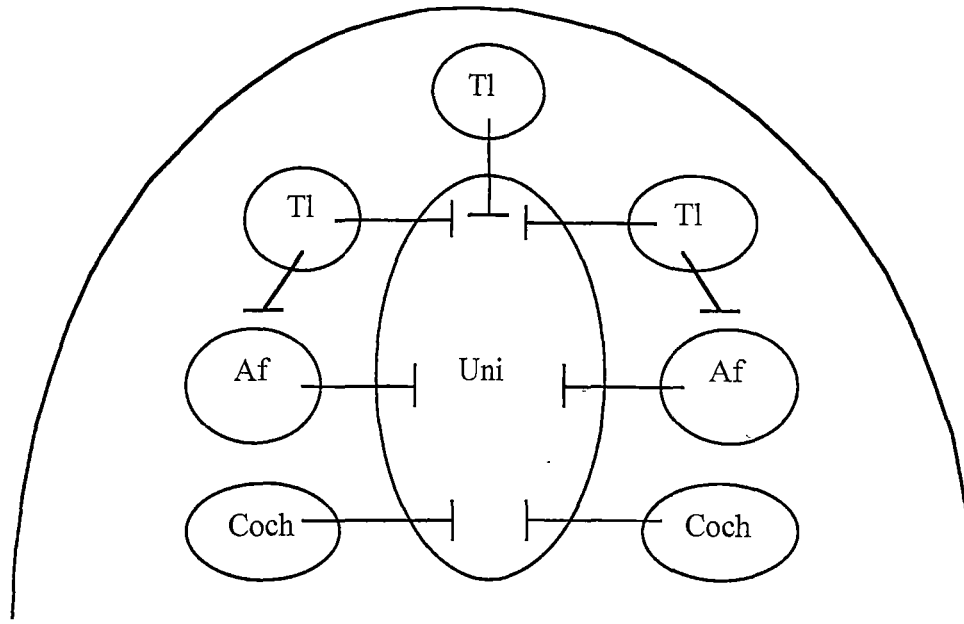
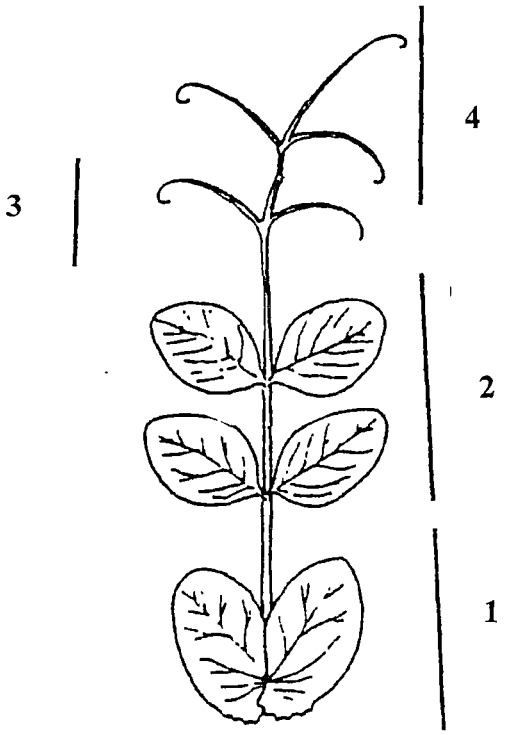


Figure 1.2 Proposed model of pea leaf primordium development (from Hofer et al., 1998). Expression of *Uni* maintains the ability of the primordium to grow and initiate lateral structures. The genes *Coch*, *Af* and *Tl* act to suppress *Uni* expression and allow the formation of determinate stipules, leaflets and tendrils, respectively. Expression of *Tl* also suppresses *Af* gene action and prevents the formation of leaflet laminae in the terminal portion of the leaf.

Figure 1.3 The four domains of the pea leaf as described by (Hofer et al., 1997). (The third terminal domain is subdivided into two domains as compared with the Marx model (1987) illustrated in Fig. 1.1.)



CHAPTER TWO

Stipule Development and Development of a Single Leaf Blade : Mutants *cochleata* and *unifoliata*

The information contained in this chapter appears in part in the publication: Yaxley J.L., Jablonski W. and Reid J.B. (2001) Leaf and flower development in Pea (*Pisum sativum L.)*: mutants *cochleata* and *unifoliata*. *Annals of Botany* **88**: 225-234

INTRODUCTION

Studies of pea leaf development have concentrated on the semi-dominant mutant *tendril-less(tl)*, the recessive mutant *afila(af)*, and the *af tl* double mutant (Fig. 1.1 b, c, d), which alter leaflet and tendril pinna identity in the proximal and distal domains (Cote et al., 1992; Gould et al., 1994; Gould et al., 1986; Lu et al., 1996; Meicenheimer et al., 1983; Villani and Demason, 1997, 1999b, 2000). The stipules and basal compartment have received little attention, with only one model of pea leaf development (Gourlay et al., 2000) considering the *cochleata (coch)* mutant, in which the stipules of the middle nodes of the plant are compound (Fig. 2.1).

To increase knowledge of genes controlling the basal domain of the pea leaf this chapter gives a detailed description of the phenotype of the *coch* mutant and investigates some of its interactions with other pea leaf genes. The interaction of *coch* with *tl* in the basal compartment of the leaf and the interaction of *coch* with the recessive mutant *stipules reduced (st)* are described. As previously described, *st* mutants have stipules that are reduced to small, narrow structures (Fig. 2.2) and the leaves of *tl* mutants have leaflets in place of terminal tendrils (Fig. 2.3). Interactions between these mutants provide information regarding domains of gene activity that are not evident in the single mutants.

The size of a meristem has been linked to the size of the structures it produces (Jackson and Hake, 1999; Lijsebettens and Clarke, 1998; Smith and Hake, 1992; Sundberg and Orr, 1996). It has been suggested that differences in meristem or primordium size could produce different pea leaf mutants (Gould et al., 1992; Young, 1983), although no size difference was found in the meristems of *af, tl* and *af tl* mutants (Cote et al., 1992; Gould et al., 1986; Meicenheimer et al., 1983). Here, early leaf development of *coch* and *unifoliata (uni)* has been examined to investigate the role of meristem size in the development of different types of leaf structures, in addition to leaflets and tendrils. Strong alleles of the *uni* mutant change the multiple leaflets and tendrils of a wild-type leaf into a single leaf blade with basal stipules (Fig. 2.4) and seem to merge the proximal and distal compartments into one. Pea shoot apical meristems were examined by

environmental scanning electron microscopy (ESEM), since this allows objects to be seen in their natural state (Danilatos, 1993; Jablonski, 1997).

MATERIALS AND METHODS

Plant Material

The lines of *Pisum sativum* L. used during this work are held in the collection at Hobart, Australia. The *uni* line JI 2171 (provided by J. Hofer, John Innes Institute, UK), is descended from the original spontaneous mutant described by Eriksson (1929). This line is maintained as a heterozygote, and in our meristem studies *uni* plants were compared with their wild-type siblings. The *coch* line used here was generated (by J.Weller) through mutagenesis of the cultivar Torsdag (Hobart line 107). Seeds were treated with 1% EMS [ethylmethanesulfonate] for six hours at 18°C. Among 1100 M_2 families, one showed the leaf-like stipules typical of *coch* mutants. This mutant line (AF99) was crossed to a *coch* type line, JI 2757 (produced by S. Blixt from the variety Parvus and provided by M. Ambrose, John Innes Institute, UK). The F_1 plants of this cross had leaves typical of *coch* plants, including leaf-like stipules in the middle nodes, indicating that the Hobart putative *coch* mutant line AF99 possessed a mutant allele at the known *cochleata* locus. AF99 has been backcrossed twice with its progenitor line (HL107).

For generation of the *coch st* double mutant, *coch* (AF99) (male parent) was crossed with Hobart multiple marker line HL31 (female parent) which is homozygous recessive for *st*. To examine the expression of *Tl* in a *coch* background, *coch* (AF99) (male parent) was crossed with Hobart multiple marker line HL111 (female parent) which is homozygous recessive for the leaf development genes *tl, af* and *st*.

Identification of *coch st* double mutant plants

Two wild-type F_1 heterozygotes from the cross of *coch* (AF99) (male parent) by *st* (HL31) (female parent) were grown and from these plants 40 F_2 seeds were sown. It was determined that the cross was successful because the F_1 plants had wild-type stipules. Yet the identity of the double mutant plants amongst the 40 plants of the F_2 generation was not immediately obvious, as both *coch* and *st* mutations have the effect of reducing stipules. The *coch st* double mutants were identified from their floral morphology and their leaf phenotype was then examined. (Homozygous *coch* plants have mutant flowers while the *st* mutation does not affect flowers). The F_3 progeny of seven F_2 plants with reduced stipules (*st st*) and wild-type flowers were grown. Two thirds of these plants were expected to be heterozygous for the *coch* mutation (*Coch coch*),

however, only one of the seven F_3 families grown showed segregation of flower phenotype. In this family there were ten plants with reduced stipules and wild-type flowers (*Coch - st st*), and two plants with both reduced stipules and compound stipules and mutant *coch*-like flowers (putative *coch coch st st* plants). The F_4 progeny from the putative double mutant plants (14 plants), and some of their heterozygous (*Coch coch st st*) siblings (16 plants), were grown to confirm the double mutant phenotype, which was clear based on flower morphology in the homozygous *st* background.

Identification of *coch coch Tl tl* plants

Four F_1 heterozygotes from the cross of *coch* (AF99) (male parent) by HL111 (female parent), were grown and from these plants 64 F_2 seeds were sown (16 from each F_1 plant). The cross was found to be successful as the F_1 plants had wild-type leaves. Leaf forms of the ten F_2 plants that were heterozygous for *Tl (Tl tl)* and had *coch* leaf-like stipules were observed. Plants heterozygous for *Tl* can be distinguished because their leaves have flattened tendrils (Villani and DeMason, 1999; White, 1917).

Growing Conditions

All plants were grown in a 1:1 mixture (v/v) of vermiculite and 10 mm dolerite chips topped with 2-3 cm of pasteurised potting mix (1:1 mixture (v/v) of coarse river sand and peat moss, with added macro and micro nutrients). Plants were grown two per pot in 14 cm slimline pots. Before planting the seeds a small section (about 3 mm^2) of testa was removed with a sharp razor blade to facilitate even germination. Seeds were then coated in Thiram 800 fungicide (active ingredient Thiram 800g/kg [Agchem, Parafield Gardens SA]) to help prevent fungal infection during germination, and planted into wet soil. The pots were watered sparingly for the first five days until the seedlings began to emerge. Watering was then three times per week for three weeks, and then daily after this time. Plants were provided with nutrient solution (1g/l Aquasol [Hortico, Australia] and 0.05 g/l iron chelate [Kendon Chemicals]) weekly. Vertical strings were used to support the plants once they reached 10 cm in height. The photoperiod was 18 hours, consisting of natural daylight extended by mixed incandescent (100 W bulbs) and fluorescent (40 W cool white tubes) light, giving approximately 25 $\mu mol\ m^{-2}\ s^{-1}$ at the pot surface. Growth of the segregating populations and of plants for examining inflorescence structure was during autumn/winter with mean maximum/minimum day and night temperatures of approximately 22°C and 14°C, respectively. Plants for microscopic examination and for all other morphological measurements were grown during spring, with mean maximum/minimum day and night temperatures of approximately 25°C and 15°C, respectively. Plants were treated with fungicide or pesticide treatments as required.

Scoring of Morphological Characters

Counting of nodes was acropetal, with the cotyledons as node zero, the two subsequent scale leaves as node one and node two, and the first true leaf as node three. The leaves at the apex are numbered using the plastochron index (Erickson and Michelini, 1957; Lamoreaux et al., 1978; Sylvester et al., 1996). Means were compared using Student's t-test.

Environmental Scanning Electron Microscopy

Three mutant and three wild-type leaves, for both the *coch* and *uni* mutants and their corresponding wild-types, were examined for each of nodes eight to twelve. To observe each successive node, groups of plants were harvested every two to three days. Ten plants of each mutant line and the corresponding wild-type were examined four weeks later to determine the typical mature leaf morphology of each node examined during development.

The whole apices of plants were harvested directly before they were to be examined. The meristem was then dissected from the apical leaves under a stereo-microscope with a minimum light intensity and with the sample surrounded by a pool of water to prevent dehydration. The apex was then attached to an aluminium stub with double-adhesive conductive tape and placed on a Peltier-effect thermoelectric cold stage (Omega CN 900A, ElectroScan) attached to an environmental scanning electron microscope (ESEM 2020, ElectroScan). The specimen chamber was closed and the temperature reduced to 2.5-2.8°C before the pressure was reduced. The specimen was localised and the primary focus and video signal level established at a water vapour pressure of 8 Torr. The pressure was then slowly lowered to 6.0-7.5 Torr (to minimise water on the specimen surface). Once an image was acquired (at 2048 x 2048 resolution) Ilford FP-4 (125) film was used to obtain a hard copy.

Using this technique the apices do not require any treatment before microscopy, thus substantially reducing the possibility of artefacts (Crang and Klomparens, 1988). The structural integrity of the specimen is maintained when the internal pressure corresponds to the saturated water pressure at a given temperature. (The surrounding medium also acts as a neutralising agent to prevent primary electron charge build-up on the surface of the specimen.) Measurements of apical meristem size were made on screen during ESEM observation.

RESULTS

Phenotype of the *coch* mutant

The *coch* mutation affects only the stipules of the leaf; leaflets and tendrils are unchanged. Stipules were mostly absent from the first few nodes (nodes 3-5). Above this (nodes 6-7) *coch* stipules were sometimes small and straplike, sometimes sessile and elliptical, or sometimes spatulate. At higher nodes (nodes 8-11) the stipules were largely compound, with proximal leaflets and distal tendrils, that re-iterated the structure of the leaf blade. Above node 11 leaf complexity was reduced and spatulate and sessile-elliptical stipule forms became common; occasionally thread-like forms occurred (Figs. 2.5, 2.6).

Approximately 25% of *coch* leaves had mis-matched stipule pairs. For example, an elliptical sessile stipule could occur opposite a spatulate stipule, or a leaf-like stipule opposite a thread-like stipule (eg. node 14, Fig. 2.5). In addition, approximately 10% of compound *coch* stipules had leaflet and tendril pairs that were not opposite each other on the stipule rachis (eg. node 11, Fig. 2.5). This occurred less commonly (approximately 2%) on the main rachis of wild-type leaves.

The stipules of *coch* mutants did not have visible axillary buds. This applied to both compound *coch* stipules and simple *coch* stipule forms. Thus, *coch* compound stipules appear to re-iterate only the proximal and distal parts of the compound pea leaf (the leaflets and tendrils). They do not have stipules or axillary buds at their bases, as occurs in true compound leaves. In addition, almost half of the *coch* plants with leaf-like stipules had no apparent axillary bud in the main leaf axil. This compares with an occurrence of approximately 3% 'blind' leaf axils in wild-type leaves.

The node at which leaves with two pairs of leaflets first appeared was similar for *coch* (11.3 ± 0.2) and wild-type (11.8 ± 0.2) plants ($0.1>p>0.05$, n=24). On average compound *coch* stipules also changed to two leaflet pairs at the same node (10.4±0.7) as the main leaf rachis (11.3 ± 0.2)($0.4>p>0.2$, n=24). However, the compound stipules exhibited more variability. Some changed to two leaflet pairs substantially earlier (node 7) than the main leaf rachis, and other compound *coch* stipules never showed more than three leaflets (one leaflet pair and a mixed leaflet-tendril pair). Flowering time and flowering node were also not significantly affected. Mutant *coch* plants flowered at node 15.5 ± 0.9 in 65.2 ± 0.9 days in spring, and wild-type plants flowered at node 16.0 ± 0.3 in 65.4 ± 0.6 days ($0.8>p>0.7$ flowering node, $0.9>p>0.8$ flowering time, n=24).

Wild-type pea flowers have five sepals, two fused keel petals, two wing petals and a standard petal (five petals in all), ten anthers (nine fused into a filament tube and one partially free) and a single central carpel (Ferrandiz et al., 1999; Tucker, 1989)(Figs. 2.7, 2.8). Flowers of *coch* mutants ranged from nearly normal in appearance (Fig. 2.7), to open flowers with super-numerary organs in each whorl, abnormal organ fusing and some organs that were a mosaic of

different organ types (Fig. 2.8 and Table 2.1). The more severely affected flowers occurred at later nodes. The first formed flowers on the main stem and on laterals were the most normal in appearance and set the most seed. Mutant *coch* flowers were largely self-sterile and *coch* plants normally produced no more than ten seeds per plant (compared with an average of approximately sixty seeds per plant for the wild-type progenitor line). Flowers of *coch* mutants were partially sterile when cross-fertilised with wild-type pollen, and the pollen from *coch* mutants also showed reduced fertility when it was used to pollinate wild-type flowers. Due to the low fertility of *coch* plants, they produced more lateral branches than the wild-type.

In *coch* flowers there was an increase in sepal number (Table 2.1) and mosaic sepal/petal organs appeared in the sepal whorl in flowers with severe mutant phenotypes. There was also an increase in the number of wing and keel petals (Table 2.1), and the keel petals did not pair and fuse properly to make a keel (Fig. 2.7). Considering petal shape and colour, some petals appeared to be a mosaic of wing and keel petal. In flowers with a weak mutant phenotype there was no change in the number of standard petals. However, in the flowers with a strong mutant phenotype two or three standard petals were common, forming a radially symmetric flower structure (Fig. 2.8). All *coch* petals were narrow at the base (Fig. 2.9) compared with those of the wild-type. Wild-type petals form an enclosing sheath around the anthers and stigma, whereas the narrowed petal bases of the *coch* flower cause the petals to fall open so the anthers and stigma are not enclosed within the keel. This means that *coch* flowers have a more open structure. Their pollen tends to dry quickly and not be deposited on the stigma(s), which contributes to their reduced fertility.

The number of anthers was also altered in *coch* flowers (Table 2.1). Occasionally less than ten anthers were present, but more commonly there were more than ten anthers (up to 17 in flowers with severe mutant phenotypes). Anthers of *coch* flowers were commonly fused together above the filament tube, and also fused to petals above the base (the outer stamens are normally basally adnate to the petals (Tucker, 1989)). In *coch* mutant flowers with severe phenotypes, most of the anthers were abnormally fused. The gynoecium remained unchanged in *coch* flowers with weak mutant phenotypes, whereas in highly disturbed flowers there were up to four carpels present (Fig. 2.8, Table 2.1).

In wild-type pea flowers there are normally two flowers per panicle. This varies somewhat at different nodes, with more single-flowered panicles at the highest nodes ((Hole and Hardwick, 1976) and Fig. 2.10). The number of single-flowered panicles also varied with planting season; a greater number of single-flowered panicles occurred during winter (Fig. 2.10). In mutant *coch* plants there was a greater occurrence of single-flowered panicles at all nodes in both plantings (Fig. 2.10). This varied with node and season in a similar manner to the wild-type, with more single-flowered panicles at higher nodes and during winter. Approximately ten percent of *coch* panicles contained one flower plus a partially developed flower or flower bud (Fig. 2.8). Occasionally inflorescences contained three flowers.

Interaction of *coch* and *st*

The *st* mutant has stipules reduced to small strap-like structures (Fig. 2.2). This morphology is the same at each node, and other leaf parts and flowers are normal (Pellew and Sverdrup, 1923). The *coch st* double mutant had *coch*-like stipules, indicating that *coch* is largely epistatic to *st* in this background. The *coch st* double mutants showed all the stipule forms typical of *coch* plants: compound, elliptical and sessile, strap-like, spatulate, and filamentous stipules. Yet, the double mutant plants had significantly fewer ($p < 0.001$) compound stipules than *coch* plants. Mutant *coch* plants had 7 ± 0.3 nodes carrying compound stipules and *coch st* double mutant plants had 4 ± 0.5 nodes with compound stipules (n=24).

Interaction of *coch* and *tl*

Leaves of the *tl* mutant have leaflets in distal positions where normally tendrils are present (Fig. 2.3). The *tl* mutation is incompletely dominant, and heterozygotes can be distinguished by the presence of flattened, rather than cylindrical, tendrils. Compound *coch* stipules showed reduced expression of the wild-type *Tl* gene compared with the main leaf rachis. Leaves of the genotype *coch coch Tl tl* had the flattened tendrils typical of *Tl tl* heterozygotes in the distal region of the leaf. Nevertheless the compound stipules had only leaflets (Fig. 2.11), like the leaf blades of the homozygous recessive *tl* mutant.

A similar reduction in *Tl* expression was seen in the compound stipules of *coch coch af af Tl tl* plants. The leaf blade of *coch coch af af Tl tl* plants had tendrils like that of an *af* mutant leaf, with the tendrils somewhat flattened at their tips due to the incomplete dominance of the *Tl* gene. Yet, the compound stipules of this genotype consisted of branched tendrils ending in small terminal leaflets (Fig. 2.12), similar to the *af tl* double mutant phenotype (Fig. 2.13).

Early leaf development of *coch* and *uni*

In the *coch* apical meristems examined, stipule primordia were smaller and less developed than the primordia of the first leaflet pair. This was apparent from the time they were first visible at P_2 (Fig. 2.14) until late stages of leaf expansion (eg. P_9, Fig. 2.23). In contrast, wild-type leaf primordia have early stipule primordia (P_2 to P_4) that are larger than the primordia of the first leaflet pair (compare Fig. 2.14 and 2.16, 2.15 and 2.17). During P_5, compound *coch* stipule primordia initiated lateral pinna. During P_6, these first stipule lateral pinnae became more dorsiventral and secondary lateral pinnae were initiated (Fig. 2.20). The apical dome diameters of *coch* mutant plants, when P_0 is node 11, were a similar size ($190 \pm 4.7\mu m$) to that of their corresponding wild-type ($200 \pm 5.1\mu m$) (n=6).

The phenotype of the *uni* mutant has been described previously (Eriksson, 1929; Hofer et al., 1997; Lamprecht, 1933). Flowers of the *uni* mutant have an incomplete sepal whorl, no petals or stamens, and an open gynoecium, with numerous iterations of axillary flowers (Hofer et al., 1997). At the nodes examined (7-13), all *uni* leaves consisted of normal stipules with a single leaf blade. Approximately half of these *uni* leaves were lobed (Fig. 2.24). This was more common at the lower nodes examined (nodes seven to nine: 70% lobed leaves, Fig. 2.25). Leaves of *uni* plants at the higher nodes examined (nodes 11-13) had longer petioles than those leaves at nodes seven to nine, which were mostly sessile (80% sessile: Figs. 2.24, 2.26).

Leaf primordia of the *uni* mutant initiated a single large leaflet primordium and two normal stipule primordia. The *uni* leaf primordia were visible in late P_1 or early P_2 (Fig. 2.18). At the same stage, the wild-type had smaller leaflet primordia with two larger lateral stipule primordia (Fig. 2.16). During P_3, *uni* leaf primordia developed grooves on their adaxial sides (Fig. 2.19), indicating the incipient midrib. This was more obvious in the P_4 *uni* leaflet (Fig. 2.19), which was clearly a laterally flattened structure, with two halves folded along a central groove. The wild-type leaflet primordia showed no lateral flattening or midrib-groove development until late P_4 (Fig. 2.17). The differences in early leaf development between wild-type, *coch* and *uni* plants are also seen when comparing the position where hairs first appear on these leaves. In the wild-type they appear on the stipules from P_3 to P_4 (Fig. 2.17), in *coch* they appear on the first leaflet pair from P_3 to P_4 (Fig. 2.15) and in *uni* they first appear on the leaf blade primordia in early P_3 (Figs. 2.18, 2.19).

The apical dome diameters of *uni* plants, when P_0 is node 11, were significantly smaller (148.5 ± 3.3 μm) than the apical domes of their corresponding wild-type siblings (193.6 ± 3.6 μm) ($p < 0.001$, $n=7$). However, the node and time of first flower initiation were similar in mutant and wild-type. Mutant *uni* plants flowered at node 22.6 ± 0.4 in 58.9 ± 0.4 days in summer, and their wild-type siblings flowered at node 21.6 ± 0.3 in 57.9 ± 0.4 days ($0.1 > p > 0.05$, $n=20$).

DISCUSSION

Phenotype of the *coch* mutant

The stipule forms displayed by the *coch* mutant vary with node of insertion. The first five to six nodes of a pea plant are formed in the developing embryo (Gould et al., 1987; Villani and DeMason, 1997). Nodes three to five usually have no stipules in the *coch* mutant. It is possible that there may be an interaction between the environment of the embryo and the *coch* mutation which results in the absence of stipules, or only small stipules forming. The vegetative nodes of the *coch* plant that develop after germination show predominantly compound stipule forms. After flowering commences (nodes 15-16), the stipule forms found in *coch* mutants are simplified. Again, it seems likely that the major change in the condition of the shoot apex is responsible for the corresponding change in stipule form. The change in stipule form parallels the increase in leaf blade complexity in the middle nodes, and the decrease in leaf complexity after flowering, seen in wild-type plants (Villani and DeMason, 2000; Wiltshire et al., 1994).

In experiments where excised pea leaf primordia were grown in culture (Gould et al., 1994), it has been shown that the two pinnae of a leaflet or tendril pair are not always determined simultaneously. The tendency for opposite pinnae to develop into different structures is increased in *coch* compound stipules. Since the development of *coch* stipules is retarded, it is possible that they remain meristematic for longer than wild-type stipules, which could lead to their greater developmental flexibility. In addition, it is more common for the pinnae of *coch* compound stipules not to be positioned opposite each other. This is a shoot-like characteristic (Lacroix and Sattler, 1994; Sattler and Rustihauser, 1992), and supports the contention that the *coch* mutation reduces determinacy in the stipules.

It seems likely that the open structure of the *coch* flower will reduce the viability of pollen and the receptive life of the stigmatic surface. However, while generating crosses in this study, it was found that *coch* flowers pollinated by hand with wild-type (*Coch*) pollen had poor seed set. This indicates that there are probably also structural and developmental defects in the gynoecium of *coch* mutants that reduce seed set. Such abnormal development of the gynoecium and of the female gametophyte in *coch* flowers was described by Molhova (1988). In the *coch* line he examined, there was sometimes no ovule formation and sometimes disturbed early formation of the embryo sac. Molhova (1988) also described the production of feminised stamens in *coch* flowers, leading to the production of multipistillate flowers. While the production of multipistillate flowers through an increase in carpel numbers was observed in this study, no feminisation of the stamens was seen. This difference could be due to the different lines examined. Molhova (1988) found the pollen from *coch* mutants showed a high percentage of sterility; this is likely to be related to the feminised stamens he also described. The pollen from the *coch* mutant utilised in this study

also showed reduced fertility when crossed onto wild-type flowers and so it is also likely to have some abnormalities.

It is noteworthy that it is the base of the wing and keel petals that is altered in all *coch* flowers, even in those with the weakest phenotypes. This suggests that there may be some homology between the base of the petals and the base of the leaves (stipules) in the action of the *Cochleata* gene. The defective petal bases may be in part responsible for the improper fusing of the keel petals and anthers. In pea the petals and stamens originate from a common primordium (Ferrandiz et al., 1999; Tucker, 1989). Incorrect separation of these organs may result in the fused stamens and petals seen in *coch* flowers. The formation of some floral organs that are a mosaic of different organ types indicates that the *coch* mutation also has an effect on floral organ identity.

The reduced formation of two-flowered panicles seen in *coch* inflorescences shows that the early development of the inflorescence is also altered by the mutation. In pea flowers, once the inflorescence meristem has formed and the meristematic region that will form the flower has become distinct, the remaining region will form another flower initial, or will revert to a vegetative state and form a small stub (Hole and Hardwick, 1976). In *coch* flowers, a second floral initial is much less likely to form (there are more single-flowered panicles). Additionally, more developmental flexibility is seen, as half-formed second flowers, or complete third flowers, are sometimes seen in *coch* mutants (Fig. 2.8), whereas these forms do not appear in the wild-type. Thus, the *Coch* gene has been shown to have pleiotropic effects on leaf and flower development, altering stipule form, floral organ number, shape and identity, fertility and inflorescence structure.

Interaction of *coch* and *st*

Double mutant *coch st* plants appeared like *coch* plants. This is in contrast to the findings of Marx (1987) and Gourlay *et al.* (2000) who reported that *coch st* double mutants had no stipules. This may be due to differences in the lines used. In this study *coch* is predominantly epistatic to *st*. It is possible that the *coch* mutation changes the fate of the stipule cells into more 'leaf-like' cells and thus prevents the action of *st*, which acts only on stipule cells. Epistasis occurs at all nodes and all stipule forms are seen in *coch st* double mutant plants. The suggestion that *coch* alters the fate of stipule cells is strongly supported by the fact that *coch* compound stipules behave genetically as if they were leaf blades (Marx, 1987). For example, the *coch af* double mutant has stipules that consist of tendrils like the leaf blade.

Gourlay *et al.* (2000) have found some compound stipules in *st af tl* triple mutants. Compound stipules are not found in *af tl* double mutants. They propose that the gene *St* may also have a role in suppressing *Uni* expression in wild-type stipules and so promoting their simple structure. Still, this proposal predicts that the *coch st* double mutant would have compound stipules, which, as mentioned previously has not been found by previous workers. The gene

interaction seen here, which produced the predicted compound stipules, lends support to this proposal of *St* as an additional weak suppressor of *Uni* gene action in the stipules, although there is no evidence that this is a direct interaction.

Interaction of *coch* and *tl*

The interaction between *coch* and *tl* suggests that there is a gradient of wild-type *Tendril-less* gene action in the leaf, with *Tl* action being reduced at the base of the leaf. This normally cannot be seen as *Tl* does not act on the stipules. Yet in the compound stipules of *coch*, the reduced action of the wild-type *Tl* gene in the base of the leaf is evident. This supports proposals that gradients of gene action could be involved in determining leaf form in pea (Hofer and Ellis, 1996; Lu et al., 1996). Lu *et al.* (1996) suggested that a gradient of *Tl* gene function would be a plausible explanation of pea leaf morphology, with *Tl* function producing a tendril-inducing or branching-inhibiting morphogen at the leaf tip. Using morphological measurement of a series of mutants with different numbers of *tl* genes, Villani and DeMason (1999) show that *Tl* acts in both the proximal and distal parts of the leaf, but has a greater effect in the distal region.

Early leaf development of *coch* and *uni* mutants

The small size of *coch* stipules and their slow growth and development from their initiation at P_2 suggests strongly that *coch* stipules are developmentally retarded compared with the leaflet primordia of the same leaf. (Early slow growth of *coch* stipules was also shown by Gourlay *et al.* (2000). However, he found no difference between *coch* and wild-type primordia until P_3. This discrepancy is possibly due to the different lines examined.) It is possible that this retarded growth and development is the primary consequence of the *coch* gene mutation. If this is the case the *coch* stipules are probably more meristematic in nature than wild-type stipules and may respond inappropriately to normal developmental signals, developing into complex compound structures. The formation of compound stipules seems to be due, at least in part, to the expression of *Uni* seen in *coch* S3 and S4 stipules, which is not seen in wild-type stipules (Gourlay *et al.* 2000). *Uni* is important in maintaining blastozone activity. However, this ectopic expression of *Uni* in compound *coch* stipules is not seen until after morphological changes have occurred in *coch* primordia (at P_2, Fig. 2.14). Also, it is seen only in the compound *coch* stipules and not in the other simpler *coch* forms. Given these facts, it seems unlikely that alteration in *Uni* expression is the primary effect of the *coch* mutation. The action of the *coch* mutation is to alter the stipule primordia in such a way as to allow ectopic *Uni* expression in some primordia leading to the formation of compound stipules at these nodes.

The effects of the mutations *coch* and *uni* seem to be apparent at an earlier stage (late P_1 to early P_2) of leaf primordia development than the mutants *tendril-less* and *afila* (apparent from late P_4 to P_6 and from late P_2 to P_3 and respectively) (Gould et al., 1986; Gourlay et al., 2000; Meicenheimer et al., 1983; Villani and DeMason, 2000), so they may act earlier in the leaf development pathway. No differences amongst pea leaf mutants in the size of shoot apical domes have previously been reported. The smaller diameter (25% reduced) of *uni* meristems may be a consequence or a cause of their altered leaf morphology. However, apical size is not always linked to leaf size (Goliber et al., 1999; Smith and Hake, 1992). The wild-type *Uni* gene is not expressed in the shoot apical meristem (Gourlay et al., 2000), so the reduction in *uni* meristem size may not be a primary effect of the mutation. It is possible that cells expressing *Uni* in the marginal blastozones normally signal to cells in the shoot apical meristem. Recent work has implicated auxin concentration as a determinant of primordial size in tomato, with increasing auxin treatment concentration increasing the number of cells recruited into primordia (Reinhardt et al., 2000).

Figure 2.1 The compound stipule phenotype of the *coch* mutant (right) compared with the wild-type pea leaf (left).

Figure 2.2 The reduced stipule phenotype of the *st* mutant (right) compared with the wild-type pea leaf (left)

Figure 2.3 The *tl* mutant leaf consists on stipules and leaflets only, with leaflets replacing tendrils in the distal region of the leaf.

Figure 2.4 The single leaf blade phenotype of the *uni* mutant (right) compared with the wild-type pea leaf (left).

Figure 2.5 The stipule morphology of a single *coch* plant. Pairs of stipules from node 5 (top right) to node 14 (bottom left) are shown.

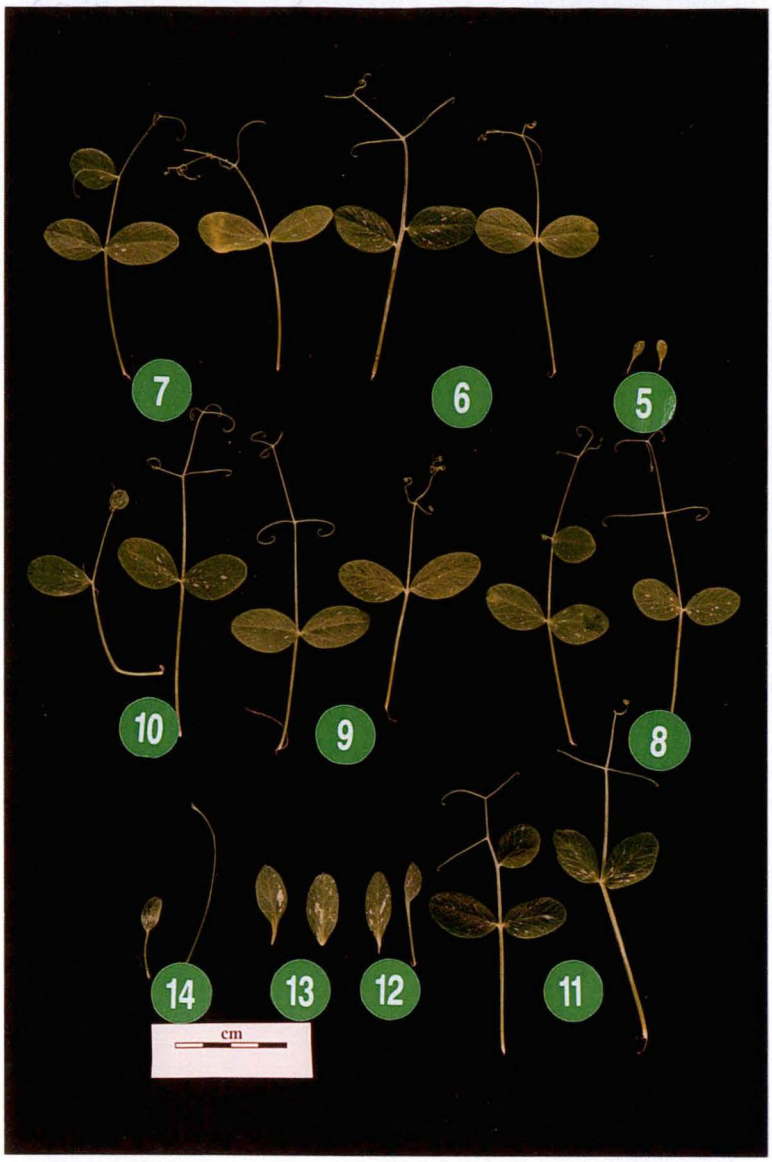


Figure 2.6 Occurence of different stipule forms at different nodes of *coch* plants (n=40)

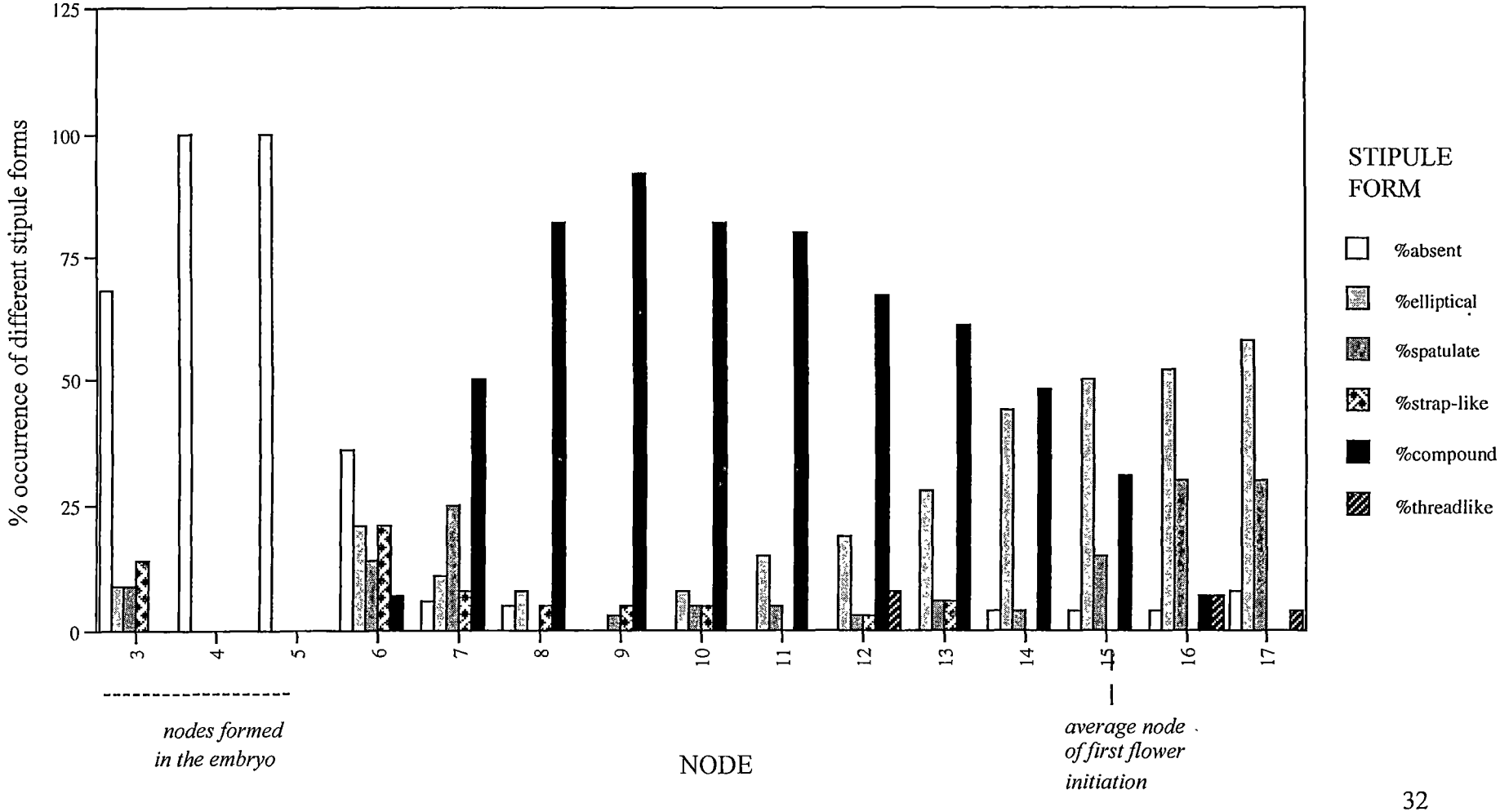


Figure 2.7. *coch* flowers with weak mutant phenotypes (a wild-type flower is on the far left). The mutant flowers with weak phenotypes have additional wing and keel petals, which become disorganised, and additional sepals and anthers.

Figure 2.8. *coch* flowers with strong mutant phenotypes (a wild-type flower is on the far left). In addition to the changes seen in Figure 3, more severe mutant flowers may have two (a,b) or three (c) standard petals and two to four gynoecia (e,f : these flowers are several days old which has allowed the carpels to grow out and become more visible). Flower (a) has an additional, less developed, flower in the panicle, and flower (b) has another small floral structure arising from its pedicel.

Figure 2.9 Mutant *coch* flowers (left) have narrowed petal bases compared with the wild-type (right). Whole flowers are shown at the top and a single standard petal at the bottom. (Petal bases are narrower in *coch* mutant flowers even when there are no extra petals in the whorl.)

Figure 2.10 Occurrence of single-flowered panicles in *coch* plants and their wild-type progenitors (cv. Torsdag).
(Panicles of *coch* mutants with one flower plus a partially developed bud were considered to be panicles with more than one flower.)

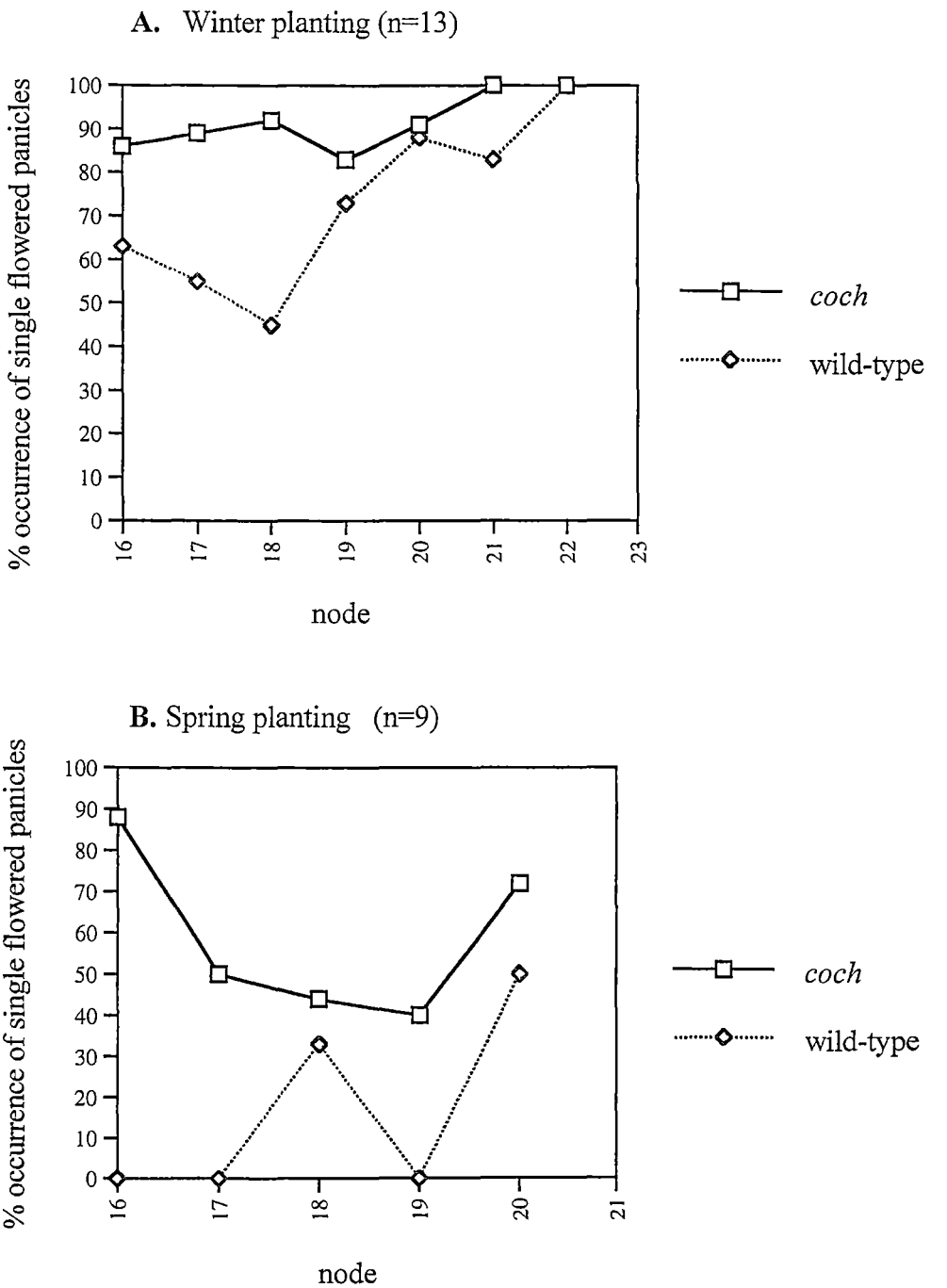


Figure 2.11 Leaf-like stipules of the genotype *coch coch Tl tl* have small terminal leaflets typical of the homozygous recessive genotype *tl tl* (arrow). The main leaf rachis has flattened tendrils typical of a *Tl tl* heterozygote.

Figure 2.12 Leaf-like stipules of the genotype *coch coch af af Tl tl* have small terminal leaflets (arrow) typical of the double recessive genotype *af af tl tl* (see Fig. 2.13). The main leaf rachis has the flattened tendrils typical of a *Tl tl* heterozygote.

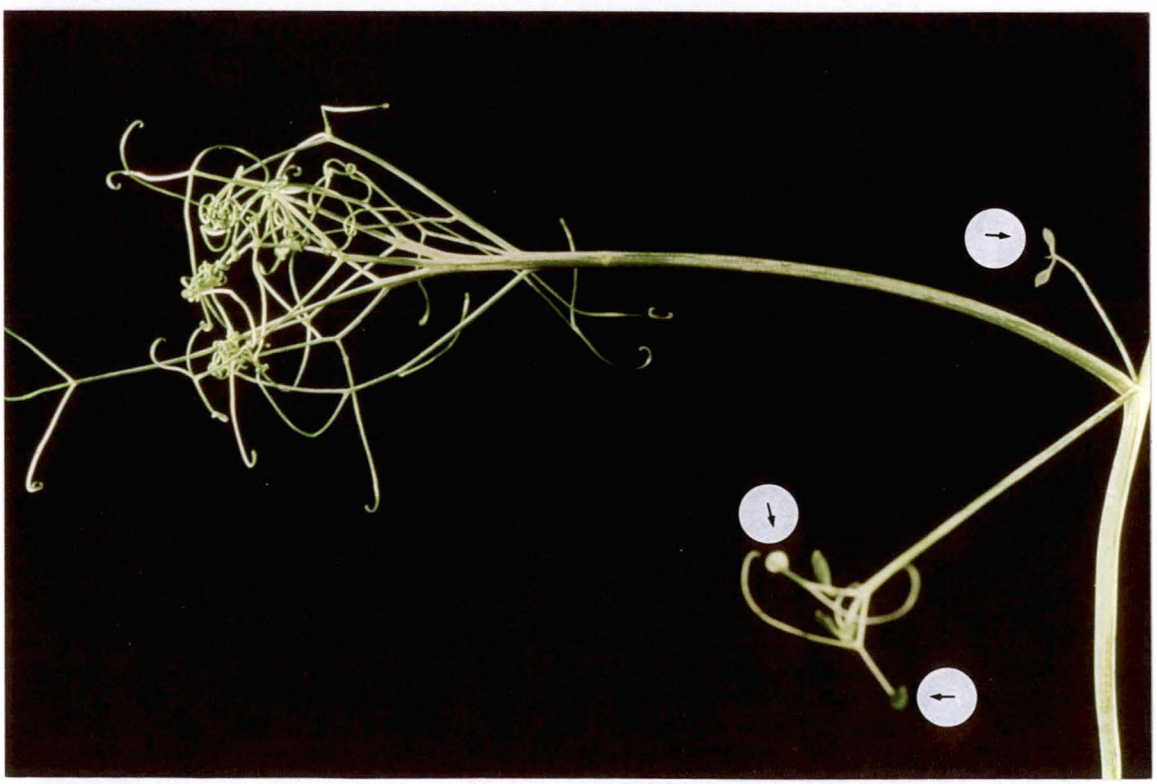


Figure 2.13 'Parsley leaf' phenotype of the *af tl* double mutant.

Figure 2.14 A *coch* apex with the largest leaf subtending node 9. The P_2 meristem has stipule primordia (S2) which are smaller than the primordia of the first leaflet pair (L2).

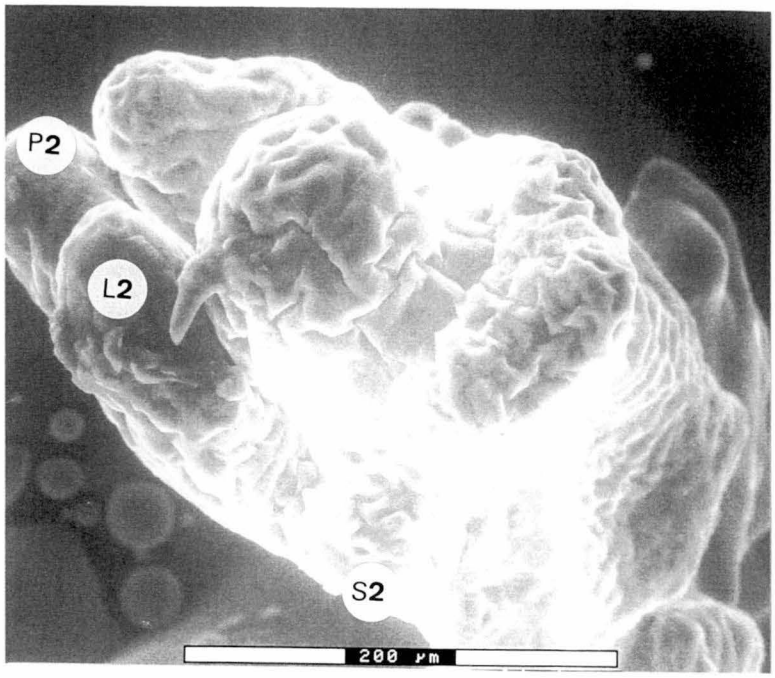


Figure 2.15 A *coch* apex with the largest leaf subtending node 12. An inflorescence meristem is also present (IF). The stipule primordia of the P_3 and P_4 leaves (S3 and S4) are small and undeveloped compared with the primordia of the first leaflet pairs (L3 and L4). The stipule primordia are also small and undeveloped compared with the wild-type stipule primordia (see 2.17).

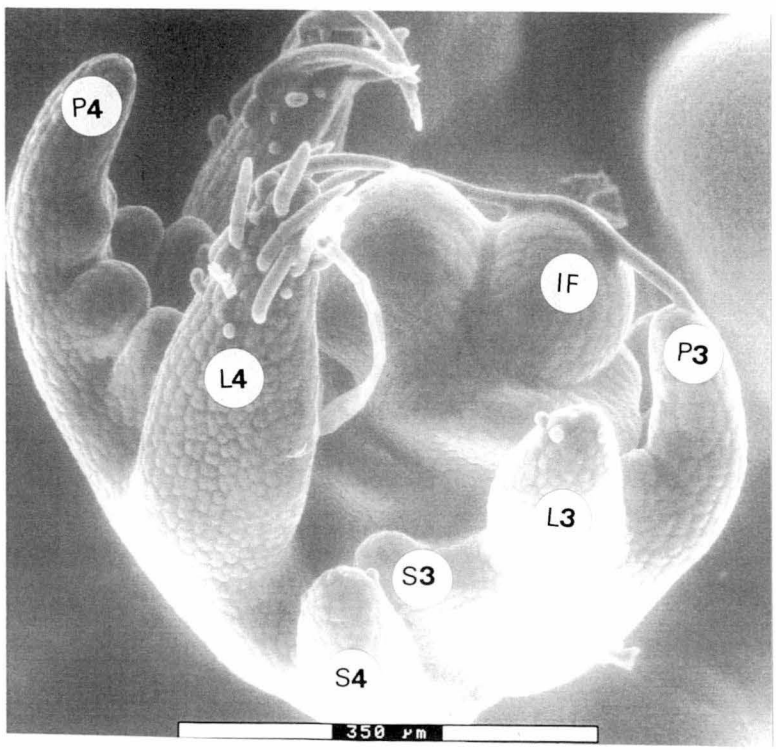


Figure 2.16 A wild-type apex with the largest leaf subtending node 12. The early P_2 meristem shows well-developed stipule primordia (S2) and bulges on its flanks due to developing leaflet primordia (L2).

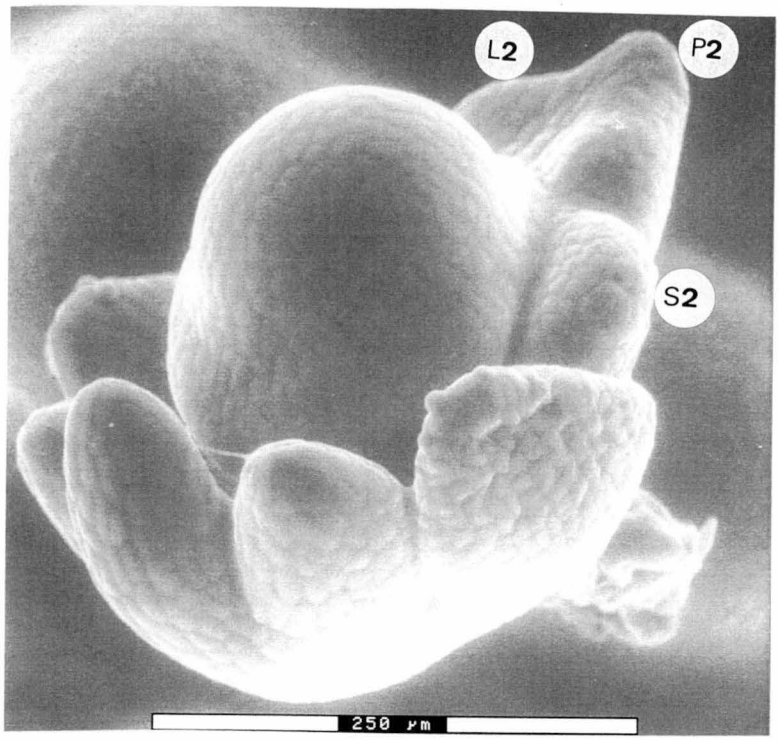


Figure 2.17 A wild-type apex with the largest leaf subtending node 12. The stipule primordia of the P_3 and P_4 leaves (S3 and S4) are larger and more developed than the first leaflet primordia (L3 and L4). The L4 leaflet primordia are just beginning to develop an adaxial groove (arrow).

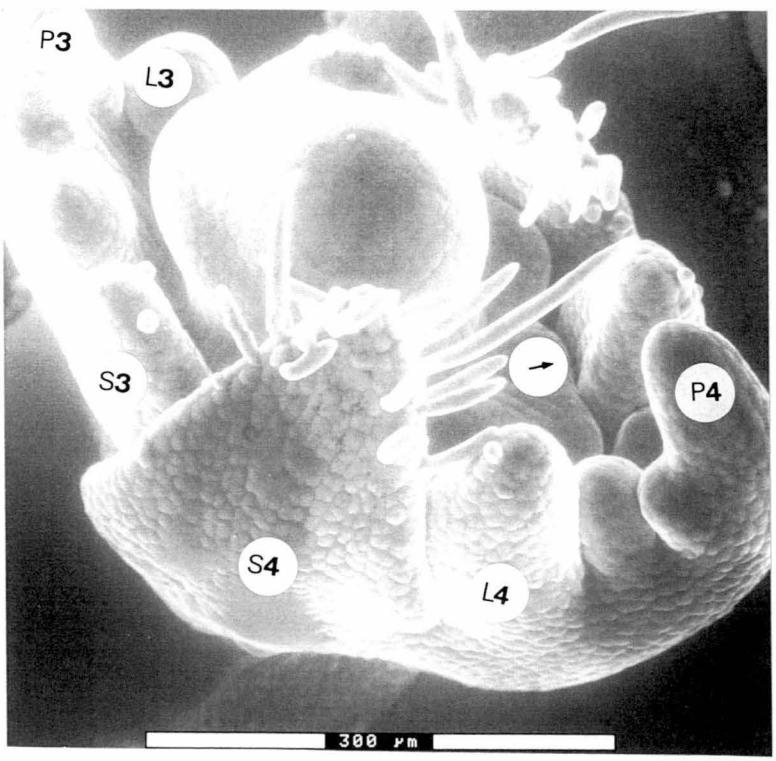


Figure 2.18 The apex of a *uni* mutant with the largest leaf subtending node 8. The P_2 primordium is forming a large single leaf blade with two stipule primordia (S2). The P_3 primordium is similar, but also has a large lobe (lo) in its leaf blade.

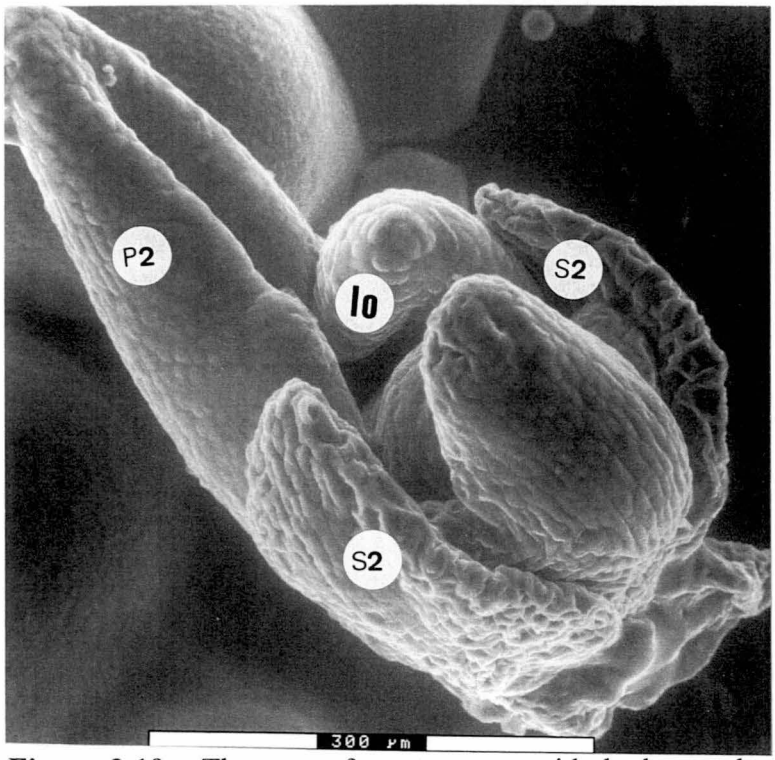


Figure 2.19 The apex of a *uni* mutant, with the largest leaf (P_4) subtending node 12. The P_3 primordium shows a single large leaf blade primordium (L3) and stipule primordia (S3). The P_3 leaf blade primordia has a central adaxial groove (arrow) where the midrib is forming. The P_4 leaf blade primordia shows a clear central channel and is developing a laterally flattened folded leaflet shape. The P_4 leaf blade primordium also shows the development of a lobe on its flank (lo).

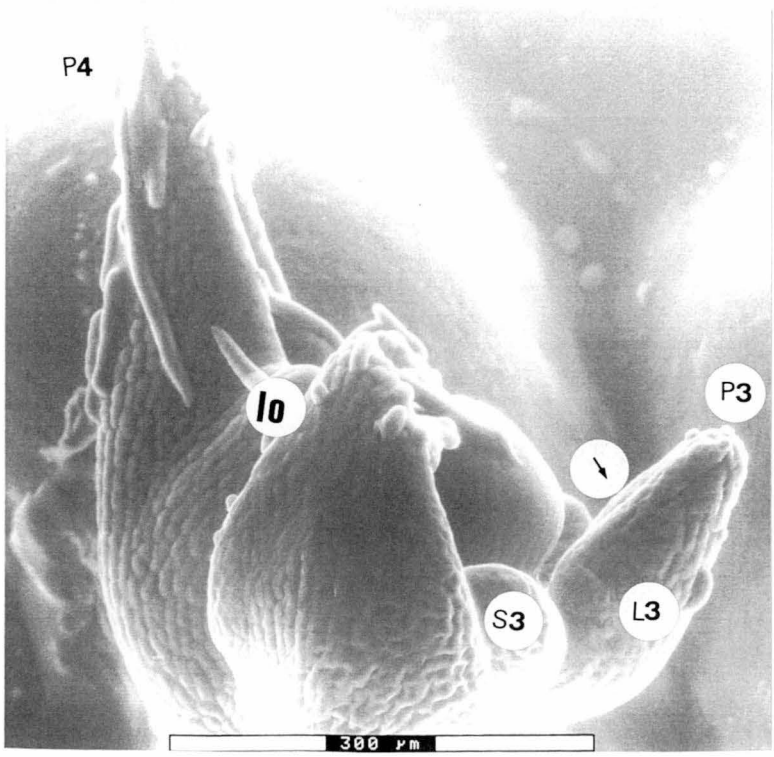


Figure 2.20 P_6 *coch* stipule primordia (S6), from node 11, showing three pairs of lateral primordia (l_1, l_2, l_3). The primary pair (l_1) is becoming flattened, indicating that they will become leaflets. The P_6 leaflet (L6), and the P_5 leaf are also indicated.

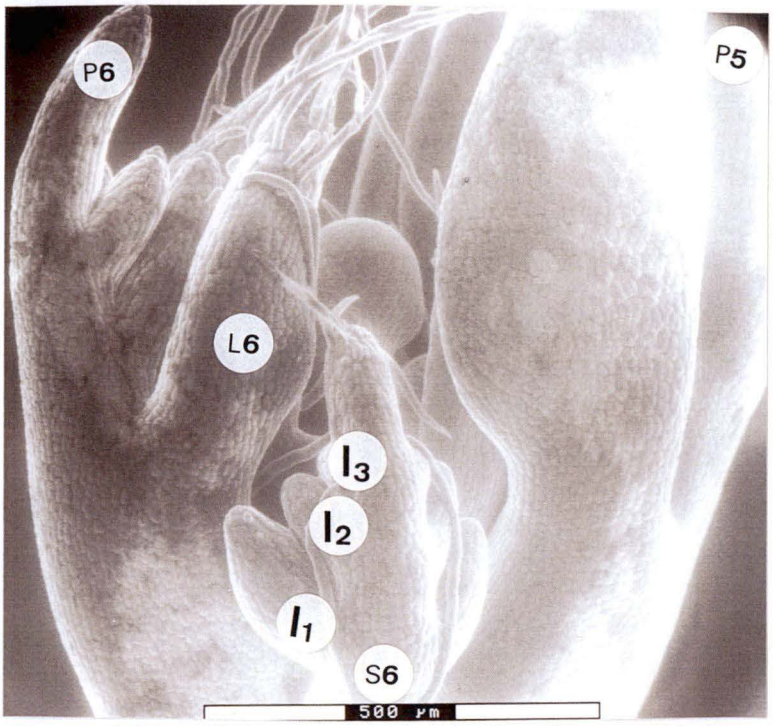


Figure 2.21 P_7 leaves at node 13 of *coch* mutant (left) and wild-type (right) plants, showing retarded development of *coch* stipules.
(The apical meristem of the *coch* mutant has been removed so the stipules can be seen.)

Figure 2.22 P_8 leaves at node 12 of *coch* mutant (right) and wild-type (left) plants, showing retarded development of *coch* stipules.
(The apical meristem of the *coch* mutant has been removed so the stipules can be seen.)

Figure 2.23 P_9 leaves at node 11 of *coch* mutant (left) and wild-type (right) plants, showing retarded development of *coch* stipules.

Figure 2.24 A lobed, sessile *uni* leaf (node 8).

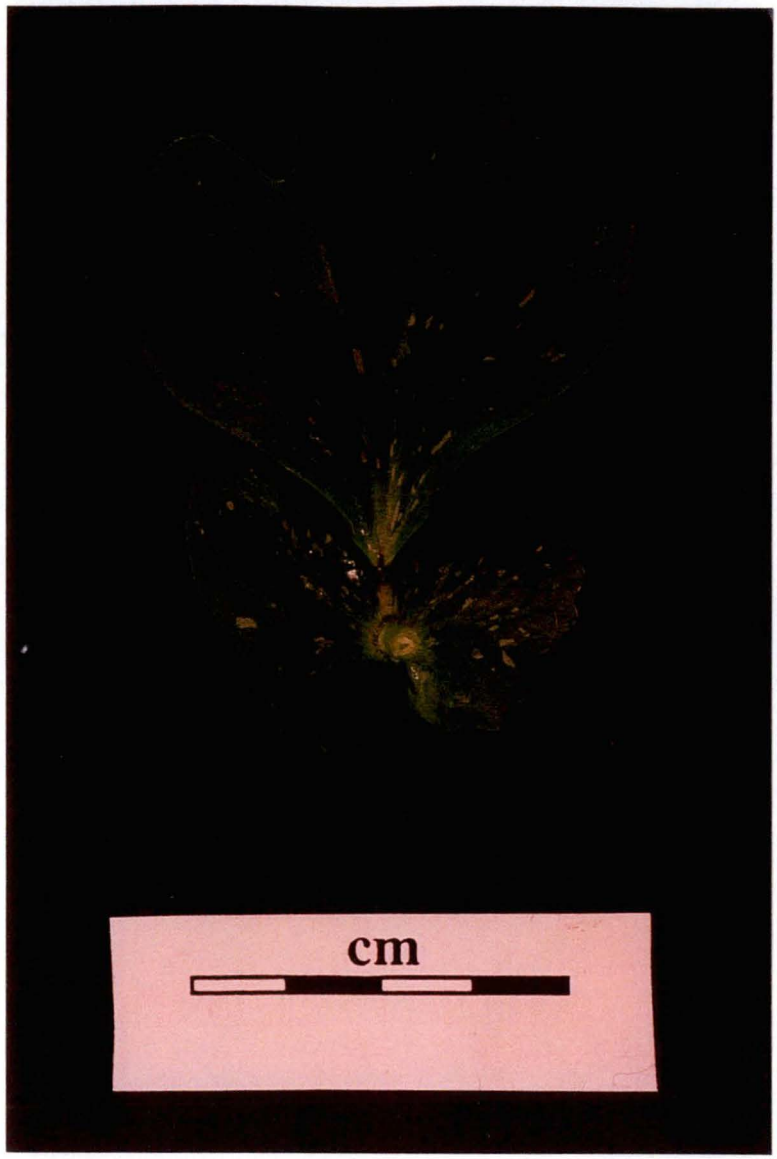


Figure 2.25 Occurrence of lobed leaves at different nodes in *unifoliata* mutant plants (JI 2171)
(Two separate plantings of ten plants each were examined to determine these percentages.)

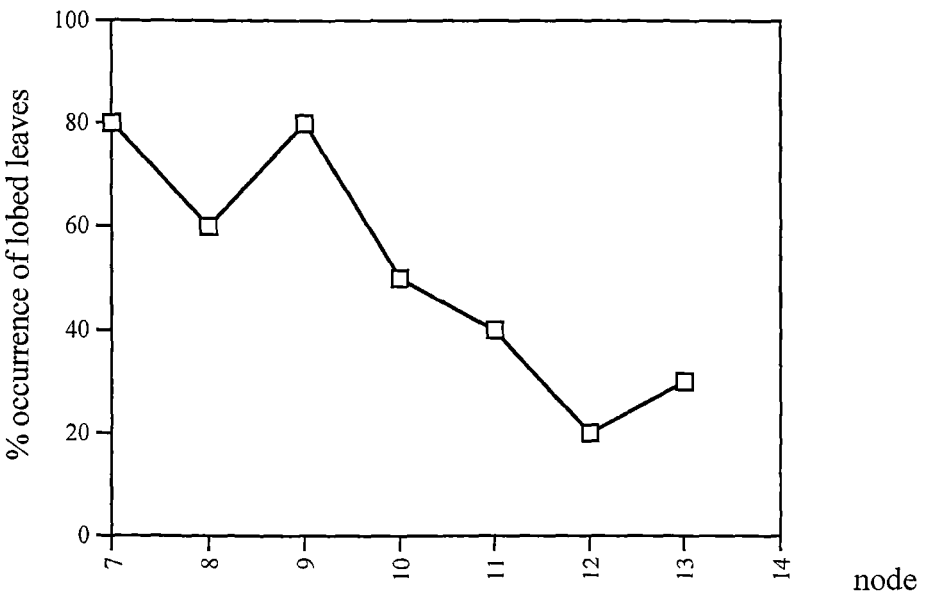


Figure 2.26 Occurrence of sessile leaves at different nodes in *unifolata* mutant plants (JI 2171)
(Two separate plantings of ten plants each were examined to determine these percentages.)

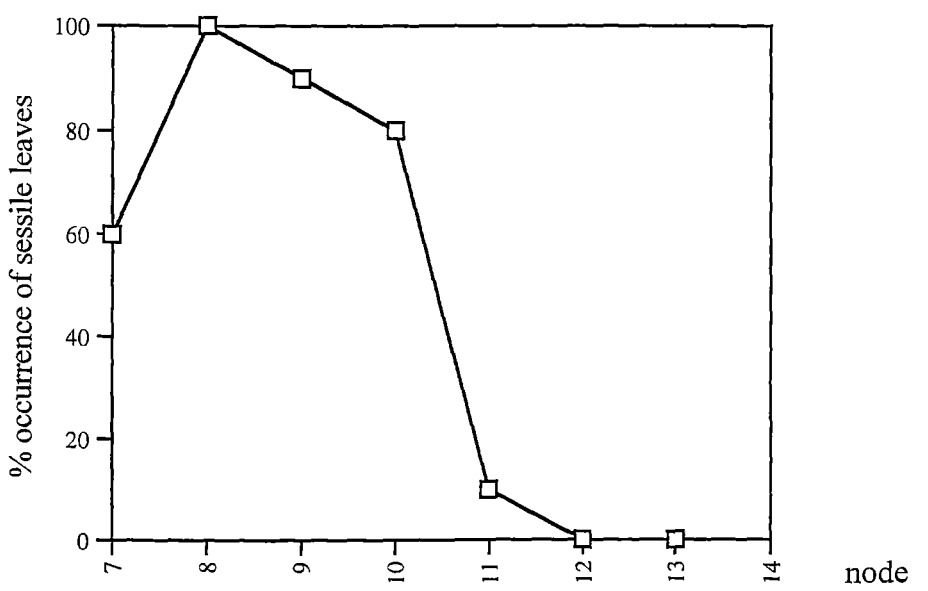


Table 2.1 Number of floral organs in wild-type and *coch* mutant flowers (Means with different letters in the same row are significantly different [$p<0.02$])

Floral organ	Average number of organs ± standard error		
	Wildtype (n=50)	*coch* mutant weak phenotype (n=20)	*coch* mutant strong phenotype (n=20)
Sepals	5.0 ± 0 a	6.0 ± 0.2 b	7.1 ± 0.4 c
Petals: standard	1.0 ± 0 a	1.0 ± 0 a	2.7 ± 0.2 b
wing	2.0 ± 0 a	2.1 ± 0.1 a	2.9 ± 0.3 b
keel	2.0 ± 0 a	2.8 ± 0.1 b	2.5 ± 0.2 b
Anthers	10.0 ± 0 a	11.8 ± 0.4 b	12.6 ± 0.8 b
Carpels	1.0 ± 0 a	1.0 ± 0 a	1.8 ± 0.6 b

CHAPTER THREE

Leaflet Tip Development: The Mutant *Insecatus*

INTRODUCTION

The *insecatus (ins)* mutant (Lamprecht, 1959) (Fig. 3.1) has an incised notch containing a tendril at the leaflet tips of some leaves. This notch with a tendril occurs mostly (90%) in the first leaflet pair, and mostly (70%) on a single leaflet of the pair. Lamprecht (1959) found that the degree of incision in the leaflet tips was variable, rather than being a sharply defined qualitative character. However, despite this difficulty in classifying phenotypes, he reported that the *insecatus* mutation was caused by a monogenic recessive gene, with some heterozygotes showing small leaflet-tip incisions. He could not demonstrate any certain linkage for the gene (Lamprecht, 1959).

A new mutant which was found to be allelic to *insecatus* (here designated *Ins-2,* with the original mutation designated *Ins-1*), was found in two different but related crosses (Fig. 3.2). The action of *insecatus* at the tip of the leaflet is similar to the action of the *sil af* double mutant at the stipule tip (Fig. 1.1i). The double mutant *af sil* has some stipule tips that are deeply incised and from which adventitious tendrils arise (Marx, 1977). This transformation is homeotic in nature; the tendrils arising from the notched stipule tips have the same form as the tendrils in the distal portion of the leaf. In the triple mutant *af tl sil* the notched stipule tips contain branched tendrils terminating in small leaflets (Marx, 1977).

This chapter describes the *insecatus* mutant in detail and examines its interactions with other pea leaf development mutants to investigate whether it is also homeotic and gives additional developmental potential at the leaflet tip.

MATERIALS AND METHODS

Plant Materials

The lines of *Pisum sativum* L. used during this work are held in the collection at Hobart, Australia. The *Ins-1* line JI 793 is from the original mutant line isolated by Lamprecht (Lamprecht, 1959) (provided by J. Hofer, John Innes Institute, UK). The first phenotype described here (Fig. 3.2) was found in a cross between Hobart multiple marker line HL111 and a branching mutant (S2-271) which has a cultivar Solara background. The second mutant phenotype (Fig. 3.3), with additional tendrils, but no notches at the leaflet tip, was found in a planting of cultivar Borek. (Both mutants were originally detected by I. Murfet.) These two mutant phenotype lines have some genetic material from S2-271 in common in their genetic background, and so they may not represent separate alleles, but may be different phenotypic expressions of the same allele in different genetic backgrounds. Therefore they have both been designated as the *Ins*-2 allele and when necessary their background line will be described to distinguish them.

The cv. Borek line has been backcrossed once to its progenitor line to remove any additional unlinked mutations that might be carried in the line. However, as the other mutant phenotype was isolated from a multiple marker line, a suitable wild-type line with which to compare the mutant is still being generated. Lines of *Ins-2* which carry different additional mutant leaf genes have been selected. Pure-breeding lines of *Ins-2 tl*, *Ins-2 af* and *Ins-2 af tl* have been generated (from the multiple marker line/cv. Solara background). Hobart line 107 (HL107, cv. Torsdag) was used as a wild-type line with which to cross *Ins-1* for checking the inheritance of the mutant gene.

For generation of the *Ins-2 coch* double mutant, *Ins-2* (from the cv. Solara background, which has the strongest mutant phenotype) was crossed with the *coch* line AF99 isolated (by J. Weller) in a mutagenesis programme using cv. Torsdag (HL107). The *Ins-2 uni* double mutant was obtained by crossing *Ins-2* (from the cv. Solara background) with a *uni* line (M1 224) generated in the same mutagenesis programme. This new *uni* allele has a weaker phenotype than that of *uni* line JI L2171 used in the previous chapter, and has a greater number of trifoliate, rather than unifoliate, leaves. The allelism of this new *uni* line was checked by S. Taylor, by crossing it with the uni^{tac} line JI L1396 (provided by J. Hofer). The uni^{tac} allele is a weaker allele with more normal flower morphology, which facilitated crossing of the two lines.

Growing Conditions and Morphological Characteristics

Preparation of pots, planting, watering and nutrition and fungicide/pesticide treatment of plants were as described in Chapter 2. To test the effect of temperature

on the growth of *Ins-2* plants, 28 plants (from the cv. Solara background) were grown on the glasshouse apron during winter. The average day temperature was 21°C and the average night temperature 14°C. The photoperiod was 18 hours of light, with natural daylight extended by mixed incandescent and fluorescent lights as described in Chapter 2. At the same time 20 plants were grown in each of two Thermoline growth cabinets (Thermoline, Australia) at 12°C and 25°C (± 1°C). Twelve plants survived at 12°C. The photoperiod in the growth cabinets was 18 hours, provided by a mix of 14 fluorescent tubes (36 W/W cool white, Thorn Australia) and 4 incandescent globes (60 W Pearl, Thorn Australia), which delivered approximately 100μmols m^{-2} sec^{-1} at the pot surface.

To test the effect of photoperiod on the growth of *Ins-2* plants (from the cv. Solara background), 20 plants were grown in a phytotron with a photoperiod of 8 hours of daylight. The plants were grown on trucks which would be positioned in the glasshouse during the day and move into dark bays (with a night temperature of 16°C) at the appropriate time. Basal laterals were removed with a razor blade as they grew out from plants grown in short-days, as the vigorous growth of basal laterals can weaken the growth of the main stem.

For examination by electron microscopy 20 *Ins-2* plants, and 20 *Ins-2- tl tl* plants (both from the cv. Solara background) were grown. Once nine leaves were expanded, three plants were decapitated every two to three days and the apex examined using environmental scanning electron microscopy (ESEM 2020, ElectroScan) as described in Chapter 2. The development of leaves at nodes 15-19 was examined, but no deviation from normal development could be detected in the small apices. In larger apices, with leaflets greater than 500μM long, the development of hairs at the tips of the leaves caused problems with electrical discharge. Therefore the experiment was repeated, but developing leaflets were examined using a dissecting microscope (Zeiss, Stemi SV6). Photographs were taken under tungsten lights (Schott KL 1500 electronic light source) using a Contax 167 MT 35mm camera body and Kodak Tungsten Ektachrome film. This second group of 20 *Ins-2*, and *Ins-2- tl tl* plants was grown during autumn when the average day temperature was 22°C, and the average night temperature was 14°C, with a photoperiod of 18 hours light, as before.

The length of leaflets of *Ins-2* plants from each of the cv. Solara and cv. Borek backgrounds was measured from 20 plants grown in the glasshouse in spring/summer (average day temperature 25°C, average night temperature 15°C) with an 18 hour photoperiod. Leaflets were measured with a ruler to the nearest mm in length.

Scoring of nodes was as described in Chapter 2. Unless otherwise stated, only leaves with a distinctive incision, or protruding tendril structure at the tip of the leaflet, were scored as showing the mutant phenotype. Node of flower initiation was counted acropetally (from the cotyledons at node 0) to the node with the first formed flower initial.

RESULTS

Inheritance and allelism of *insecatus* and its putative new allele

Inheritance of *insecatus*

The *insecatus* mutant was reported as recessive by Lamprecht (Lamprecht, 1959), but observations suggested that it may be dominant. Both F_1 plants of the cross HL107 (cv. Torsdag) by *ins* strongly expressed notched leaflet tips at nodes 24-30, suggesting that the mutant allele is dominant. However, only 30% of the 64 F_2 plants (32 from each F_1 parent) of this cross expressed notched leaflet tips, suggesting that the mutant allele is recessive. To examine its inheritance further the progenies of twelve F_2 plants expressing notched leaflet tips and twelve F_2 plants with wild-type leaves from the cross above were grown, making 188 F_3 plants in all. If the mutant allele was recessive then all the F_2 plants with notched leaves would have offspring with notched leaves only, and generate no wild-type F_3 segregates. If the mutant allele was dominant, but with poor penetrance, then the F_2 plants expressing notched leaflet tips would generate in total one sixth wild-type progeny. One twelfth of the F_3 plants from F_2 parents with the mutant *ins* phenotype were wild-type in appearance. This was less that the one sixth expected if *ins* was dominant, but this was probably because only F_2 plants strongly expressing the mutant phenotype were chosen to grow progeny from, so more could have been homozygous than heterozygous for the mutation. The offspring of these wild-type F_3 plants from F_2 plants with notched leaves were grown, and all retained the wild-type phenotype. This indicated strongly that *ins* is a dominant allele with variable penetrance depending upon genetic background. If the *ins* allele was recessive, plants with the *ins* mutant phenotype could not have wild-type offspring that bred true. In light of this, the gene symbol for the original mutation could be changed to *Ins-1* to indicate its dominant nature, but the penetrance will be variable depending on the genetic background of the plants in question. Hereafter in this chapter, the allele designated *ins* by Lamprecht (Lamprecht, 1959), will be referred to as *Ins-1*.

Allelism testing

The *Ins-1* line was crossed with the putative new *Ins-2* mutant (from the Solara background, as it has the strongest phenotype) to determine if the two similar mutants were allelic. If the two mutants are allelic, the F_2 progeny should all express the mutant notched leaflet tip phenotype. If the two mutants are not allelic then one sixteenth wild-type plants would be expected in the F_2 progeny as *Ins-1* is shown above to probably be a dominant allele. The three F_1 plants all expressed notched leaflet tips. An F_2 population of 181 plants was grown. All of these expressed notched leaflet tips, apart from 32 that had a long point at the leaflet tip (see Figs. 3.4, 3.5 and 3.6) and 29 that were wild-type in appearance. However, this did not necessarily indicate that the two genes *Ins-1* and *Ins-2* were not alleles, as *Ins-1* had been shown to have poor penetrance.

To examine this possibility the offspring of all the wild-type F_2 plants and all the F_2 plants with pointed leaf tips were grown. All of these F_2 plants had F_3 progeny that had definite notched leaflet tips, indicating that they were carrying the *Ins-1* or *Ins-2* gene, but had not expressed the notched trait in the F_2 generation. The only situation in which all of these 61 F_2 families would express the mutant *Insecatus* phenotype in the F_3 generation is if the *Ins-1* and *Ins-2* genes are allelic. If they were not allelic, some proportion of pure-breeding wild-type plants would be expected. Thus, *Ins-1* and *Ins-2* are allelic, as was strongly indicated by their similar phenotypes. This result also lends support to the proposition that the abnormally long point at the tip of a leaflet is a weak form of expression of the *Ins* phenotype.

Inheritance of *Ins-2*

Ins-2 plants from both the Solara and Borek backgrounds have been crossed twice each with the wild-type line HL107 (cv. Torsdag) and on both occasions all eight F_1 plants showed typical *Ins-2* mutant phenotypes. This indicates that the *Ins-2* allele is dominant. This is consistent with the evidence above that the *Ins-1* allele is dominant.

Phenotype of *Ins-2*

On close observation, it was seen that mutant *Ins-1* and *Ins-2* plants all produced a range of phenotypes. There were leaflets with a small protuberant tendril at the tip, leaflets with two or three short tendril-structures at the tip, and leaflets with small or large notched incisions at the tip that contained protruding tendrils (Figs. 3.4, 3.5, 3.6). It was clear that the protruding tendril tissue in the mutant leaflets is tendrillar in nature, rather than being similar to the mid-vein or veins, because it is thigmotropic and commonly seen wrapping around other leaves and stems.

In those leaves with the strongest mutant phenotypes, there was a branched, rather than a single, tendril in the notched incision at the leaflet tip (Fig. 3.5). Very large 'notches' also tended to have overlapping rounded edges (Fig. 3.5). Smaller 'notches' commonly had thigmotropic tendril-like tissue on each side of the 'notch' incision in the leaflet tip (Fig. 3.7).

In the case of *Ins-1* and *Ins-2* in the Solara background, the notched incision at the leaflet tip containing the protruding tendril (Figs. 3.1, 3.2), was the most common form of mutant phenotype. It consisted of approximately 95% of mutant leaflet tips in the case of *Ins-2* in the Solara background. For *Ins-2* in the Borek background, the most common mutant phenotype was a 'trident' of tendril-like structures at the leaflet tip (Fig. 3.8 and Fig. 3.6). This accounts for approximately 90% of mutant leaflet tips for *Ins-2* plants in this background. Incisions at the leaflet tips are much less common in *Ins-2* mutant plants in the Borek background than in the other *Ins* lines.

Mutant leaflets do not occur at every node in *Ins*-1 and Ins-*2* mutant plants. On average there are approximately three leaflets per plant that display the mutant phenotype. The leaflets that express the mutant phenotype are most commonly those that occur at nodes near the flowering node, and are almost always confined to the first pair of leaflets in a leaf.

The expression of the *Ins-2* allele is affected somewhat by temperature and day-length (Fig. 3.9, 3.10, 3.11). The typical form of the mutant leaflet tip is not affected, but the node and frequency of occurrence of mutant leaflet tips are affected. This is not surprising as the form of the wild-type plant is also affected by day-length and temperature.

To test the effect of temperature *Ins-2* plants (from the Solara background) were grown at 12°C, 21°C and 25°C (Fig. 3.9). The optimum growing temperature was 21°C, with plants flowering earlier and having fewer nodes at this temperature (Table 3.1, Fig. 3.9). At each temperature the mean node for the occurrence of a notched leaflet tip was approximately one node below the node of initiation of the first flower (Table 3.1). The occurrence of the notched mutant phenotype was less common at 21°C, with an average of 1.3 notched leaflet tips per plant (n=28), than at 12°C, with an average of 2.1 notched leaflet tips per plant (n=12), or at 25°C, which had the highest average of 2.5 notched leaflet tips per plant (n=20).

To test the effect of a short day photoperiod *Ins-2* plants (from the Solara background) were grown in 8 hours of light, rather than the normal 18 hours of light, at 21°C (Fig. 3.10). Under short-day conditions, as for wild-type plants, plant growth was extended, flowering was delayed and there was a greater number of nodes (Table 3.1). Notched leaflet tips occurred over a greater range of nodes than in long-day conditions (Fig. 3.10). However, there were fewer mutant leaflet tips per plant in the short day photoperiod, with an average of only 1.0 notched leaflet tips per plant (n=20), as compared with 1.3 mutant leaflet tips per long day plant (n=28). Node of first flower initiation and total number of nodes also showed a greater standard deviation under short-days than under the long-day photoperiod (Table 3.1). The mean node of occurrence of the notched leaf phenotype was three nodes below the node of first flower initiation under short days, rather than one node below as occurred in long days (Table 3.1). This could be due to the mutant phenotype spreading over a greater number of nodes than is seen in short days (Fig. 3.10). However, in both long and short days approximately 70% of the number of total nodes occur before the average node of *Ins-2* expression.

Structure of *Ins* leaflet primordia

No change in the structure of *Ins-2* leaflet primordia is visible when the primordia are less than 5 mm long. Careful examination of the emerging primordia using environmental scanning electron microscopy (as per meristem examination in

Chapter 2) showed that wild-type and *Ins-2* leaflet primordia appear identical in the early stages of development.

Notched incisions in the leaflet tips of *Ins-2* plants became visible under a dissecting microscope when the leaflet primordia were 9 to 10 mm long. Both the incised notches with protruding tendrils of *Ins-2* plants (Fig. 3.11), and the lobed leaflets in the rounded notch of *Ins-2 tl* plants (Fig. 3.12) are visible at this stage. Thus, alterations in the leaflet primordia to produce the *Ins-2* phenotype occur at a late stage of leaf development.

Length of mutant *Ins-2* leaflets

The length of *Ins-2* leaflets (from the Solara background) expressing the mutant phenotype was compared with the length of leaflets that were wild-type in appearance. Leaves on mutant plants in which one leaflet of the first leaflet pair was mutant in appearance and the other leaflet of the pair was wild-type in appearance were examined. It could therefore be ascertained whether the incision in the mutant leaflet tips was likely to be due to reduced growth or additional growth at the leaflet tip.

It was found that leaflets of the *Ins-2* mutant which express the mutant phenotypes were longer (not including the protruding tendril) than the leaflets opposite them that did not have altered tips (Table 3.2). So, the notch structure at the mutant leaf tips seems to be due to additional growth at the leaflet tip. However, the length from the base of the leaflet to the base of the incision in a mutant leaflet, was shorter than the length of leaflets with a wild-type phenotype. It follows that the mutant notch structure is not just a simple addition to a normal wild-type leaflet tip. These results were confirmed by measuring leaflet length in a second independent planting of *Ins-2* mutants (data not shown).

Similarly, it was found that the lengths of mutant *Ins-2* leaflets from the Borek background, were mostly longer than the opposing leaflets of wild-type phenotypes (Table 3.3). When the length of the tendril-structures at the leaflet tip was also included in the length of the mutant leaflets, they were significantly longer at all nodes than the leaflets with wild-type phenotypes (Table 3.3). Thus, there is additional growth at the leaflet tip of *Ins-2* mutant leaflets in both backgrounds. This additional growth occurs in the laminar tissue of the leaflet tip at most nodes, as well as the additional tendril-structures that appear.

For both *Ins-2* backgrounds, the length of leaflets of the first leaflet pair in leaves not expressing the mutation was measured. These lengths were compared with the leaflets that were wild-type in appearance, but found opposite mutant leaflets. There was no significant difference in length between leaves that were wild-type in appearance whether they were opposite an expressing or a non-expressing leaflet in a pair. Thus, the mutant phenotypes of *Ins-2* leaflets appear to be due to additional growth at the leaflet tip, rather than reduced growth or cell death. A non-

expressing leaflet that is opposite the leaflet with the mutant phenotype, is not made shorter than normal by its mutant partner.

Interaction of *Ins-2* with other pea leaf mutants

Interaction of *Ins-2* and *tendril-less(tl)*

As one line of *Ins-2* was originally isolated from multiple marker line HL 111, progeny from this material provided the opportunity to examine the double mutant *Ins-2 tl*, as HL 111 carries the *tl* mutation. When *Ins-2* is present with the homozygous *tl* mutation, a leaflet appears in the incised notch at the end of the leaflet tip (Fig. 3.13), rather than the tendril that appears in *Ins-2* plants. The 'notch' at the leaflet tip of these double mutant plants is always in the form of two overlapping rounded edges. Tendril tissue does not appear at the edge of the incised notch as it does in *Ins-2* plants (see Fig. 3.7).

In double mutant *Ins-2 tl* plants with strong mutant phenotypes, branched leaflets with a petiole appear in the notch at the leaflet tip of expressing leaves (Fig. 3.14). These double mutant plants with strong phenotypes also have a red 'dot' of anthocyanin pigment at the base of the petiole in the notch at the leaflet tip (Fig. 3.15). A dot of pigment normally appears at the petiole base of wild-type leaflets in plants carrying the appropriate pigment genes (Fig. 3.14). This provides evidence that the distal leaf portion is being re-iterated in the notch at the leaflet tip.

Plants heterozygous for *tl* (with the genotype *Tl tl*) have flattened tendrils. Mutant plants in the segregating population with the genotype *Ins-2 Tl tl* also had flattened tendrils appearing in the incised notches at the mutant leaflet tip. Thus, the form of the tip of the leaf is reflected at the tip of the *Ins* leaflet.

Interaction of *Ins-2* and *afila(af)*

Multiple marker line HL 111 also carries the *afila* mutation. Initial observation of segregating populations showed no apparent change to *af* mutant plants that carried the *Ins-2* mutation. Also there were no apparent changes to the tiny leaflets present in *af tl* double mutant plants (Fig. 2.13) when the plants also carried the *Ins-2* gene. Thus, it seemed that *af* was epistatic to *Ins-2*. To further examine this observation two pure-breeding lines of *Ins-2 af* and *Ins-2 af tl* were selected. Twenty plants of each line were grown at 12, 21 and 25°C, and in photoperiods of 8 and 18 hours. All plants were identical to the *af* or *af tl* double mutant phenotypes, respectively. Thus, *af* is epistatic to *Ins-2*; it appears that 'normal' leaflet tissue must be present for *Ins-2* to act and that the tiny 'leaflets' in *af tl* plants behave differently from normal-sized leaflets.

Interaction of *Ins-2* and *cochleata(coch)*

To examine the interaction of *Ins-2* and *coch*, a strongly expressing *Ins-2* plant (from the Solara background) was crossed with the new *coch* allele (AF99), isolated in Hobart by J. Weller. Five of the eleven F_1 plants expressed strongly

incised leaflet tips, and the other F_1 plants were wild-type in appearance. This could be due to the impenetrance seen in a cultivar Torsdag background (AF99 has a Torsdag background), or it is possible that the *Ins-2* parent plant was heterozygous. Sixty four seeds were planted from each of two F_1 plants with strong *Ins-2* mutant phenotypes. Plants in the F_2 progeny that were homozygous for the *coch* mutation were obvious because of their compound stipules. These compound stipules reflect the form of the main leaf rachis. However, of the twelve double mutant plants observed, eleven had strongly notched first leaflets in the main leaf rachis, but no notched tips present in the leaflets of the leaf-like *coch* stipules (Fig. 3.16). One double mutant plant with notched leaflets in the main rachis had small indentations in the tips of the leaflets of the *coch* leaf-like stipules (Fig. 3.17). This may represent a weak form of *Ins-2* expression in the leaf-like stipules of the *coch* mutant.

Interaction of *Ins-2* and *unifoliata (uni)*

Ins-2 (from the Solara background) was crossed with a new *uni* allele isolated in Hobart (M1 224) by J. Weller. This *uni* line has both unifoliate leaves with a single leaf blade and trifoliate leaves. *Ins-2* pollen was crossed onto the male sterile *uni* flowers and two F_1 seeds were formed. These F_1 plants were wild-type in appearance and 180 F_2 progeny were grown from them. Plants in the F_2 population that carried *Ins-2,* but were not homozygous for the *uni* mutation had incised notches in their leaflet tips containing protruding tendrils as usual. The 40 plants in the F_2 population which were homozygous for the *uni* mutation had some heart-shaped leaves with small indents at their tips, and some lobed leaves, but such leaf forms also occur in normal *uni* mutant plant populations (Fig. 2.24). Hence, it was not clear if these small indentations seen at the end of the *uni* leaves were due to *Ins-2* expression or not. However, two *uni* mutant plants in the F_2 population had trifoliate leaves that had definite incised notches with rounded edges, which occurred at the tip of the leaflet at the distal end of the leaf (Fig. 3.18). These notches each contained a leaflet-like structure. Notably, this expression seen in the trifoliate *uni* leaflet is at the distal tip of the *uni* leaf, and not in the side leaflets. This indicates that in *uni*, *Ins-2* expression may occur at the end of the simple leaf, rather than at the tips of leaflets, at the side of the compound leaf.

A low level of expression of the *Ins-2* notched leaf phenotype was found in both *Ins-2- Uni-* plants and *Ins-2- uni uni* plants in this F_2 population. This suggests that, again, *Ins-2* has poor penetrance in a cultivar Torsdag background, such as that of the *uni* line used here (M1 224). Other genes segregating in the population, such as *uni* and *le,* showed the correct proportion of mutant phenotypes (data not shown) indicating that the segregation of the cross was normal.

DISCUSSION

Inheritance and allelism of *Ins-1* and *Ins-2*

It has been determined that *Ins-1* is very likely a dominant gene with poor penetrance, as mutant *Ins-1* plants in segregating populations will generate wild-type progeny, which is not possible if the gene is recessive (as suggested by Lamprecht in his original description of the mutant (Lamprecht, 1959)). It is possible that Lamprecht did not grow advanced generations of his crosses to confirm his conclusion, but it seems likely that mutant plants with smaller incised notches at their tips are mostly heterozygotes as he suggested. The penetrance of *Ins-1* was not good (40-45%) in a cultivar Torsdag background, so it would be worthwhile examining other backgrounds to see if penetrance is increased. As the *Ins-2* line with the strongest phenotype was isolated from a multiple marker line, some additional work is also needed to develop comparable mutant and wild-type non-segregating lines for these alleles and to provide appropriate genetic material for future studies.

All but one of the other mutations affecting pattern formation in the pea leaf are recessive in nature. The *Scarlaris forma(Td)* mutant shows incomplete dominance and produces leaves with dentate stipule and leaflet margins above node 5 or 6 (Marx, 1987; Wellensiek, 1925). *Ins* is now the only known dominant homeotic pea leaf development mutant.

The discovery of a new allele at the *Ins* locus is valuable because the study of several alleles can reveal much more about the action of the gene than if a single allele only is known. It is possible that the phenotype of the weakest allele represents the most fundamental action of the mutant gene. The phenotype of the *Ins*-2 mutant in the Borek background probably represents the weakest phenotype of the *Ins* gene. The major phenotype of the *Ins-2* allele in the Solara background is an incised leaflet tip with a protruding tendril, and that of the *Ins-2* allele in the Borek background is a 'trident' of three short tendrils at the leaflet tip with no incision. It is possible that this typical 'trident' form is a type of short branched tendril and that *Ins-2* in the Borek background affects the leaflet tip later in development, or more distally in position than does the *Ins-1* allele. Gene isolation and molecular analysis of these alleles would reveal a great deal more about their mode of action, which would complement this classical genetic study. In particular it would be revealing to compare the sequences and expression patterns of the alleles and to try and create a loss-of-function mutant.

Phenotype of *Ins-2*

Neither temperature nor day-length substantially affects the *Ins-2* mutant phenotype, which suggests that the developmental processes controlled by this gene do not have a strong environmental input. However, it is apparent that ontogeny is

important to the action of *Ins*, as in all environments examined the *Ins-2* mutant phenotype appeared in the leaves when 70-75% of the plant's leaves had been expanded. It is possible that the action of *Ins* is connected with the physiological changes that lead to flowering or with changes in leaflet size that occur in relation to it. The largest leaflets occur just before the node of first flower initiation, and this is where the mean node of *Ins* expression also occurred. This correlates with the reduced *Ins* expression, or lack of *Ins* expression seen in the small leaflets of compound *coch Ins* stipules, or in the tiny distal leaflet of *af tl* double mutants. However, it is also possible that this reduced expression is due to altered gene expression in these leaflet structures. For instance, we know that *Uni* is expressed in developing compound *coch* stipules (Gourlay et al., 2000) and in the small distal *af tl* leaflets (Hofer et al., 1997), whereas it is not expressed in wild-type leaflets (Gourlay et al., 2000).

The phenotype of the *Ins* mutants shows an important change in structure from mid-vein to tendril. The thigmotropic tendril which protrudes from the incised notch, or from the leaflet tip, grows directly out of the mid-vein of the leaflet. While not part of this study, this change in structure and function between mid-vein and tendril would be worthwhile examining at the tissue level, and may provide insight into the development of thigmotropism.

The structure of *Ins-2* leaf primordia and the length of *Ins-2* leaflets

The phenotype of *Ins-2* leaf primordia is wild-type until the developing leaflets are 9 to 10 mm long. Thus, it seems likely that *Ins* acts late in the leaf developmental pathway. Leaflets of *Ins-2* plants that are expressing the mutant phenotype are also consistently longer than leaflets that are wild-type in appearance. This strongly suggests that *Ins* acts by adding new tissue to the leaflet tip, rather than producing a 'notch' at the leaflet tip through selective cell death or reduced growth.

Interaction of *Ins-2* with other pea leaf mutants

The interaction of *Ins-2* and *tl* as seen in the double mutant *Ins-2 tl* shows that the action of *Ins-2* is homeotic in nature. In a *tl* background the *Ins-2* mutation adds a leaflet-like structure to the mutant leaflet tips, rather than a tendril as in the wild-type. This also occurs in the *Ins-1 tl* double mutant (O. Smirnova, personal communication).

It appears that the *Ins* alleles require the presence of the wild-type *Af* gene in order to express. In the double mutant *Ins-2 af* and the triple mutant *Ins-2 af tl* the *af* mutation is epistatic to *Ins-2*, and the plants appear exactly like *af* and *af tl* mutants, respectively. It seems likely that normal-sized leaflet lamina tissue must be present for *Ins* to express. This could be confirmed by examining the *Ins-2 af uni* or *Ins-2 af*

uni^{tac} triple mutants, as these plants have reasonably large leaf lamina areas as well as carrying the *af* mutation. However, establishing a higher level of *Ins-2* expression in a *uni* line than was achieved in this study would be a prerequisite to producing these triple mutants. There are several other genes that require the presence of *Af* to be expressed (Marx, 1987). These are the wax distribution genes *wachslos(wlo)* (Nilsson, 1933) and *vix-cerata(wb)* (Vilmorin, 1913) and the gene *apulvinic(apu)* (Harvey, 1979) which produces leaflets on long petioles. If *af* is epistatic to *Ins-2*, then we can surmise that *Ins-2* acts after *af* in the genetic pathway of pea leaf development.

It is possible that the leaflets of *coch* leaf-like stipules may be too small to express *Ins-2* mutant leaflet tips. (As discussed above, the same may be true of the small *af tl* leaflet-like structures, but examining the triple mutant with *uni* should help to test this hypothesis.) It is also possible that the leaflets of *coch* leaf-like stipules may not be competent to respond to the *Ins-2* mutation; that is, *coch* may be epistatic to *Ins-2*. This would mean that the leaflets of *coch* leaf-like stipules are not the same as the leaflets of the main leaf rachis in their genetic control. This second possibility does not seem likely as *coch* leaf-like stipules reflect the phenotype of the main leaf rachis in all other mutant combinations where the second mutation does not affect the stipules (Marx, 1987), and *Ins-2* does not affect the stipules. It seems likely that the very small notches, that were seen at the end of the leaflets borne on the compound stipules of a double mutant *Ins-2 coch* plant, represent *Ins-2* expression in a *coch* background. The *Ins-2* mutation probably had a low level of expression in this cross as the *coch* line used has a cv. Torsdag background, in which *Ins-1* showed low penetrance. To confirm the double mutant phenotype it would be necessary to produce more *Ins-2 coch* plants. Crosses with different *coch* alleles on various backgrounds may improve the penetrance of the *Ins-2* phenotype.

The double mutant *Ins-2 uni* also showed low levels of expression of the putative double mutant. The phenotype of the double mutant needs to be confirmed by crossing *Ins-2* with other *uni* lines to try and improve expression of *Ins-2*. It is possible that a *uni* line with a more severe mutant phenotype of mostly unifoliate leaves, or in contrast the use of the weak allele *uni*^{tac}, may be a more useful genetic background. If the putative double mutant phenotype, of a notch containing a leaflet at the tip of the *uni* leaf, is correct, then this has significant implications for our understanding of the form of the *uni* leaf. It implies that the *uni* leaf (whether unifoliate or trifoliate) is the equivalent of a wild-type leaflet, with *Ins-2* expressed at its tip, and that the *uni* mutation stops the expression of genes that lead to the formation of multiple leaflets and tendrils.

The confirmation of double mutant phenotypes (especially in the case of *Ins-2- uni uni*) would be aided by the mapping of the *Ins* gene, because then it would be possible to use the segregation of a nearby marker to confirm the presence of the mutant *Ins* gene in a putative double mutant plant.

General Discussion

Ins is a dominant mutation, and hence probably represents a gain-of-function. Its action is to allow further development of the leaflet tip, through the addition of new material, late in leaf development. The *Ins* mutation gives the leaflet tip more developmental potential than it normally has. One could speculate that *Ins* may maintain the leaflet tip in a more meristematic state than the wild-type leaflet tip, so that it can respond to additional developmental signals. If the leaflet tip remains in a meristematic state, then it is possible that the phenotype of *Ins* is the result of the leaflet tip responding to signals that it cannot normally perceive, which are produced to control development of the distal tip of the leaf. That is, the gain of function may result from the loss of the normal shut down of development at the leaflet tip. The fourth terminal compartment of the compound pea leaf, as suggested by Hofer and Ellis (1998), may be the 'unit' that is repeated in the incised notch of the *Ins* leaflet tip.

Most leaves differentiate from the tip downwards (Lyndon, 1983). This is certainly so for maize (Freeling et al., 1988), tobacco (Poethig and Sussex, 1985), cotton (Dolan and Poethig, 1998) and *Arabidopsis* (Telfer and Poethig, 1994), (Lijsebettens and Clarke, 1998), although tomato leaflets develop marginal lobes acropetally (Sinha, 1999). The basipetal pattern of maturation probably applies to pea leaflets, as epidermal hairs first appear at the tip of the leaflet or stipule (Fig. 2.17), although observation of cell division patterns would be needed to confirm this. If this is the case, then the tip of the pea leaflet would normally be one of the most mature parts of the leaflet. Thus, it would be possible for *Ins* to give the leaflet tip greater developmental potential by delaying its maturation.

Figure 3.1 Leaf of the original *Insecatus(Ins)* mutant described by Lamprecht (JI 793), on the left, showing incised leaflet tip with protruding tendril, and a typical wild-type leaf on the right (cultivar Torsdag).
(JI 793 also has reduced stipules. These appear to be due to an allele of the mutant *stipules reduced(st)*, and are inherited independently of the leaflet-tip phenotype [data not shown].)

Figure 3.2 Mutant leaflet tip of an *Ins-2* mutant from the cultivar Solara background, showing a similar mutant phenotype to the original *Ins-1* line, with incised leaflet tips containing a protruding tendril. A leaflet tip with a wild-type phenotype from the same plant is shown on the left.
(*Ins-2* mutant plants have normal stipules.)

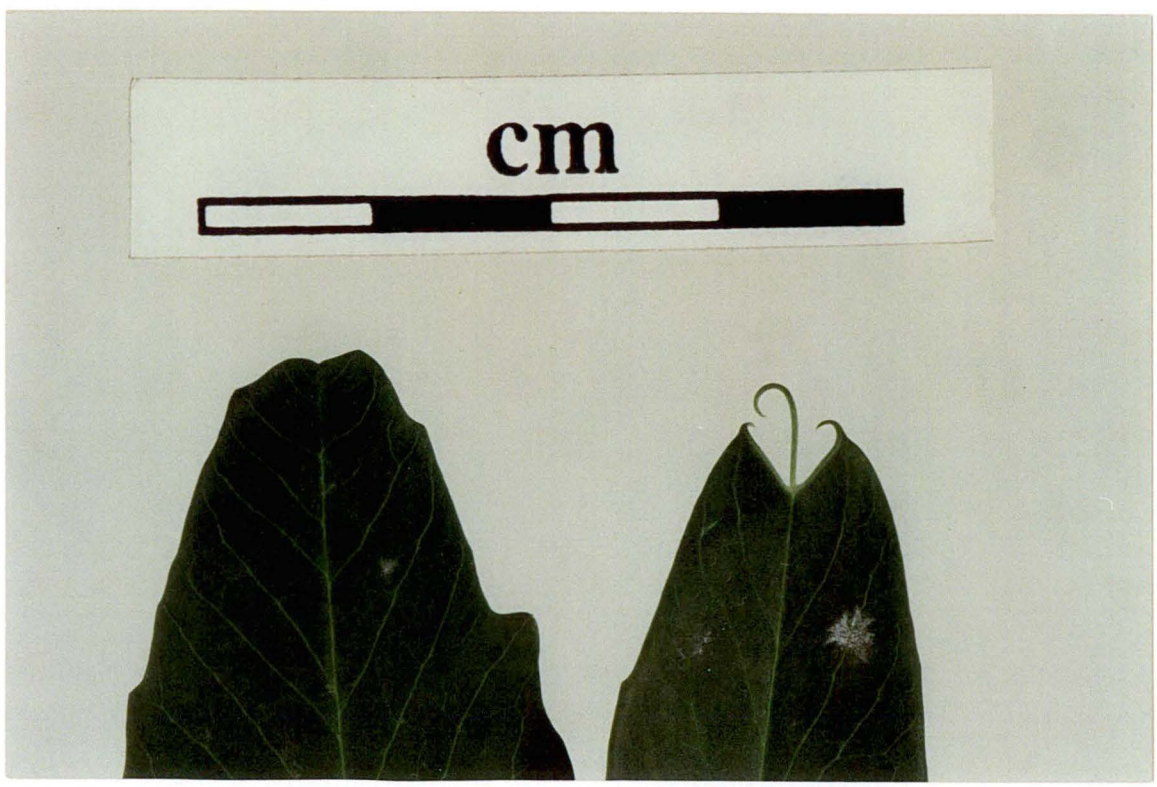


Figure 3.3 Mutant leaflet tip of an *Ins-2* mutant from the cultivar Borek background, showing a typical 'trident' structure of three tendrils at the leaflet tip, but no leaflet-tip incision (right). A leaflet tip with a wild-type phenotype from the same plant is shown on the left.

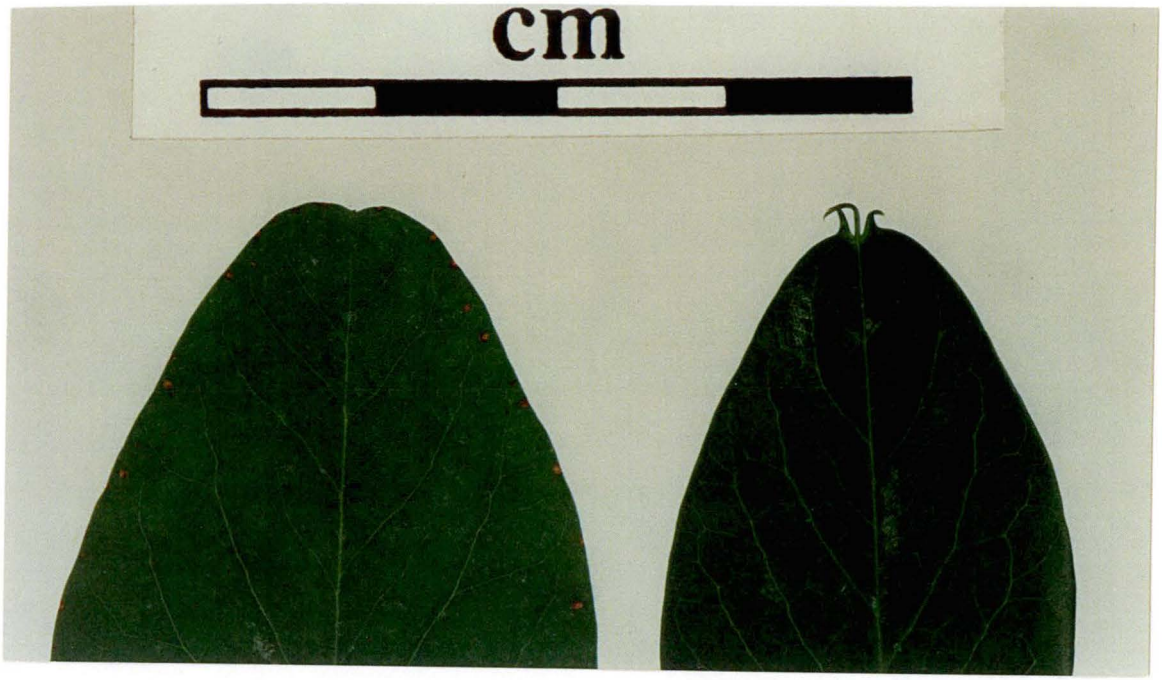


Figure 3.4 Mutant leaflet tips of the *Ins-1* mutant showing a typical range of mutant phenotypes. A leaflet of wild-type phenotype is shown on the top left, with mutant leaflet phenotypes then increasing in severity from a extra-long pointed leaflet tip, to a deep notched incision with a long protruding tendril at the bottom right.

Figure 3.5 Mutant leaflet tips of the *Ins-2* mutant from the cultivar Solara background showing a typical range of mutant phenotypes. A leaflet of wild-type phenotype is shown on the bottom left, with mutant leaflet phenotypes then ranging from a pointed leaflet tip with a protruding tendril, to a deep leaflet-tip incision with a protruding branched tendril in a lobed leaflet at the bottom right.

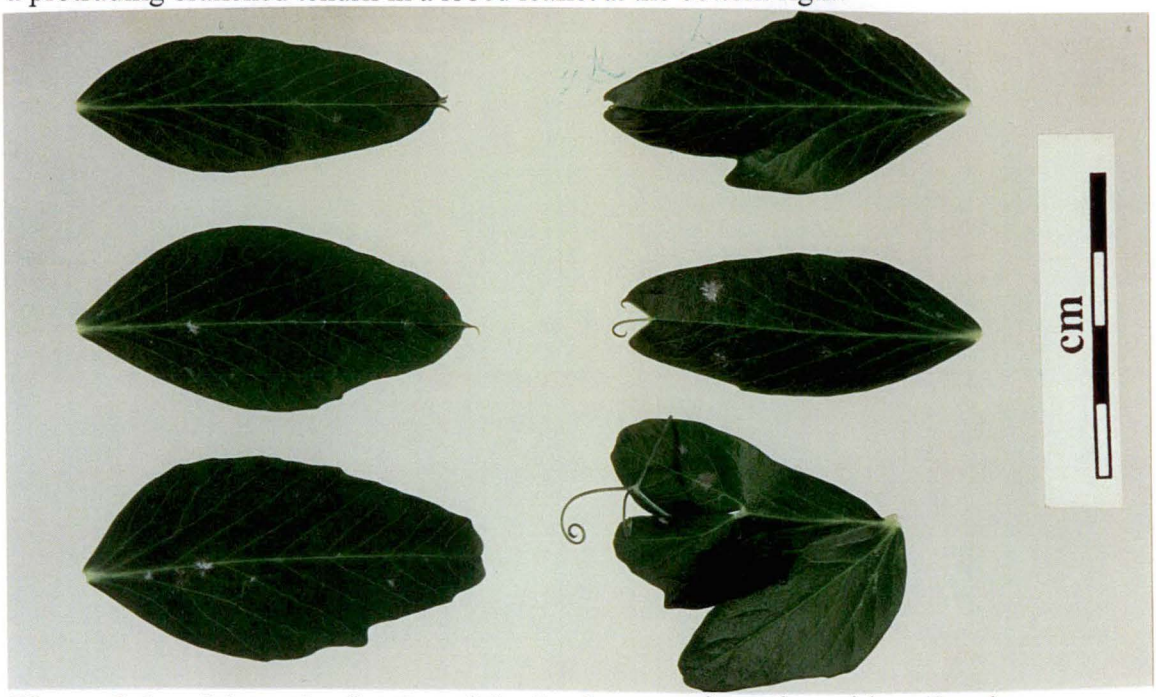


Figure 3.6 Mutant leaflet tips of the *Ins-2* mutant from the cultivar Borek background showing a typical range of mutant phenotypes. A leaflet of wild-type phenotype is shown at the bottom left, with mutant leaflet phenotypes then increasing in severity from a pointed leaflet tip with a protruding tendril, through leaflet tips with two or three protruding tendrils, to the most severe form with a incised notch at the tip with a protruding tendril in the incision at the top right.

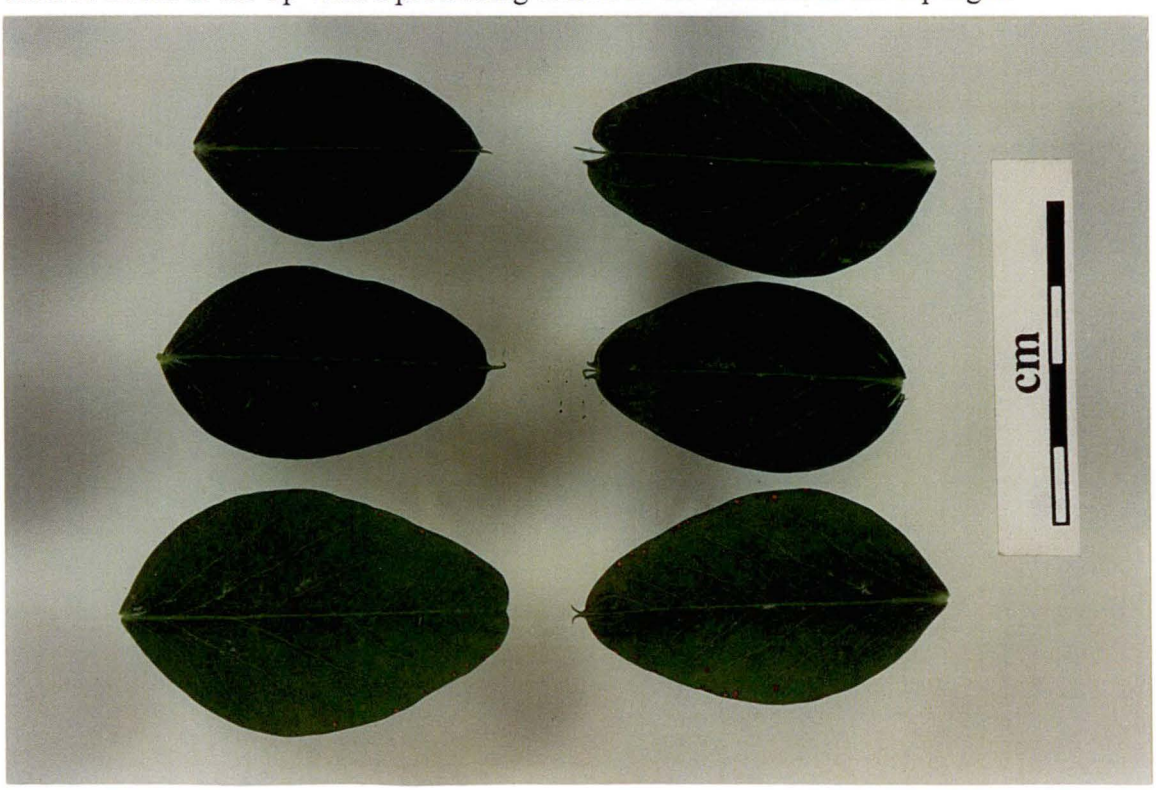


Figure 3.7 Leaflet tip (front and back) of an *Ins-2* mutant (from the cv. Solara background) showing the incised notch at the tip with protruding tendril-like tissue both within the 'notch' and along the sides of the notch. This is the most common mutant phenotype in this line.

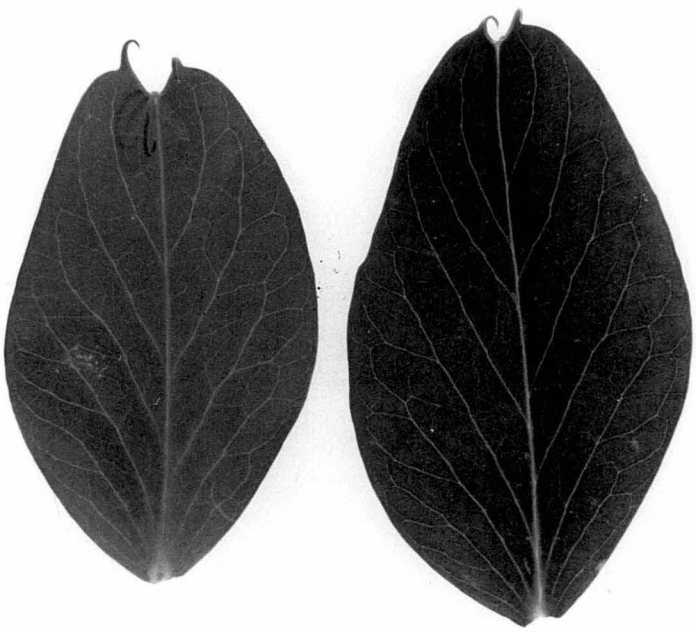


Figure 3.8 A 'trident' of tendril-like structures at the leaflet tip is the most common mutant phenotype was *Ins-2* in the Borek background.

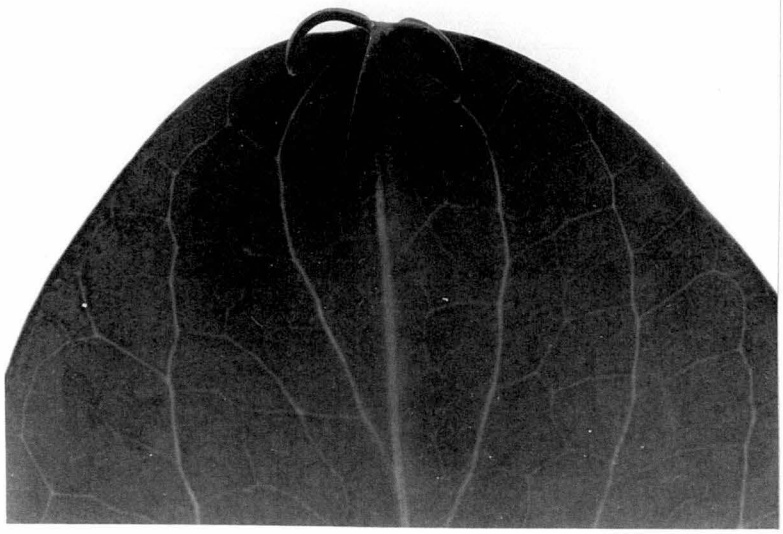


Figure 3.9 Occurrence of mutant leaflet tip phenotype in *Ins-2* plants (from the cv. Solara background) at 12 (n=12), 21 (n=28) and 25 °C (n=20).

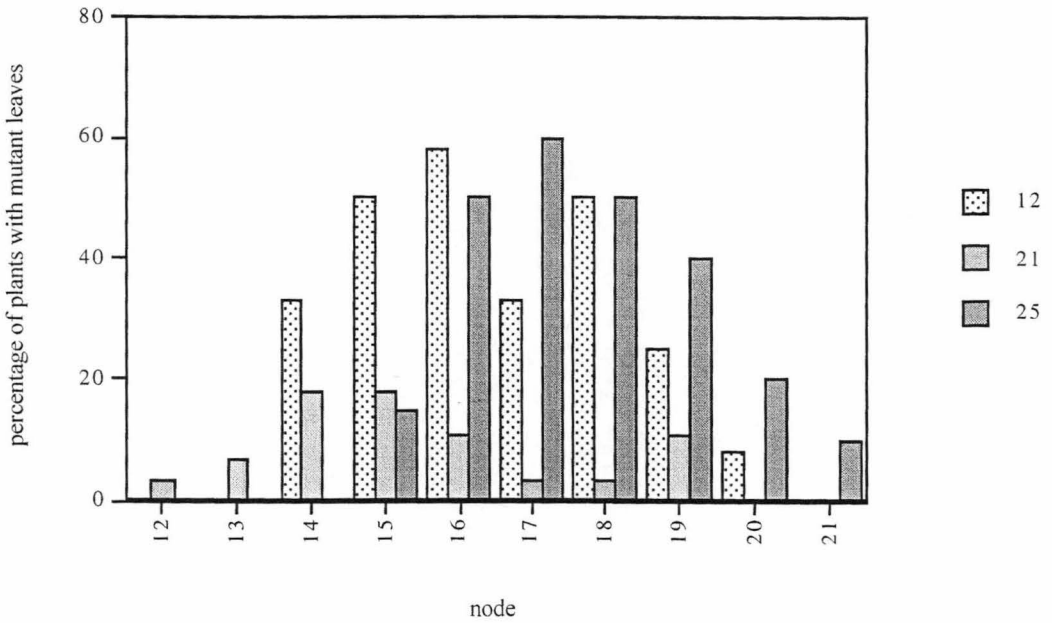


Figure 3.10 Occurrence of mutant leaflet tip phenotype in *Ins-2* plants (from the cv. Solara background) grown under a long-day (LD) photoperiod of 18 hours (n=28), or a short-day (SD) photoperiod of 8 hours daylight (n=20).

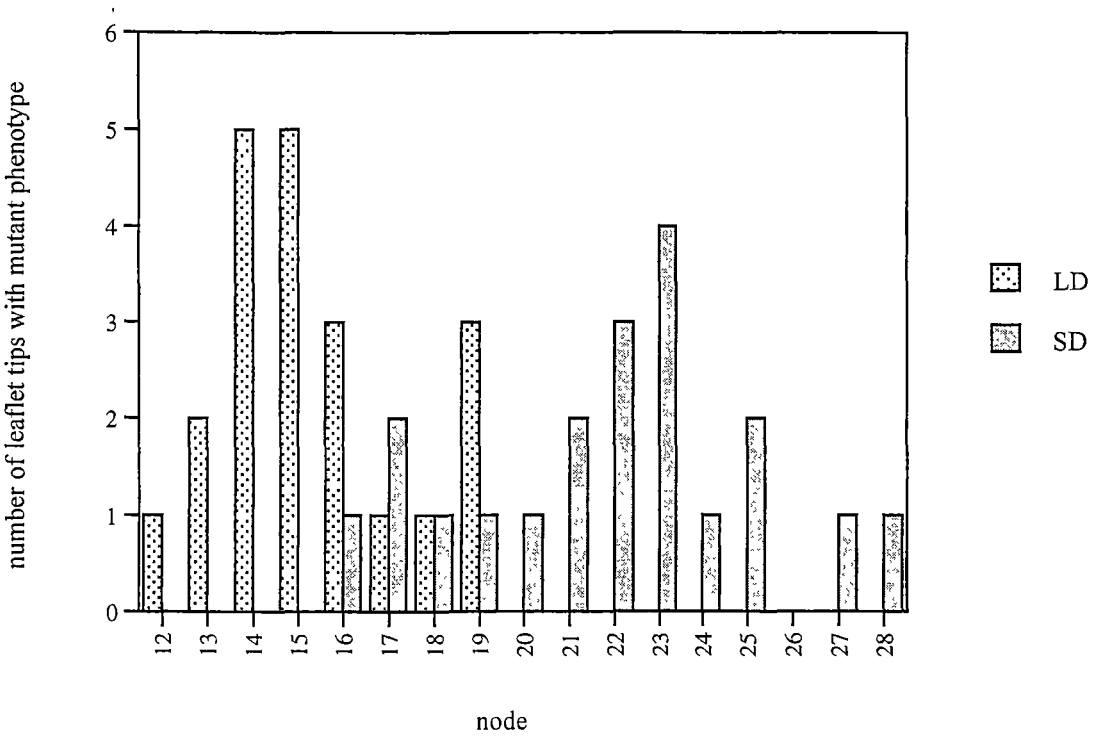


Figure 3.11 Developing leaflets of *Ins-2* plants with mutant tips are shown on the middle and right, with a developing *Ins-2* leaflet with the wild-type phenotype on the left. (Divisions in the scale bar are 5 mm.)

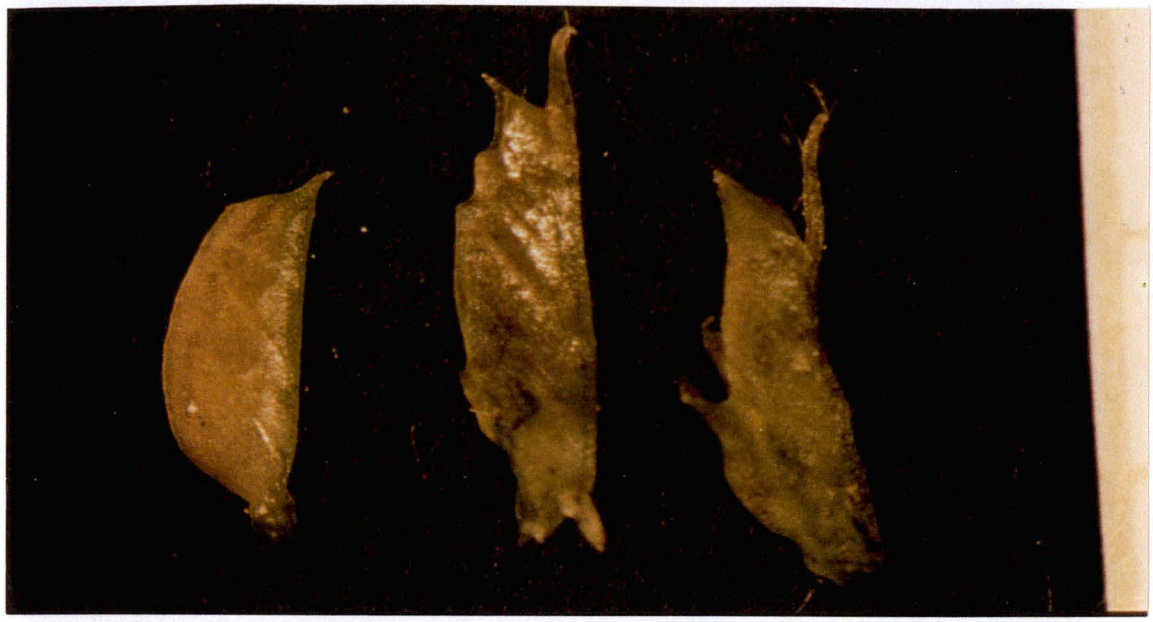


Figure 3.12 Developing leaf of an *Ins-2 tl* double mutant plant. The left leaflet (facing) of the primary leaflet pair shows a leaflet-like structure (arrow) developing in a notch at the tip of the leaflet. (Divisions in the scale bar are 5 mm.)

Figure 3.13 Mutant leaflet tip of the double mutant *Ins-2- tl tl,* showing a leaflet-like structure within the notch at the leaflet-tip, rather than the protruding tendril seen in *Ins-2* mutant plants (see Fig. 3.2).

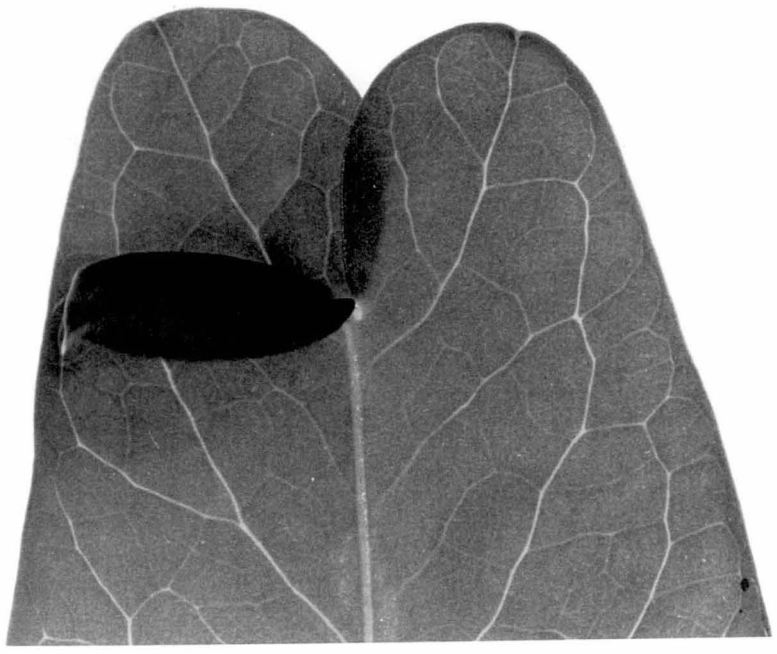


Figure 3.14 Leaf of an *Ins-2 tl* double mutant plant showing a strong mutant phenotype with branched leaflet-structures present in the incised notches at the tips of both leaflets of the primary leaflet pair. The structures in the notches at the mutant leaflet tips resemble the end of the leaf.

Figure 3.15 At the base of the incised notch at the mutant leaflet tip of the *Ins-2 tl* plant shown in Fig. 3.10 there is a dot of red anthocyanin pigment where the petiole of the branched leaflet structure in the notch joins the notch. This is reminiscent of the dots of anthocyanin pigment that appear where the petiole of a leaflet joins the main leaf rachis (as seen in Fig. 3.10).

Figure 3.16 Leaf of an *Ins-2 coch* mutant. The primary leaflet pair on the main leaf rachis show the incised notches in the leaflet tips containing a protruding tendril which is typical of the *Ins-2* mutant. However, the leaf-like stipules show no incisions or tendrils at the tips of their leaflets.

Figure 3.17 One double mutant *Ins-2 coch* plant in the F_2 population grown showed small 'notches' at the tips of the leaflets of the leaf-like stipules, reflecting the incised notches seen in the primary leaflet pair of the main leaf rachis.

Figure 3.18 A trifoliate leaf and flower of the double mutant *Ins-2 uni*. At the end of the leaf there is an incised notch containing a lobed leaflet-like structure. The position of this notch is in contrast to its position in a compound leaf, where notches are found at the side of the leaf in the first pair of leaflets (Fig. 3.1).

Table 3.1 Mean of total nodes, node of first flower initiation and node of mutant leaflet tip expression for *Ins-2* (from the cv. Solara background) mutant plants grown under different temperature and daylength regimes.

Day length (hours)	**Temperature °C**	**Number of plants**	**Node of flower initiation**	**Total nodes**	**Mean node of mutant leaf phenotype occurrence**
18	12	15	17.4 ±0.3 (σ= 1.3)	23.2±0.4 (σ= 1.4)	16.5±0.3 (σ= 1.7)
18	25	20	18.3±0.2 (σ= 0.9)	23.3±0.3 (σ= 1.2)	17.6±0.2 (σ= 1.5)
18	21	28	16.4±0.3 (σ= 1.4)	21.1±0.4 (σ= 1.9)	15.6±0.4 (σ= 1.9)
8	21	20	24.8±0.5 (σ= 2.3)	32.0±0.6 (σ= 2.9)	21.6±0.7 (σ= 3.2)

Table 3.2 Length of first-pair leaflets in *Ins-2* plant leaves (from the cv. Solara backgound). The length of a leaflet expressing the mutant phenotype is compared with the length of a leaflet opposite it, which is wild-type in phenotype. The length of the mutant leaflet does not include the protruding tendril, but consists of the lamina only. (The length of the mutant leaflet from the base of the leaflet to the base of the notched incision is also compared with the wild-type phenotype leaflet length.)

Node	**Average length (mm) (n=20)**	**t (compared with wild-type leaflet)**	**Probability <**
14 wild-type leaflet	49.7±0.5		
mutant leaflet	52.7±0.7	3.32	0.05
mutant notch base	45.0±0.9	4.32	0.02
15 wild-type leaflet	54.3±0.7		
mutant leaflet	58.3±0.7	5.18	0.01
mutant notch base	50.3±0.3	5.18	0.01
16 wild-type leaflet	61.3±0.3		
mutant leaflet	65.0±0.5	6.80	0.01
mutant notch base	57.7±1.0	3.54	0.05
17 wild-type leaflet	63.0±0.8		
mutant leaflet	66.7±1.0	2.89	0.05
mutant notch base	53.7±1.1	6.82	0.01
18 wild-type leaflet	45.3±0.3		
mutant leaflet	50.3±0.3	12.9	0.001
mutant notch base	40.0±0.9	5.40	0.02
19 wild-type leaflet	37.0±0.9		
mutant leaflet	51.0±1.7	7.2	0.01
mutant notch base	35.0±0.94	1.5	N.S.
20 wild-type leaflet	34.0±0.9		
mutant leaflet	38.3±1.0	3.16	0.05
mutant notch base	25.3±1.2	5.76	0.01

Table 3.3 Length of first-pair leaflets in *Ins-2* plant leaves from the cv. Borek background. The length of a leaflet expressing the mutant phenotype is compared with the length of a leaflet opposite it, which is wild-type in phenotype. The length of the mutant leaflet does not include the protruding tendril-structures, but consists of the lamina only. (The length of the mutant leaflet plus the tendril-structures is also included for comparison.).

Node	Average length (mm) (n=20)	t (compared with wild-type leaflet)	Probability <
15 wild-type leaflet	41.3±0.3		
mutant leaflet	43.7±0.5	3.95	0.02
mutant leaflet + tendrils	46.0±0.9	8.64	0.001
16 wild-type leaflet	44.3±0.3		
mutant leaflet	45.0±0.0	2.57	N.S.
mutant leaflet + tendrils	50.0±0.0	21.0	0.001
17 wild-type leaflet	46.0±0.8		
mutant leaflet	49.3±0.3	3.85	0.02
mutant leaflet + tendrils	54.7±1.4	5.25	0.01
18 wild-type leaflet	50.0±0.5		
mutant leaflet	53.0±0.5	4.50	0.02
mutant leaflet + tendrils	55.3±0.5	7.36	0.01
19 wild-type leaflet	45.0±0.0		
mutant leaflet	45.0±0.0	0	N.S.
mutant leaflet + tendrils	49.7±0.5	8.64	0.001

CHAPTER FOUR

Conclusions: Leaf Development in *Pisum*

Cochleata and *Uni*

The most recent model of pea leaf development (Hofer and Ellis, 1998) proposes that *Uni*, with one or more other genes, acts to maintain an indeterminate fate in the leaf meristem, with the inhibition of *Uni* by *Af* and *Tl* producing determinate leaflets and tendrils. They suggest that *Coch* acts to inhibit *Uni* at the base of the leaf to distinguish determinate stipule cells from the partially indeterminate rachis. Recent molecular evidence upholds this model. Gourlay et al. (2000) show that *Uni* is expressed in the leaf primordium when leaf primordia are initiated and is down regulated when they become determined. The loss of *Uni* gene function results in a prematurely determined leaf primordium that produces a simple leaf form. In keeping with this, there is prolonged *Uni* expression in the primordia of the complex leaf forms seen in the *afila* and *afila tendril-less* mutants.

This model is largely supported by the evidence from mutant phenotypes reported here. A loss of deteminacy is seen in the formation of compound stipules rather than simple ones in *coch* plants, and some loss of determinacy in the flowers resulting in the production of supernumerary organs. However, the primary effect of the *coch* mutation seems to be to retard the development of the stipules. This then seems to allow expression of *Uni* in some stipule primordia, leading to their development into compound structures. The retarded development of the stipules seems to allow them to behave more like leaf blades. This is shown by the way *coch* mutant stipules mimic the form of the leaf blade (Marx, 1987), and is supported by the epistasis shown here between *coch* and *st*.

This thesis also shows reduced action of *Tl* in the basal compartment of the leaf, which supports the existence of a gradient of *Tl* gene action from high in the distal compartment to low in the basal compartment, as proposed by Lu et al. (1996), but not included in the model of Hofer and Ellis (Hofer and Ellis, 1998). Other new knowledge is that *uni* mutants have reduced shoot apical meristem diameters, which could result in their simpler leaf form. However, *Uni* is not expressed in the shoot apical meristem (Gourlay et al., 2000), so it may act indirectly to maintain shoot meristem size.

If, as suggested by Hofer and Ellis (1998), the pea leaf can be regarded as a determinate lateral shoot, then the wild-type *Coch* gene reduces the number of 'shoots' from three to one (with two subtending stipules), and the *Uni* gene increases the number of shoots from none (a simple determinate leaf) to one (a compound leaf rachis with two subtending stipules). Thus, these two gene products seem to have opposing effects on the determinacy of the pea leaf. However, they act on different

parts of the leaf and do not appear to act in the same pathway. The double mutant *coch uni*^{tac} phenotype is reported to be additive (Marx, 1987). If the mutant condition (no gene function) represents an original ground-state condition (as proposed by Coen and Meyerowitz (1991) and Lu et al. (1996)), then the phenotype of the *coch* mutant suggests that perhaps pea stipules were originally derived from other leaves, which have subsequently been reduced and modified.

There has been considerable debate about whether stipules are an integral part of leaves (Cote et al., 1992; Goliber et al., 1999; Marx, 1987; Rustihauser and Sattler, 1986). There is substantial variation in anatomy and morphology of different taxa, even in closely related groups (Lacroix and Posluszny, 1989). Pea stipules have a more complex vascular connection to the stem than the rest of the leaf (Cote et al., 1992; Mitra, 1949; Sachs, 1977) and primordia that are initially more flattened and triangular than the domed leaflet primordia. In other respects, stipules are very similar to leaflets (Vienne de and Gottleib, 1990). Some consider that genetic evidence favours the view that stipules in peas are an integral part of the leaf (Goliber et al., 1999; Marx, 1987). However, while the stipule is part of the compound leaf, the stipules seem in other ways to be fundamentally genetically different from the other lateral pea leaf organs - tendrils and leaflets, which supports the idea that they may have a different origin. Tendrils can appear in the place of leaflets, and *vice versa*, but stipules never appear in other positions on the leaf. Similarly, the transformation of a leaf part into a re-iteration of the whole leaf blade only occurs in the stipules. The stipule appears to have a separate genetic control from the rest of the leaf that is mediated by the *Cochleata* gene, because mutations in this gene are sufficient to transform the stipules into organs like compound leaves which respond genetically in the same way at the main leaf rachis. This points to the origin of stipules in *Pisum sativum* L. as leaves which subsequently became modified, rather than as part of the leaf which has later become elaborated. Evidence presented here clearly suggests that pea stipules cannot be considered simple homologues of leaflets because they have additional leaf-like features.

Insecatus

The dominant mutant *Insecatus* shows a gain-of-function to the leaflet tip, allowing it to become more meristematic in nature. *Insecatus* can be compared to the *KNOTTED* homeobox genes in maize. The dominant *KN1* mutant shows ectopic expression of the *KN1* gene in cells of the epidermis, where it does not normally occur. This causes those cells in which it is expressed to become more meristematic in nature (Freeling and Hake, 1985; Hake, 1992). However, these 'meristematic' cells form a 'knot' of unspecialised cells in the epidermis, so this is a different mode of action from *Ins* , which rather results in new differentiated structures being formed at the leaflet tip. The *Lax midrib* (Schichnes et al., 1997) mutation in maize may also have a similar mode of action to *Ins*. This dominant mutation causes the formation of ectopic leaf-like structures on the leaf blade, and its mode of action is suggested to be

that of a delayed signal from the veins to "make the organ which you are competent to make".

It cannot be determined whether *Ins* is a homeotic or a heterochronic mutant until the gene is isolated. Then gene expression can be examined to determine whether the primary change is in the timing (heterochrony) or the position (homeosis) of gene expression. I have called *Ins* a 'homeotic' mutation in the sense that the mutant phenotype is one of an 'organ in the wrong place'. This change in pattern formation could be caused by a position, a timing change, or it could be the result of the presence of too much gene product causing gene expression to be above a certain critical threshold.

The leaf tip in cotton (*Gossypium barbadense*) showed some unusual development in a study of clonal sectors in cotton (Dolan and Poethig, 1998). In this study Dolan and Poethig showed that veins are important in determining boundaries of clonal sectors. They found that clonal sectors rarely crossed veins, except at the tip of the leaf. The explanation proposed was that the vein at the leaf tip does not differentiate until late in leaf development. This is despite the fact that cotton shows cell division in the leaf that ceases at the tip first and then at the base. If the vein at the cotton leaf tip is relatively slow to develop, then this could also be the case for the pea leaflet. Other studies have also pointed to the importance of the mid-rib in leaf development. In maize the cells in the mid-vein act as organisers for leaf development; they act as origins of position dependent information (Scanlon et al., 1996), (Harper and Freeling, 1996). The mid-rib axis is the first leaf domain to be identified (Hudson and Waites, 1998). Thus, it is probably significant that the mutant structures at the tip of *Ins* leaflets are structured around the mid-vein, and that the novel tendril structures grow directly out from the mid-vein.

Ins shows the potential of the leaflet tip for further development. In a similar way, the *sinuate leaf (sil)* (Marx, 1977) mutant shows that the stipule tip also has further developmental potential. The *sil* mutant has undulating stipule and leaflet margins, but the *sil af* double mutant has some stipule tips that are deeply incised and from which arise adventitious tendrils (Marx, 1977). This sounds similar to the effect *Ins* has on the leaflet tip, and in the same way it is also homeotic, for example, the triple mutant *sil af tl* has branched adventitious tendrils terminating in small leaflets arising from the stipule tips, which reflect the form of the mutant main leaf rachis.

However, there are two substantial differences between *sil* and *Ins*. Firstly, the homeotic effects of *sil* are not seen until it is combined with *af*. The incised stipule tips of *Ins* are present in the single mutant alone, and furthermore *af* is epistatic to *Ins*. Secondly, *sil* is recessive while *Ins* is a dominant mutation. Thus, *Ins* must act after the wild-type *Afila* gene and requires its presence to be active, while *Sil* and *Af* must interact in some way that affects the developmental potential of the stipule tips; possibly, the *sil* mutation allows the *af* mutation to affect the stipule tips.

This idea is supported by the effect *sil* has when combined with the wax production mutants *wlo* and *wb*. These two mutations have contrasting effects. In

wlo mutants, only the upper leaf surfaces are waxless, and in *wb* mutants only the upper leaf surfaces retain wax. (In wild-type pea leaves both leaflets and stipules have wax on both upper and lower surfaces.) The *sil wlo* double mutant has both stipule tips and upper leaflet surfaces waxless, and the *sil wb* double mutant has both stipule tips and upper leaflet surfaces waxy, with the lower surfaces waxless (Marx, 1977). Here *sil* is allowing the stipule tip to behave genetically like a leaflet, and shows a link between the development of the stipule tip and the upper leaflet surface in particular. Perhaps this is related to the recently discovered gradients of dorsal and ventral gene activity in leaves (Hudson, 1999; Scanlon, 2000; Tsukaya, 1998). The generation of the triple mutant *Ins sil wlo* or *Ins sil wb* could be used to confirm that *Ins* and *sil* mutations operate in different genetic pathways.

The mutant *crispa (cri)* produces crinkled leaflets and stipules (Lamm, 1949). When combined with *sil* ,the double mutant plant has stipules which have an outgrowth of laminar tissue from their tips (Marx, 1987). Thus, it seems that the wild-type *Sil* gene has the effect of limiting the development of the stipule tip, in combination with other genes. This is in line with the suggestion that pea stipules may be reduced leaves. The question of whether *Sil* acts after *Coch*, or is in a different developmental pathway, could be addressed by examining the *sil coch* double mutant and the *sil coch af* triple mutant.

The normal effect of *Ins* gene is difficult to determine as it is likely to be a gain of function mutation. Its effect in the mutant is to add a developmental potential to the leaflet tip that is not normally present, probably due to the incorrect expression of a wild-type gene controlling differentiation. *Ins* produces a link between the development of the leaflet tip and the development of the distal tip of the whole leaf. It is possible that the *Ins* gene could be the unknown gene 'X', referred to in the Hofer and Ellis model (1998), which acts, weakly, with *Uni* to maintain blastozone activity in the pea leaf.

CHAPTER FIVE

Precocious Germination: The *viviparous* Mutant

INTRODUCTION

Two novel allelic viviparous mutants of *Pisum sativum* L. have been isolated. Mutant seeds germinate precociously in the pod (Fig 5.1). These are the first mutants of this type described in the garden pea and the gene symbol *vip* has been assigned to the locus. Normally, seeds do not germinate during development on the mother plant, but rather undergo a period of maturation and then desiccation before being shed. However, there is considerable evidence to suggest that the embryo within the seed becomes capable of germinating about halfway through seed development, and that an active process is invoked to suppress the germination potential of the embryo until maturation is complete (Kermode, 1990; Paek, 1998). In the mutant *viviparous* a vital part of this active suppression process has been disabled.

Cultivated pea seeds are non-dormant. That is, the dry seeds are quiescent; they are able to survive long periods in a dry state, but will germinate when provided with appropriate moist conditions. Wild peas in the Israel to Asia Minor region (ssp. *elatius, humile* and *fulvum*) have semi-hard to very hard testas, in comparison with the soft coats of white-flowered modern domestic lines (Ian Murfet, personal communication). These harder seeds would require more sustained moisture for imbibition, and hence germination, than modern domesticated pea lines. In many other types of seeds, including *Arabidopsis* (Koornneef et al., 1998), there is a period of dormancy. During this time the embryo cannot germinate despite favourable conditions. After a period of after-ripening occurs, they gain the capacity to germinate (Holdsworth et al., 1999; Li and Foley, 1997).

In most species studied, abscisic acid (ABA) plays an important role in the prevention of precocious germination. Many ABA-deficient and ABA-insensitive mutants show reduced seed dormancy, and some have viviparously germinating seeds (Ackerson, 1984; Bewley and Black, 1994; Black, 1991; Hilhorst and Karssen, 1992; Holdsworth et al., 1999). There are several ABA-deficient mutants that have reduced tolerance to water stress and reduced seed dormancy. In *Arabidopsis*, there are the *aba* mutants (*aba1, aba2* and *aba3*) (Koornneef et al., 1982; Neill et al. 1986). In tomato, the *flacca (flc)* and *sitens (sit)* mutants show wilting and reduced seed dormancy (Groot and Karssen, 1992). Vivipary is shown by the maize *vp* mutants, which have low levels of ABA and carotenoids: *vp2, vp5, vp7, vp8, vp9, white seedling3 (w3), albescent (all)* and *yellow9 (y9)* (Robertson, 1955; Neill et al., 1987;

McCarty, 1995). The biochemical lesions in the ABA synthesis pathway, causing ABA deficiency, differ between these mutants (Koornneef et al., 1988; Giraudat et al., 1994; McCarty, 1995). The *Arabidposis aba* mutants do not show viviparous germination, but seeds of the tomato *sit* mutant, which have thin testas, will show viviparous germination in over-ripe fruits (Groot et al. 1991).

There are also several well-known ABA-response mutants that are insensitive to ABA. They were initially isolated in *Arabidopsis*, by growing mutagenised seed on media containing ABA (Koornneef et al., 1984). These ABA-insensitive mutants (*abi)*; *Abi1, abi2, abi4* and *abi5*, resemble *aba* mutants, and yet have wild-type ABA levels (Koornneef et al., 1984; Koornneef et al., 1988; Leung and Giraudat, 1998). The *abi3* mutant is different in that is has a seed specific phenotype, with normal stomatal closure (Koornneef et al., 1984; Koornneef et al., 1989). Severe alleles of *abi3* have seeds that remain green and show viviparous germination inside the siliques (Nambara *et al.*, 1992). A similar, and probably homologous, seed-specific mutant, *vp1,* occurs in maize. It shows germination of immature embryos precociously on the ear and is ABA-insensitive (Robertson, 1955; Robichaud et al., 1980; Robichaud and Sussex, 1986; Neill et al., 1987; McCarty et al., 1989). The *Abi1* and *abi2* genes encode protein phosphatases that presumably play roles in ABA signal transduction (Leung et al., 1997). The *vp1* and *abi3* genes encode seed-specific transcription factors (McCarty et.al., 1991; Giraudat et al., 1992).

When defects in both ABA synthesis and ABA responsiveness are combined in the *Arabidopsis aba abi3* double mutant, the seeds remain green and germinate viviparously late in development (Koornneef et al., 1989). In species that exhibit vivipary as part of their normal development, ABA responses have also been shown to be affected. In the case of the mangrove *Rhizophora mangle* L., unusually high concentrations of ABA are required to inhibit the growth of excised embryo-seedlings (Sussex, 1975).

Gibberellins (GAs) have been shown to promote germination in several species (Bewley and Black, 1994; Hilhorst and Karssen, 1992; Holdsworth et al., 1999). In these species, the ratio of GA to ABA during seed development is thought to be important in the suppression of germination and onset of seed maturation (Hilhorst and Karssen, 1992; Koornneef et al., 1998). A GA deficiency early in seed development has been shown to suppress the vivipary of some ABA-deficient viviparous seeds (White et al., 2000). Additionally, there is a report of a spermine-resistant mutant in *Arabidopsis* that shows viviparous germination under some conditions (Mizra and Rehman, 1988). This suggests there may also be a role for spermine in seed dormancy in some species.

Embryos of many species, including pea (Cook et al., 1988), display the ability to germinate precociously on culture media when removed from the ovule, without desiccation, after pattern formation is complete (Goldenberg et al., 1994; Kermode, 1990; McCarty, 1995; Ren and Bewley, 1999). Such precocious germination can be prevented in many cases by adding ABA to the culture medium (Black, 1991; Butler and Cuming, 1993; Kermode, 1995). However, ABA may not have this role in pea. The pea ABA synthesis mutant *wilty (wil)* does not exhibit

vivipary, despite having one fifth the ABA level of wild-type seeds (De Bruijn et al., 1993). The *wil* mutant loses turgor and wilts dramatically if exposed to moisture stress and/or hot dry wind or direct sun (Fig. 5.2) (Marx, 1976). It has reduced ABA levels in its shoot and leaves and is unable to produce sufficient ABA to close its stomata quickly and effectively (Wang et al., 1984). In addition, it has been reported that the precocious germination of pea embryos in culture cannot be fully inhibited by ABA (Barratt et al., 1989).

There are also other pathways which have a role in controlling precocious germination, in addition to those concerned with hormonal regulation. The *Arabidopsis* genes *FUSCA3 (FUS3)* and *LEAFY COTYLEDON1 (LEC1)*, are transcriptional regulators which help to control precocious germination in *Arabidopsis* (Wobus and Weber, 1999). Their embryo-specific mutants show vivipary and desiccation-intolerance which appear to be due to heterochronic changes (Holdsworth et al., 1999). Mutant *fus3* embryos have ectopic trichomes and accumulate anthocyanins in the cotyledons, traits which are normally only seen in leaves (Keith et al., 1994). The cotyledons of *lec1* mutant embryos appear to be intermediate in structure between cotyledon and leaf cells; they are rounded and also have ectopic trichomes (Meinke et al., 1994; West et al., 1994). In both *fus3* and *lec1,* storage gene expression is reduced and vascular differentiation is advanced (Keith et al., 1994; McCarty, 1995; West et al., 1994). Analysis of double mutants between *abi3, lec1* and *fus3* show that these loci regulate separate, but overlapping, developmental pathways (Parcy et al., 1997). Integration of their functions prevents precocious embryo germination and promotes seed maturation. The *ABI3, VP1* and *FUS3* genes share several highly conserved domains (Luerssen et al., 1998). Biochemical and molecular studies have shown that the *VP1* protein activates some promoter activities and represses others (Hoecker et al., 1995). Similarly, the double mutant *abi3* fus3 shows sets of both up-regulated and down-regulated genes (Nambara et al., 2000). In this way these transcription factors may repress germination and promote seed maturation.

There is also increasing recognition of the role of metabolites, such as sugars, as important regulators of seed development (Weber et al., 1998; Wobus and Weber, 1999). In a recent paper, sugars have been shown to alter the sensitivity of *Arabidopsis* seed to inhibition of germination by ABA (Finkelstein and Lynch, 2000) and the previously described *ABSCISIC ACID INSENSITIVE -4* gene in *Arabidopsis* has been found to be identical to the *SUCROSE UNCOUPLED-6* gene which shows a reduced sensitivity to hexose sugars for germination (Huijser et al., 2000). Changes in carbohydrate state during *Vicia faba* seed development changes gene expression patterns and seems to control developmental programmes (Weber et al., 1997). In legumes, the seed coat modulates nutrient supply to the growing embryo (Weber et al., 1998). During early seed development the high ratio of hexose sugars to sucrose, created by the activity of invertases, seems to promote growth by cell division (Weber et al., 1996). Alternatively, feeding sucrose to young pea and bean cotyledons promotes storage rather than growth activity (Wang and Hedley, 1993; Weber et al., 1996). The link between sugar status, amino acid import and cell

proliferation and storage protein production during seed development is an active area of current investigation (Borisjuk et al., 1998; Smeekens, 2000).

Aims and significance

This new viviparous mutant represents an important tool for greater understanding of the involvement of ABA in the precocious germination of peas, in particular, and of the switch between the seed maturation and germination programmes in general. This study of the viviparous mutant at the anatomical, genetic, biochemical and molecular biological levels seeks to determine how development in perturbed in this mutant, which in turn should allow us to understand how precocious germination is normally suppressed in the wild-type plant.

Pea seeds are an important protein source for both humans and animals worldwide (Casey et al., 1993; Eeuwens and Schwabe, 1975). The advantages of using pea as a material to study seed development are that it has a large embryo which is easy to visualise and manipulate, and provides enough material for molecular and biochemical studies (Weber et al., 1998). The study of development in pea has been aided by the large number of mutants available (Wang and Hedley, 1993). However, until now there have been no mutants affecting seed dormancy in pea.

There is substantial commercial significance in understanding the control of precocious germination, as an understanding of the germination process should allow improved control of pre-harvest sprouting and seed dormancy. Pre-harvest sprouting is prevalent in certain cultivars of wheat and barley in cool damp, or humid, growing conditions. It is a significant world-wide problem (Holdsworth et al., 1999; Li and Foley, 1997). In addition, dormancy is one of the major factors contributing to the persistence of weeds in agricultural systems (Li and Foley, 1997). The factors that control seed gene expression are highly conserved in different species (Goldberg et al., 1989). Therefore, there is good reason to believe that the knowledge derived from pea will be relevant to other species.

This thesis will analyse the defect present in these novel *viviparous* mutants. This will include morphological description of the mutant, analysis of its pattern of inheritance and determination of the position of the locus on the *Pisum* gene map.

The levels of ABA in *vip* seeds will be examined to see if low ABA allows their precocious germination, as it does in some mutants of other species. These ABA levels will be compared with those present in the *wil* mutant of pea, which has low ABA, but does not have viviparously germinating seeds. Levels of ABA in *vip* shoots and their response to droughting will also be investigated, and the *vip wil* double mutant generated, to locate other potential lesions in the ABA production pathway. We will also culture both mature germinating and immature (non-germinating) seeds on media containing ABA to determine whether *vip* seeds are insensitive to ABA, rather than having a defect in ABA synthesis.

The *lh-2* mutant of pea has reduced bioactive GA levels in the shoot and seeds. It is a dwarf with reduced seed weight and increased seed abortion (Batge et al., 1999; Swain et al., 1993, 1995). Work on this mutant has led to the conclusion that GAs are important during seed development in *Pisum* (Swain et al. 1997). If the *vip* mutant is germinating due to high GA levels at a critical point during development, then it is likely that the *vip lh-2* double mutant will not have seeds which show precocious germination. However, many legume seeds have very high GA levels for the major part of development and do not show precocious germination (Quatrano 1987; Reid 1990; Karssen 1995) . To test the hypothesis that high GA levels are important for the precocious germination of *vip* seeds, the *vip lh-2* double mutant will be generated and the levels of bioactive GAs in germinating *vip* seeds will be examined.

The expression of well-known pea seed maturation-related gene products will be examined to determine whether there is a 'switch' between maturation and germination programmes (as has been suggested by Fernandez, (1997)), or whether the two programmes are loosely integrated and can overlap during precocious germination (as is more commonly imagined (Kermode, 1995)). Typically, storage products are deposited in late embryo development, and these are then broken down and used by the germinating seedling as an initial food source. Other proteins which are abundant in late embryo development are related to the acquisition of desiccation tolerance by the seed . The expression of the pea homologue of the *ABI3* gene (Giraudat et al., 1992), will also be examined. *ABI3* is a seed-specific transcription factor that regulates many genes involved in seed maturation (Parcy et al., 1994), and has a role in the control of precocious germination in *Arabidopsis* seeds.

MATERIALS AND METHODS

Mutant Description and Genetic Analysis

In a mutagenesis programme (by J. Weller), cv. Torsdag (Hobart line 107) was treated with the alkylating agent EMS (ethylmethanesulphonate) at 1% for 6h at 18°C. Among 1100 M_2 families screened, two showed segregation for viviparous seed. Dry *vip* seeds are not viable, so the two mutants were recovered via M_2 heterozygous siblings, as these selfed heterozygous siblings again produced viviparous seeds in the M_3. The two original mutant lines were designated as A303 and A353. These lines were crossed to each other and the F_1 seeds were viviparous, indicating that both mutations are at the same locus. The mutant alleles were named *vip-1* (A303) and *vip-2* (A353), respectively.

The best survival rates of *vip* seedlings were obtained by removing germinating seeds from a drying pod in which the body of the pod was browning, but the sutures still remained green. The germinating seed was then positioned with the extending radicle downwards into the soil and the top of the cotyledons (about 0.5 cm) exposed above the surface of the soil. For the first two weeks the soil around the seedling was gently watered, rather than the seedling itself being directly watered, as this reduced rotting. Germinating seeds with obviously damaged or dehydrated roots would not usually grow into healthy, normal sized plants.

Preparation of pots, planting, watering, nutrition and fungicide/pesticide treatment of plants were otherwise as described in Chapter 2. During winter the average day temperature was 21°C and the average night temperature 14°C, and during summer the average day temperature was 25°C and the average night temperature 15°C. The photoperiod was 18 hours of light, with natural daylight extended by mixed incandescent and fluorescent lights as described in Chapter 2. Scoring of nodes was as described in Chapter 2. Morphological measurements, including seed weights, were taken from summer-grown plants and seeds.

To determine the mode of inheritance of the *vip* mutation, wild-type pollen from the progenitor line (HL107, cv. Torsdag) was crossed onto *vip* plants. This produced non-germinating seeds in those pods, indicating that the crosses had been successful, and that it was the genotype of the embryo controlling the precocious germination. These F_1 seeds were then planted and the clearly segregating F_2 seeds in their pods were scored. This cross was carried out on several occasions for both *vip* alleles (see Table 5.2).

For mapping the *vip* locus, *vip-2* was crossed with the multiple marker line HL111. The *vip-2* allele was used rather than *vip-1,* as *vip-2* mutants tend to have better seedling survival rates. Some significant linkage was found between *vip* and loci on linkage group five, so a further round of crosses was performed with both *vip-1* and *vip-2* alleles and other loci on linkage group five. The *vip-1* mutant was crossed with HL106 which carries the *cerise (ce)* mutation, and the *vip-2* mutant was crossed with HL21 which carried the *crispa (cri)* mutation.

Double mutants

For generation of double mutant lines with *vip*, the *vip-2* allele was used as it showed better seedling survivorship than *vip-1*. HL233, which carries the *wil* mutation, was obtained from G. Marx (Cornell University, NY, USA); it originated as a mutant isolated by L. G. Cruger of the Del Monte Corporation, California (USA) (Marx, 1976). Crosses were performed with *vip-2* as both the maternal and paternal parent. When *vip-2* was the maternal parent, the success of the cross could be established by the production of non-precociously germinating F_1 seed. When *wil* was the maternal parent, the success of the cross could be determined by the production of tall F_1 plants, as HL233 (*wil*) is a dwarf line. 'Wiltiness' of plants homozygous for the *wil* mutation could be scored easily when the plants were observed after they had recently been exposed to direct sunlight (see Fig 5.2).

The dwarf *lh-2* line (NGB5843, previously *lh*^{t}) was derived from cv. Torsdag (HL107) by Dr K. Sidorova (Novosibirsk, Russia) (Reid, 1986; Swain and Reid, 1992). The cross of *lh-2* and *vip-2* was also performed with *vip-2* as both the maternal and paternal parent. Again, if *vip-2* were the maternal parent, the success of the cross was determined by the formation non-viviparous F_1 seed, and if *vip-2* were the paternal parent, the success of the cross was indicated by tall F_1 plants.

ABA levels in seeds and shoots

Seeds of *vip-1, vip-2*, their wild-type progenitor cv. Torsdag (HL107) and *wil* (HL233) were analysed. Plants were grown in summer and in winter and seeds from these plants were harvested at five day intervals, from five to thirty days after flower opening. Harvested tissue was weighed and immersed in approximately 5 ml/g of cold methanol (-20°C) containing butylated hydroxytoluene (BHT). It was then stored at -20°C for one hour. The tissue was homogenised and then the methanol concentration reduced to 80% by addition of distilled water. It was then stored at 4°C for 24 hours. The sample was filtered (Whatman No. 1 filter paper), and $^{2}H_{3}$-ABA internal standard (provided by S. Neil, University of Bristol, UK) was added to the filtrate.

The filtrate was dried under vacuum and dissolved in 3 ml of 0.4% (v/v) acetic acid in water. It was then loaded through a 0.45 µm filter onto a Sep-Pak C18 cartridge (Millipore) which had been pre-conditioned with three washes of 1 ml of 0.4% acetic acid (as above). Following this the cartridge was washed with a further 2 ml of 0.4% acetic acid, and then with 10 ml of 20% methanol in 0.4% acetic acid (in water). ABA was eluted with 10 ml of 50% methanol in 0.4% acetic acid (in water).

The eluate was dried under vacuum, then dissolved in 200 µl of methanol and methylated with 750 µl of etheral diazomethane. The methylated product was then dried under a stream of nitrogen gas.

The same method was used to extract ABA from three week old shoot tissue grown in summer for the determination of ABA levels in control and droughted shoots. Droughted shoots were not watered for five days (controls were watered daily).

Quantification of endogenous ABA was performed using gas chromatography mass spectrometry-selection ion monitoring (GC-SIM), using a Kratos concept ISQ mass spectrometer (described in Hasan et al., 1994). High resolution mode was used (R = 10 000). Ions m/z 193.0817 and 190.0629 were monitored for quantification of endogenous ABA. The identification of peaks was confirmed by retention time and by monitoring the additional ions 165.0868 and 162.0680. Endogenous ABA levels were calculated from peak areas after correction for the presence of unlabelled ABA in the internal standard.

Gibberellin levels in seeds

Gibberellin levels were measured in seeds of *vip-1* and *vip-2* mutant alleles and their wild-type progenitor cv. Torsdag. Seeds were harvested at five day intervals from five to thirty days after flower opening. Gibberellins were extracted using the Sep-Pak method described above for ABA, except that the cartridge was washed with 25% methanol, rather than 20% methanol, and the GAs were eluted with 35% methanol, rather than the 50% methanol used for ABAs. Deuterated (d_2) internal standards for GA_1, GA_3, GA_8, GA_{19}, and GA_{20} were provided by L. Mander, Australian National University.

Immediately before the samples were analysed by GC-SIM the gibberellins were trimethylsilated by addition of 3 µl of dry pyridine and 10 µl of *N,O*-bis-trimethylsilylacetamine, and then incubated at 80°C for 15 to 20 minutes.

During quantification by GC-SIM the ions monitored were; 506 and 508 for GA_1, 504 and 506 for GA_3, 594 and 596 for GA_8, 434 and 436 for GA_{19} and 418 and 420 for GA_{20}. Identification was confirmed by retention time and the presence of additional ions. Endogenous GA levels were calculated on the basis of peak areas, after correction for the contributions of naturally occurring isotopes and for the presence of unlabelled GAs in the internal standards (Lawrence et al. 1992, Ross et al. 1995).

Growth of mature seeds and immature embryos in media containing ABA

Seeds of the *vip-1* allele were chosen for these experiments as they had a slightly more severe seedling phenotype than *vip-2*, and so would be more likely to show a significant effect.

Growth of mature seeds

Precociously germinating 25 day old summer-grown *vip-1* seeds were grown in media containing ABA. Mature seed of the wild-type progenitor (cv. Torsdag) which had been germinated in moist sterile conditions for 72 hours were used for comparison. At this age, radicle extension was about 1 cm in both *vip* and wild-type germinating seeds.

Dry wild-type seeds with no holes in their testas were surface sterilised in 70% ethanol for 2 minutes, then rinsed thoroughly in sterile distilled water. Sterile seeds were then grown in sterile (autoclaved) moist vermiculite for 72 hours in ambient laboratory conditions. Drying pods of the *vip-1* mutant were sterilised in 1.5% (w/v) sodium hypochlorite solution for ten minutes. Pods were then washed thoroughly with sterile distilled water. Seeds were then transferred into sterile media under sterile conditions in a laminar flow cabinet.

The culture media was Murashge and Skoog basal medium (Sigma), plus 3% (w/v) sucrose (Sigma) with 1% (w/v) Phyta-gel agar (Sigma). Treatments with final

ABA concentrations of 0, 3, 15, 30 and 100 μM ABA were used. The ABA (Sigma, mixed cis/trans isomers) solutions were sterilised by filtration through a 0.2 μm filter (Millipore). The remaining constituents were sterilised by autoclaving. The pH was adjusted to pH 6.0.

Seedling were grown in large glass test tubes (25 cm long by 4 cm diameter) sealed with Parafilm. Forty millilitres of media was placed in each tube, which was then allowed to set on a slope to aid root penetration. Seeds were grown on the media at 20°C under 25 μmol $m^{-2}s^{-1}$ fluorescent lights for 22 days. Five seeds of each genotype were grown at each different ABA concentration.

Growth of immature embryos

Summer-grown mutant *vip-1* embryos and their wild-type progenitors from cv. Torsdag were harvested at 12 days after flower opening. At this age the mass of each embryo was approximately 20 mg. Fifteen to twenty seeds of each genotype were used for each of three treatments: 2% sucrose (w/v), 13% sucrose (w/v) and 2% sucrose (w/v) with 200 μM ABA.

The 13% sucrose (w/v) medium was selected as a high osmotic pressure medium following the work of Cooke et al. (1988) who showed high osmotic pressure media of 5 to 15% sucrose inhibited precocious germination of immature pea embryos. Similarly, Barratt et al. (1989) showed inhibition of precocious germination of immature pea embryos in a high osmotic pressure medium (5% sucrose) also containing ABA (100μM). The effect of sucrose as a carbon source or as a developmental signal was not tested here.

Pods were harvested and surface sterilised by agitation in 1.5% (w/v) sodium hypochlorite solution for ten minutes. Pods were then washed thoroughly with sterile distilled water. The pods were opened and embryos extracted from the seeds under sterile conditions in a laminar flow cabinet. Each healthy embryo was transferred to a single well in a 24-well plate (see Fig. 5.20 to 5.25).

The culture media was Murashige and Skoog basal medium (Sigma), plus 750 mg/l L-glutamine (Sigma) with 0.7% (w/v) Phyta-gel agar (Sigma). Where necessary sucrose (Sigma) or ABA (Sigma, mixed cis/trans isomers) were added. The L-glutamine and the ABA solutions were sterilised by filtration through a 0.2 μm filter (Millipore). The remaining constituents were sterilised by autoclaving. The pH was adjusted to pH 5.6. One ml of media was placed in each well.

The edges of the plates were sealed with plastic film and the embryos incubated at 20 °C under 82% shade cloth (ambient light approximately 25 μmol $m^{-2}s^{-1}$) for 10 days.

Expression of seed maturation genes in vip

Preparation of radioactive probes

Plasmids containing cDNAs of different pea seed genes were kindly provided by Trevor Wang, John Innes Institute, UK (*ABR-17*; Iturriaga et al., 1990), T.J. Higgins, CSIRO Plant Industry, Australia (albumin [PA2]; Higgins et al., 1987, and lectin [pS15-50]; Higgins et al., 1983), Masumi Robertson, CSIRO Plant Industry, Australia (dehydrin [pSB12]; Robertson and Chandler, 1992), Clare Domoney, John Innes Institute, UK (legumin [pCD43]; Domoney and Casey, 1985; Domoney et al., 1986) and Jerome Giraudat, CNRS, Gif-sur-Yvette Cedex, France (*ABI3*; Giraudat et al. 1992). Suitable gene fragments were amplified using appropriate primers in a Perkin Elmer Gene Amp 9600 thermal cycler. The desired fragment was isolated by gel electrophoresis and purified using a QiaQuick™ column (according to the manufacturer's instructions; Qiagen). Probes were prepared and labelled with α^{32}P-dCTP (Bresatec) with approximately 25 ng of template, using a Gigaprime DNA labelling kit (according to the manufacturer's instructions; Bresatec). Unincorporated radiolabelled cCTP was removed by chromatography on a Sephadex™ G-50 column. Incorporation was estimated using a Geiger counter. Probes were denatured by boiling prior to their use in hybridisation reaction.

Isolation of total RNA and Northern transfer

Total RNA was isolated from 0.5 g samples of seed or shoot tissue using a Plant RNeasy kit (according to the manufacturer's instructions; Qiagen). RNA was quantified by measuring absorbance of the solution with a GBC UV/VIS 916 spectrophotometer (1 unit at 260 nm = 40 μg/ml).

Total RNA was electrophoresed through a formaldehyde denaturing gel, as described by Fourney et al. (1988). Samples containing 10 μg of total RNA were run on a 1.2% agarose gel with 0.66M formaldehyde in MOPS buffer (20 mM MOPS [3-(N-morpholino)propanesulphonic acid], 5 mM sodium acetate and 1 mM EDTA), with MOPS as the running buffer. Before loading RNA samples were denatured by heating at 65°C for 15 minutes in a MOPS solution containing 15% formaldehyde and 45% formamide. Ethidium bromide (1 μg) and 1% bromophenol blue (w/v) were then added to each sample. Equal loading of RNA in each lane was confirmed by ethidium bromide staining of the rRNA bands. Only gels with equally loaded RNA were used for blotting and hybridisation.

Gels were blotted onto ZetaProbe membrane (Bresatec) overnight by capillary action in a transfer solution of 50 mM sodium hydroxide. The membrane was then washed with SSC buffer (3M sodium chloride and 0.3M sodium citrate) and the RNA cross-linked to the membrane by heating at 80°C for 30 minutes.

Hybridisation and signal detection

RNA blots were hybridised using ExpressHyb™ Hybridisation Solution (according to the manufacturer's instructions; Clontech). Incubation was carried out in glass tubes in a Hybaid Micro 4 oven. Radioactive membranes were exposed to autoradiography film (Kodak) at -80°C between two intensifying screens (Kodak).

RNA blots were stripped of probes for re-use by incubation in 0.5% SDS solution at 90 to 100°C for 10 minutes.

RESULTS

Mutant Description and Genetic Analysis

Viviparous germination of all seeds of the *viviparous (vip)* mutant occurs 20 to 25 days after flower opening (depending on the season), without a period of quiescence (Figs. 5.4, 5.5). This is soon after contact point is reached (ie. all liquid endosperm has been absorbed). In the mutant the radicle emerges and splits the testa, while in the wild-type (cv. Torsdag) the seed is beginning to yellow, dry and become quiescent (Torsdag carries the gene *I* and so has yellow cotyledons). As maturation continues the *vip* seeds remain green (Figs. 5.5). The seeds of *vip* mutants also appear to have more rapid early development (10 days after flower opening) than the wild-type progenitor line (cv. Torsdag) (Fig 5.4).

The radicle of viviparously germinating seeds reaches one to two centimetres in length and in about 10% of seeds the plumule and scale leaves begin to emerge. Growth of the germinating *vip* seeds continues until it is halted by dehydration as the mother plant senesces and the pod dries (Fig. 5.6). The *vip* seeds desiccate and die if they remain on the mother plant, but they survive and grow to maturity if removed from the pod and planted before they dehydrate. Growth of young *vip* seedlings is somewhat weaker and slower than that of wild-type seedlings (Fig. 5.7), and they show a high level of seedling mortality (10-15%). However, after this initial lag period those surviving grow into healthy plants with the same habit as their wild-type progenitor (HL107, cv. Torsdag) (Fig. 5.8) (Table 5.1)

Two alleles of the *vip* locus have been produced, here designated *vip-1* and *vip-2*. Their phenotypes are similar. Seedling survival is slightly greater for the *vip-2* allele. In crosses with the wild-type, the segregations were in agreement with the expected 3:1 wild-type:mutant ratio, indicating that the *vip* mutants are Mendelian recessives. However, the combined data from several crosses indicated a significant and consistent paucity of *vip* seeds for both alleles, indicating a reduced fertilisation by *vip* gametes (Table 5.2). There does not seem to be increased seed abortion in *vip* pods. The pods of *vip* mutant plants have a similar number of seeds per pod as the wild-type pods, with each line having approximately 3 seeds per pod (Table 5.3). However, *vip* plants produce fewer pods per plant (Table 5.3); plants of the *vip-1* and *vip-2* mutants have approximately three pods per plant, while the wild-type (cv. Torsdag) has five to six pods per plant. This is largely because *vip* plants usually have one pod per peduncle, whereas the wild-type plants carry two (Figs. 5.9).

Seed fresh weight, dry weight and water content were compared for the *vip-2* mutant and its wild-type (HL107). The fresh weight of wild-type and *vip-2* mutant seed was similar. However, the dry weight of the *vip-2* seeds was approximately 20% less than the wild-type seeds from 15 days after flowering (Fig. 5.10). In this trial viviparous germination of *vip-2* seeds occurred at around 20 days, so the *vip-2* mutant appears to have an increased seed water-content compared with the wild-type before the onset of vivipary, and before contact point is reached.

The precocious germination phenotype of *vip* mutant seeds is determined by the genotype of the embryo. The maternal genotype of the testa and pod enclosing the embryo does not affect the expression of the *vip* mutant phenotype. When a cross between *vip-2* and *wil* was performed (see following section) *vip-2* was chosen as the maternal parent. None of the F_1 seed germinated precociously, although the pod and testa had the homozygous mutant genotype. The F_2 seed showed segregation of precociously germinating, and non-precociously germinating types within the F_1 pods (Fig 5.11) although the pods were heterozygous for the *vip* mutation (*Vip-2 vip-2).* Thus, if the embryo is carrying two *vip* mutant alleles, then the seed will germinate precociously regardless of the maternal genotype.

Mapping the *vip* gene

The *vip* gene has been found to be in group V of the pea linkage map, between the morphological markers *crispa (cri)* and *cerise (ce)*, which reduces anthocyanin pigmentation and has 'cerise' coloured flowers. In the first cross with multiple marker line HL111, significant linkage was found between *vip-2* and *green pods (gp)*, but none between *vip-2* and *tendril-less (tl)* at the other end of linkage group V (Table 5.4). In subsequent crosses of *vip-2* with *cri* (HL21) and *vip-1* with *ce* (HL106), to confirm the location of *vip* on group V and more accurately define its position, significant linkage was also found with each of these two loci (Table 5.3), particularly to *cri* (RCV= 3% ± 6). The present data indicates a map order of: *.tl...*40*...gp...*10*...cri...*3*...vip...*17*...ce.* (Fig. 5.12).

ABA levels in *vip* seeds and shoots

To determine whether the viviparous phenotype of the *vip* mutant was due to a defect in ABA biosynthesis, the level of free ABA in seeds of both mutant alleles (*vip-1, vip-2*) and the wild-type (HL107, cv. Torsdag) was measured at five day intervals during development, from five days after flower opening until the mature wild-type seed was dry. Seeds of the pea ABA-deficient mutant *wil* were also assayed for comparison. These measurements were made on two separate occasions, one during winter (Fig. 5.13) and one during summer (Fig. 5.14).

In both experiments, free ABA levels in wild-type seeds rose during seed development, reaching a peak in mid-development and then declining. The winter-grown wild-type seeds showed a peak in ABA levels in the very young seed at 5 days old (Fig. 5.13), which is not seen in the summer-grown wild-type seed (Fig. 5.14). The 30 day old summer-grown seed shows low levels of ABA, as did the germinating mature wild-type seed which had been germinating for three days (Fig. 5.14).

Both *vip* alleles showed somewhat altered ABA levels. Seeds of *vip-1* show a reduced ABA peak during mid development in both summer and winter grown seeds. Levels of ABA in the *vip-2* seeds reached similar levels to the wild-type, but in the summer-grown seeds, show a peak of ABA later in development than the wild-type. In contrast, the ABA content of *wil* seeds was reduced substantially, to less than 10% of wild-type levels, throughout seed development, with ABA levels rising very gradually with time in both summer and winter-grown seeds. Yet, *wil* seed is not viviparous. (Note that the *wil* mutant is on a different genetic background from the other lines, so that ABA levels are only qualitatively comparable between *wil* and the other genotypes.)

Changes from wild-type ABA levels in the *vip* mutants appear between 10 and 15 days after flower opening in summer, and between 15 and 20 days after flower opening in winter, which is approximately 10 days before precocious germination is visible in the mutant seed. It is also notable that ABA levels in germinating wild-type seed are low (Fig. 5.14), whilst in *vip-2* plants ABA levels are relatively high when precocious germination begins.

The ABA content of *vip-1* shoots grown under normal conditions, or after drought stress (5 days withdrawal of water) is not different from wild-type shoots (Fig. 5.15). Similar results were obtained for the *vip-2* allele.

Phenotype of the double mutant *vip-2 wil*

No difference was seen between the double mutants generated, irrespective of whether *wil* was the female or male parent in the cross. The *vip-2 wil* double plants showed an additive phenotype; their seeds were viviparous and the plants showed 'wiltiness' in high temperatures. There were no significant differences between the double mutant plants and *vip*-2 and *wil* lines in growth habit or seed set.

GA_1 levels in *vip* seeds

No GA_1 was detected (<0.5 ng/g[FW]) in germinating *vip* seeds or in maturing wild-type seeds of the same age. GA_1 levels were also undetectable in germinating wild-type seeds (72 hours after imbibition).

The 2β-hydroxylated catabolite of GA_1, GA_8, was present at low levels (≅0.5 ng/g[FW]) in both *vip-1* and *vip-2* seeds during later development, whereas it was undetectable in the wild-type at this stage. The presence of this GA_8 suggests that GA_1 was previously present in the *vip* seeds (Ross et al. 1995), although it is possible that GA_8 could be formed by other routes, for instance 3β hydroxylation of GA_{29}.

The levels of some other GAs were altered in *vip* mutants. These data are not shown in detail as these compounds are not biologically active *per se*, and as such their significance is difficult to interpret.

Phenotype of the double mutant *vip-2 lh-2*

With regard to the specific effects of the mutant alleles, the double mutant *vip-2 lh-2* has an additive phenotype. That is, they are dwarfs with viviparous seeds. However, the double mutants are 60% shorter (total length = 25.5 ± 3.5 cm, n=10; very small plants not included, see below) than the dwarf parent *lh-2* (total length = 45 ± 1.5 cm, n=20) (Fig. 5.16). They have both shorter internodes and fewer nodes (Fig 5.16). They also show higher seedling mortality, at 20-25%, than the *vip* parent and approximately one third of those seedlings that do survive go on to produce very small plants (<10 cm) which do not set seed (Fig. 5.16).

Sensitivity of *vip* seed to exogenous ABA

Germinating *vip* seeds and germinating wild-type seeds showed similar inhibition of growth in the presence of ABA. When germinating *vip-1* seeds were removed from the pod and placed on ABA-containing media, their subsequent growth was reduced in a manner dependent on the ABA concentration (Figs. 5.17, 5.18). Germinating wild-type seeds were used for comparison. Mature, dry, wild-type seed were imbibed for 72 hours in sterile conditions (until their radicles were a similar length to those of the precociously germinating *vip* seeds), and then placed on ABA media. Germinating wild-type and *vip* mutant seeds both showed similar concentration-dependent inhibition of growth by ABA.

The shoot growth data was analysed by a two-way analysis of variance using the program SAS (The SAS Institute). ABA was found to have a significant influence on growth rate (P=0.0157), but neither genotype (P=0.1483), nor genotype-environment interaction (P=0.2061) were found to have a significant effect.

Expression of the pea dehydrin gene (provided by M. Robertson), which is inducible by ABA in the shoot (Robertson and Chandler, 1992), was increased in the wild-type and *vip-1* seedlings grown in the presence of ABA from the germinating *vip* and wild-type seeds described above (Fig. 5.19). In the wild-type, the expression of the pea dehydrin gene increased with increasing ABA concentration in the growth media from zero to 15 μM ABA. At the highest ABA concentrations of 30 to 100 μM, a different pattern of dehydrin gene expression was seen; unexpectedly, there was a decrease in wild-type expression of dehydrin and additional hybridising band appeared. It is likely that these high ABA concentrations represent higher ABA levels than would be found endogenously in seedlings.

The *vip-1* material also showed concentration dependent induction of the dehydrin transcript in response to exogenous ABA. However, the *vip*-1 tissue was less responsive to the exogenous ABA than the wild-type, with a lower dehydrin mRNA abundance, and higher exogenous ABA concentrations required to detect the dehydrin mRNAs (Fig. 5.19).

When twelve day old developing wild-type and *vip-1* embryos (with a mass of approximately 20 mg) were removed from their seeds and cultured in media containing 200 μM ABA , their subsequent growth and germination showed *vip-1* seeds were somewhat less sensitive to inhibition by ABA than the wild-type (Figs. 5.20, 5.21). Approximately 10% of the *vip-1* embryos on the ABA medium showed root elongation, although 75% died (Fig 5.20). In comparison all of the wild-type embryos shrivelled and browned, 20% showed a small amount of callus growth (Fig 5.21).

Embryos of both genotypes were able to grow and germinate on a medium containing 2% sucrose (Figs. 5.22, 5.23). In both genotypes approximately 40% of embryos died, but approximately 10% were able to produce organised roots and shoots. Embryos that did not form roots and shoots showed growth of disorganised leafy green tissue and root tissue from callus (Figs. 5.22, 5.23).

Embryos of both genotypes also showed growth inhibition in a medium with a high osmotic pressure (13% sucrose) (Figs. 5.24, 5.25). The *vip-1* embryos were less severely affected than the wild-type embryos. All the wild-type embryos swelled, bleached and died, although many produced some callus along the edges of the cotyledons before dying. Of the *vip-1* embryos, only 50% died, with the surviving embryos remaining green and some showing a limited degree of axis elongation. Similar results were obtained in a repeat experiment.

Expression of ABI3 in *vip* seeds

The homologous pea *ABI3* gene shows no gross changes in *vip-1* seeds of different ages (Fig. 5.26). It has a similar size and level of expression in all samples. These samples include those before and during the times at which precocious germination occurs in the *vip* mutant. Note that the pea *ABI3* transcript is smaller (approximately 2.7 kb) that the *Arabidopsis* transcript (approximately 3.1 kb). There are also other low molecular weight hybridising bands that could be a related gene family in pea.

Expression of messenger RNAs typical of maturing seeds in *vip* seeds

To examine how the *vip* mutation affects gene expression during seed maturation, the expression of five genes (*ABR-17,* albumin [PA2], dehydrin [pSB12], lectin [pS15-50] and legumin [pCD43]) whose transcripts are abundant in mid to late seed development were examined by northern blotting.

Expression was examined at several stages throughout seed development, from the young seeds 5 days after flower opening (DAO) [seed mass≅50 mg], to mature drying seeds 25 DAO (seed mass≅500 mg). Wild-type seeds which had been imbibed and germinated for 72 hours at room temperature were also included for comparison. At this stage the wild-type seeds showed a similar amount of radicle extension (approximately 1 cm) to the precociously germinating *vip* seeds. Seeds of the ABA-deficient pea mutant *wil* were also included to determine whether reduced ABA levels would affect gene expression. Seed ABA levels in *wil* are approximately 10% of wild-type levels (Figs. 5.13, 5.14). (It should be noted that *wil* seeds have a different genetic background from the other genotypes, and so expression patterns in *wil* seeds are only qualitatively comparable with the other lines). In the following northern blots, germination (as determined by visible radicle extension) begins between 17 and 21 DAO, and is evident at 21 days and 25 DAO in the *vip* seeds.

Abscisic Acid Responsive-17 (ABR-17)

ABR-17 encodes an ABA responsive pea protein similar to disease resistant proteins (Iturriaga et al., 1994). In the wild-type *ABR-17* mRNA and protein are synthesised late in seed development with abundance increasing as the seed ages (Iturriaga et al., 1994). Expression is not confined to the seed; its expression is increased during severe desiccation and in embryo cultures *in vitro* in response to exogenous ABA (Iturriaga et al., 1994). However, the roles played by this protein, and another similar protein *ABR-18*, are currently unclear. They may play a defence role. *ABR-17* and *ABR-18* show a lack of homology with other late embryo abundant proteins which suggests they are a unique group of stress-induced proteins (Iturriaga et al., 1994).

ABR-17 mRNA expression is similar for wild-type and *vip* seeds with levels increasing during maturation and on germination (Fig. 5.27). The highest levels are seen in germinating mature wild-type seeds. *ABR-17* mRNA levels are strongly reduced in the seeds of the ABA-deficient mutant *wil*, although there is a similar pattern of expression with increased expression in the 25 DAO *wil* seeds (Fig. 5.27).

Albumin (PA2)

The major pea seed albumin PA2 is abundant relatively late in seed development (Higgins et al., 1987). Its physiological role is unclear because it is unusual. It undergoes no major post-translational modification, and is located in the cytosol rather than in storage vesicles. It is relatively resistant to breakdown on germination, possibly due to its location (Higgins et al., 1987).

Expression of PA2 increases in the wild-type between 5 and 17 DAO and remains high during late seed development (Fig. 5.28). Levels of mRNA drop steeply upon germination in the wild-type . However, seeds of both *vip* alleles continue to accumulate PA2 mRNA while they germinate. Seeds of the *vip-2* mutant have particularly high levels of PA2 at 17 to 25 DAO (Fig 5.28). Seeds of the *wil* mutant accumulate similar levels of PA2 mRNA to the wild-type, and show increasing expression with age (Fig 5.28).

Dehydrin (pSB12)

Pea dehydrin accumulates in the seed during mid to late embryogenesis (Robertson and Chandler, 1992). It is most actively synthesised at maximum seed fresh weight and forms 2% of the protein in mature cotyledons. Dehydrin mRNA is reversibly induced by ABA and dehydration and is also expressed in the stressed vegetative plant (Robertson and Chandler, 1992). Dehydrin proteins are produced in many plant species in response to stresses with a dehydrating component (Campbell and Close, 1997; Chandler et al., 1994); although their precise role is unknown they may act as an intracellular stabiliser, in particular for membranes (Mao et al., 1995).

In the wild-type, dehydrin mRNA increased from 5 to 17 DAO and is high from 17 to 25 DAO, but is significantly reduced in germinating wild-type seeds (Fig. 5.29). Expression of dehydrin mRNA reaches similar levels in all of the four genotypes examined (Fig 5.29). In contrast to wild-type seeds, the *vip-1* and *vip-2* seeds continue to accumulate dehydrin mRNA even though they are germinating.

When expression of dehydrin mRNA in young germinating seedlings was examined (Fig 5.30), it was seen that expression of dehydrin mRNA is low in young germinating wild-type seedlings. However, expression levels remain high in young germinating *vip-2* seedlings, although they do show a gradual decline over seven days.

Lectin (pS15-50)

Lectins have binding sites for specific carbohydrates and for Ca^{2+} and Mg^{2+} ions (Higgins et al., 1983). They have the ability to agglutinate cells, or precipitate polysaccharides or glycocongugates. They constitute approximately 2% of total mature pea seed protein. Several biological functions have been suggested for pea lectins. So far roles in host specificity of *Rhizobium* nodulation and in defence against seed eating insects have been supported (Casey et al., 1993).

In the wild-type, lectin mRNA levels rise from 5 to 13 DAO, they are high from 13 to 25 DAO and drop steeply on germination to very low expression levels (Fig. 5.31). Seeds of the *vip* mutants continue to accumulate lectin mRNA although they are germinating (Fig. 5.31). Mutant *vip-2* seeds show very high levels of expression of lectin mRNA from 17 to 25 DAO. Seeds of the *wil* mutant show a similar lectin expression pattern to the wild-type seeds (Fig. 5.31).

Legumin (pCD43)

Legumins are a major pea seed storage protein (legumins and vicilins make up 25% of dry pea seeds by weight (Casey et al., 1993)). Pea legumin is an oligomeric protein, consisting of a hexamer of disulphide-bonded subunits each of which has an acidic (α) and a basic (β) polypeptide of M_r 40 000 and 20 000, respectively Legumin molecules are the product of a family of homologous genes (there are at least eight legumin genes in three classes in *Pisum* (Domoney and Casey, 1985)). These genes are highly regulated during seed development and are only expressed at this time (Hauxwell et al., 1990).

Here we have examined the expression of the legumin cDNA clone pCD43, a legumin A class gene which selects mRNA for legumin precursors of M_r 60 000 (Domoney et al., 1986). In the wild-type, expression of this legumin mRNA shows two peaks of expression, one at 10 DAO and a second at 25 DAO (Fig. 5.32). In the wild-type there is a substantial increase of lectin pCD43 expression on germination. A similar pattern of lectin mRNA expression to the wild-type is seen in *vip-2* mutant seeds (Fig. 5.32), while *wil* seeds show only the second peak in lectin mRNA expression at 25 DAO (Fig. 5.32). The *vip-1* seeds show only the first peak in lectin mRNA expression at 13 DAO and show low levels of expression during germination from 21-25 DAO.

DISCUSSION

Mutant Description and Genetic Analysis

The *vip* gene has been located in group V of the pea linkage map, between *crispa (cri)* and *cerise (ce)*. It is closely linked to *cri* (RCV = 3% ± 6%). There are no known quantitative trait loci (QTL) or any other genetic markers relating to germination on group V.

The *vip* mutant appears to be chiefly seed specific. The effects on early seedling growth are most likely due to disturbed metabolism in the viviparous seed and/or damage to the seedling root, which occurs in the pod due to twisting and/or desiccation of the root tip. The effects on inflorescence architecture could be due to alterations in resource allocation. Reduced *vip* fertility may also be due to changes in the flower or gametes. The reduced number of *vip* seeds in crosses is unlikely to be due to perturbed early seed development as rates of seed abortion are not increased. Thus, the *Vip* gene would appear to be a regulatory element that prevents precocious germination in developing pea seeds, but also has some role (direct or indirect) in the flower, assisting inflorescence and gamete formation.

We know that it is the genotype of the embryo that determines the expression of vivipary, as it is possible to obtain viviparous embryos in wild-type pods and vice versa, by crossing. Thus, the actual mechanism responsible for the prevention of precocious germination must reside within the embryo itself. The mutant *vip* seed remains green and shows increased water content compared to the wild-type; thus, substantial changes in the seeds' composition must occur. The seed water content differed between *vip* and wild-type from five days before radicle emergence was visible. It would seem that these changes are due to the initiation of the germination process, although it cannot be ruled out that early changes in seed metabolism subsequently lead to precocious germination.

The faster early growth of *vip* seedlings (Fig. 5.4) suggests that perhaps their precocious germination is due to a speeding-up of development, or acceleration. This

is seen in some other mutants showing precocious germination, such as *lec1* and *fus3* (Keith et al., 1994; Meinke et al., 1994) . However, other evidence of peramorphic change (increased adult characteristics), such as leafy cotyledons, or accelerated phase change (Table 5.1), is not seen in *vip* seedlings. Provascular traces in pea do not normally develop into functional vasculature until germination, at which point the procambium tissue gives rise to mature xylem and phloem elements within the first two days (Liu et al., 1996). Examination of the maturation of the provascular elements in the cotyledons could provide more evidence to show whether *vip* mutant seeds have accelerated maturation. There is currently debate in the literature about whether viviparous mutants affect specification of the maturation pathway or affect developmental timing, as both points of view have validity and both modes of action are represented among the available mutants (McCarty, 1995).

ABA and GA levels in *vip* seeds and shoots

ABA levels in pea seeds are affected by the length and temperature of their growth period (Figs. 5.13, 5.14). The winter-grown seeds show a double peak of ABA, the first early in development probably at around 5 days after flower opening (DAO), and the second when the seed reaches maximum size at around 20 days DAO (Fig 5.13). In the summer-grown seeds there is a single ABA peak only, at about 15 DAO in the wild-type (Fig 5.14). The seeds mature faster in the warmer summer temperatures, so the initial ABA peak has probably occurred before the first sampling age at 5 DAO. For the same reason, the 30 DAO wild-type, winter-grown seed has high levels of ABA because it is not yet dry, whereas the 30 DAO wild-type seed grown in summer has dried substantially and has low ABA levels (Figs. 5.14, 5.15).

Wang et al. (1987) found a biphasic ABA distribution, similar to the winter-grown seed seen here. The seeds in the Wang et al. (1987) study were grown in relatively cold temperatures of 15 to 17°C, and took 65 days to mature. They measured ABA levels in different parts of the seed and found that the first peak seen was due to ABA in the testa, and the second due to ABA in the maturing embryo. Other studies of ABA levels in legume seeds have produced different results. In soybean, King (1982) found a single ABA peak, maximal at the greatest seed fresh weight. Eeuwens and Schwabe (1975) found, using bioassay in peas, a single rise in embryo ABA at the end of seed growth. Similarly, Browning (1980) similarly found a single maximum ABA level peak in pea at maximum seed fresh weight, using GC-MS/SIM, for seed grown at 29 and 20°C. These last three studies all had seeds maturing over 25 to 30 days. This is similar to the results for the summer-grown seed in this chapter. Browning (1980) also analysed ABA levels in seed grown at 13°C, and these showed a large primary peak and a smaller secondary peak. It seems that when pea seeds mature slowly at lower temperatures two peaks in ABA levels are distinguishable, related to peak levels in the testa and in the embryo. However,

when the seeds mature more quickly in higher temperatures a single peak in ABA levels is seen.

The ABA levels seen in *vip* seed do differ somewhat from the wild-type. In the winter-grown seeds (Fig 5.13), *vip-1* seeds have lower ABA levels than the wild-type after 15 DAO, and they do not show the large peak in ABA levels at 20 DAO. However, the seeds of the *vip-2* allele are similar to the wild-type. In the summer-grown seeds (Fig 5.14), *vip-1* again shows lower ABA levels than the wild-type, while *vip-2* shows similar levels, although with a later occurring peak. The different responses of *vip-1* and *vip-2* show that there are metabolic differences between the two alleles. Significantly though, seeds of the *wil* mutant have only about 10% of the ABA level of wild-type seeds, and yet do not show precocious germination. Therefore, the precocious germination seen in *vip* mutants is most likely not due to reduced ABA levels. However, it could also be important to examine the localisation of ABA within the seed, as there is evidence that ABA is not evenly distributed within the seed, or within cells (Kermode 1995). Thus, it is possible that *vip* may alter the availability of ABA within the seed without changing overall levels.

The results shown here would also seem to discount the possibility that an overproduction of the bioactive GA (GA_1) is causing viviparous germination in the *vip* mutant. Levels of GA_1 are not detectable in both mature and germinating seeds of the *vip* mutant and the wild-type. In maturing wild-type seeds, GA_1 is detected during early and mid-development, but is not detected in late development (Swain et al., 1993; Batge et al., 1999). No previous studies have attempted to measure GA_1 in germinating wild-type seeds. It is possible that a gibberellin peak does not play a role in growth of the germinating embryo in pea as it does in some other species (Karssen, 1995). Investigation of a range of GAs at different stages of seed development in *vip* and wild-type seeds would help to determine this.

There are GA-deficient mutants of Arabidopsis and tomato which will not germinate without application of GA (Groot and Karssen, 1987; Koornneef and van der Veen, 1980). Evidence from the *lh*-2 mutant suggests that GAs are not required in pea seed development after contact point, when all the liquid endosperm has been absorbed (Swain, 1993). The differences in GA metabolites seen here between the *vip* mutant and wild-type probably result from altered seed metabolism in the mutant, rather than via a direct effect. In the example given, the increased GA_8 levels in *vip* are probably due to the embryonic axis, where 2β-hydroxylation occurs (Ross et al., 1995) - which is active in the *vip* mutant seeds at this time, while the wild-type is quiescent.

Double mutants with other hormone mutants

The phenotype of the *vip-2 wil* double mutant is additive, producing a 'wilty' plant with viviparous seeds. This makes it highly likely that these two genes operate in different pathways, and taken together with the similar ABA levels seen in *vip* and wild-type seeds and shoots seem to rule out the direct involvement of the *vip* gene in ABA synthesis.

Likewise, the *vip-2 lh-2* double mutant phenotype is largely additive, being a dwarf plant with viviparous seeds. Given this, it is unlikely that *vip* seeds show precocious germination due to high bioactive GA levels. However, the double mutant combination *vip lh-2* had non-specific effects on the overall growth and vigour of the plant, producing plants 60% shorter than the dwarf parent and with increased seedling mortality. It is possible that the *vip* mutation has an indirect action on the GA pathway; hormone levels in the double mutant seeds and shoot could be examined to investigate this, but it unlikely that the phenotype of *vip* seeds is due to the role of GAs. It is also interesting to note that young *lh*-2 seeds have three to four fold increased ABA levels (Batge et al., 1999), but this did not prevent precocious germination of the *vip-2 lh-2* double mutant seeds, providing further evidence that low ABA levels do not cause the precocious germination seen in *vip* seeds. Investigation of the ABA levels in the *vip-2 wil* double mutant seeds could provide further information about the relationship between ABA levels and vivipary.

Sensitivity of *vip* seed to exogenous ABA

Mutant *vip-1* seeds and their shoots appear to have somewhat reduced sensitivity and response to exogenous ABA. Both mature *vip-1* and wild-type seeds have their growth inhibited similarly by culture in ABA-containing media (Figs. 5.17, 5.18), and both show induction of dehydrin (an ABA-inducible gene), in these ABA containing culture media (Fig. 5.19). However, the *vip-1* seedlings grown in the ABA culture media had reduced levels of dehydrin expression compared to the wild-type seedlings (Fig 5.19). In addition, immature *vip-1* seeds showed less inhibition of precocious germination by ABA than the wild-type (Figs. 5.20 and 5.21). There is also evidence that the *vip-1* embryos showed faster earlier development than the wild-type (Fig. 5.4 a,b). It is possible that this early insensitivity to ABA allows *vip* embryos to begin germination in the pod, with these changes occurring from 12 to 15 days after flower opening. It is also possible that accelerated development leads to their germination, and the insensitivity to ABA seen in the embryo is an indirect effect of this.

The effect of sucrose in the growth media seen here was consistent with the work of Cook et al. (1988), who showed precocious germination of immature pea embryos occurred on media with 2% sucrose, but was increasingly inhibited on media containing 5 to 15% sucrose. Yet, this is the first time inhibition of precocious germination in immature pea embryos by ABA has been shown. The work of

Barratt et al. (1989) found that some 20% of immature pea embryos (mass 1 to 200 mg) could still germinate on media containing 100μM ABA, and that only a combination of ABA and high sucrose (5%) could inhibit root and/or shoot extension. The higher ABA concentration (200μM) and small (20 mg) embryos used here may explain the more efficient inhibition of wild-type precocious germination seen.

Expression of seed maturation genes in *vip*

Expression of the *ABI3* gene was not changed in the germinating *vip* mutant seeds when compared with wild-type seeds, indicating that the *vip* mutation does not occur in the pathway of *ABI3* gene expression in the seed. The putative pea *ABI3* homologue was 0.4 kb smaller than the *Arabidopsis* transcript.

There was a similar pattern of expression of *ABR-17* in wild-type and *vip* seeds, showing increasing expression levels with maturation and germination. This is likely to be related to the ABA levels in these tissues. Likewise, the low levels of ABA in *wil* seem to be reflected in low levels of *ABR-17* expression.

Expression of albumin (PA2), dehydrin (pSB12) and lectin (pS15-50) all showed similar patterns in wild-type and *vip* seeds. In the wild-type, levels increased during early to mid-development and remained high in late development, then dropped in germinating seeds. In *vip* seeds, the transcripts also accumulated during development but, in contrast, these transcripts remained high in *vip* seeds during late development, even though these *vip* seeds were germinating precociously at this age. Thus, *vip* seeds are expressing genes characteristic of maturation and germination simultaneously. The change that has enabled them to respond precociously to germination cues has not halted normal seed storage gene expression.

The expression of legumin (pCD43) is harder to interpret, but shows broadly similar patterns in wild-type, *vip* and *wil* seeds. Some differences in legumin expression patterns are seen between the two different *vip* alleles. Expression of *ABR-17*, albumin, lectin, legumin and dehydrin mRNA is somewhat reduced in *vip-1* compared with *vip-2*. This correlates with the poorer seedling survival seen in *vip-1* compared with *vip-2* seeds and suggests that *vip-1* appears to be a more severe allele with greater disruption to normal seed metabolism.

ABR-17 expression is reduced in *wil* seeds compared with the Torsdag wild-type. Although these lines have different genetic backgrounds, this supports the role for ABA in the upregulation of expression of these this gene (Iturriaga et al., 1994). Thus, the reduced ABA levels seen in *wil* seeds do affect ABA-related gene expression. However, these *wil* seeds do not show precocious germination. So, control of precocious germination is unlikely to be related directly to control of gene expression by ABA in pea. With regard to expression of albumin, lectin and to a lesser extent legumin, *wil* seeds and wild-type seeds have similar mRNA expression patterns, indicating that *wil* seeds show some aspects of normal seed development, presumably unrelated to seed ABA levels. Unexpectedly, expression of pea dehydrin was not substantially reduced in *wil* seeds. This means that in this case

dehydrin is not being induced by high ABA levels, but its induction could be due to increasing dehydration in the maturing seeds. Thus, the induction of dehydrin in pea by ABA and by dehydration seems to be at least partially separable; a finding which has also been suggested by other workers (Robertson and Chandler, 1994).

Both seed maturation and seed germination seem to proceed simultaneously in the *vip* mutant at the whole seed level (as shown by northern blotting), which contradicts the idea that developmental and germinative programmes are mutually exclusive with a simple switch between them (Kermode, 1995; Kermode, 1990). Simultaneous expression of both developmental and germinative events has also been seen in a precociously germinating line of Chinese cabbage (*Brassica rapa* ssp. Pekinensis) (Ren and Bewley, 1999) and in precociously germinating *in vitro* cultured embryos of *Brassica napus* (Finkelstein and Crouch, 1984), soybean and tobacco (Jakobsen et al., 1994). However, it may be the case that these pathways are segregated at the cellular level (Bisgrove et al., 1995; Fernandez, 1997). In any case, the simultaneous expression of both catabolic germination processes and anabolic maturation processes in precociously germinating *vip* seed would account for their high seedling mortality and poor early seedling growth. In *vip* seeds resources are being diverted towards two different and conflicting outcomes, rather than being separated to allow successful maturation and then germination and early seedling growth.

General Discussion

In many species the plant growth regulator abscisic acid plays an important role in the prevention of precocious germination. It was thought initially that *vip* would be an ABA mutant. However, investigations so far have shown that *vip* has normal ABA synthesis and some insensitivity to exogenous ABA in mid-development of the seed and in gene expression in the seedling . However, there is still a strong argument to be made that perception of ABA levels does not normally prevent precocious germination in pea seeds. This is because the *wil* mutant, with substantially reduced ABA levels in seeds, does not show precocious germination. Barret et al. (1989) also found that treatments which reduced ABA levels in wild-type seeds did not cause them to germinate precociously. It is possible that the effect of ABA on the control of germination has become less important in pea due to modifications during domestication to remove seed dormancy.

The hormone gibberellic acid (GA) is important during the onset of germination, especially in cereals. However, we have seen that *vip* and wild-type seeds contain very low levels of active GA_1. The double mutant of *vip* and the GA-deficient mutant *lh-2*, also has an additive phenotype. Hence, the factor missing in *vip* mutants, which controls precocious germination, appears to act independently of the two major hormone groups thought to control germination. This is consistent

with the fact that there are no other aspects of the phenotype of the *vip* mutant that suggest the metabolism of these hormones has been disrupted.

Another major factor thought to be responsible for suppressing germination in mature embryos is desiccation (Kermode, 1995; Welbaum et al., 1998). However, desiccation seems not to be the primary factor in this case as *vip* seeds germinate before desiccation would be due to commence in wild-type seeds. The seeds of some species (eg. tomato, tobacco) are prevented from completing germination because the embryo is constrained by its surrounding structures (Bewley, 1997). This does occur in domestic peas, and there is no substantial difference between the testae of wild-type and *vip* seeds.

An examination of the occurrence and expression in *vip* of the *Arabidopsis* genes, *LEC1* and *FUS3* (which cause vivipary when mutated) will demonstrate whether these *Arabidopsis* genes are related to the *vip* mutation. The *Arabidopsis* mutants have other phenotypes which are not seen in *vip* (eg. accumulation of anthocyanin (*fus3*) and leaf-like cotyledons (*lec1*)). However, as these mutants are seed specific and do not seem to have altered hormone levels, it is possible that *vip* is an homologous mutation, and that the differences in phenotypes are a species specific effect.

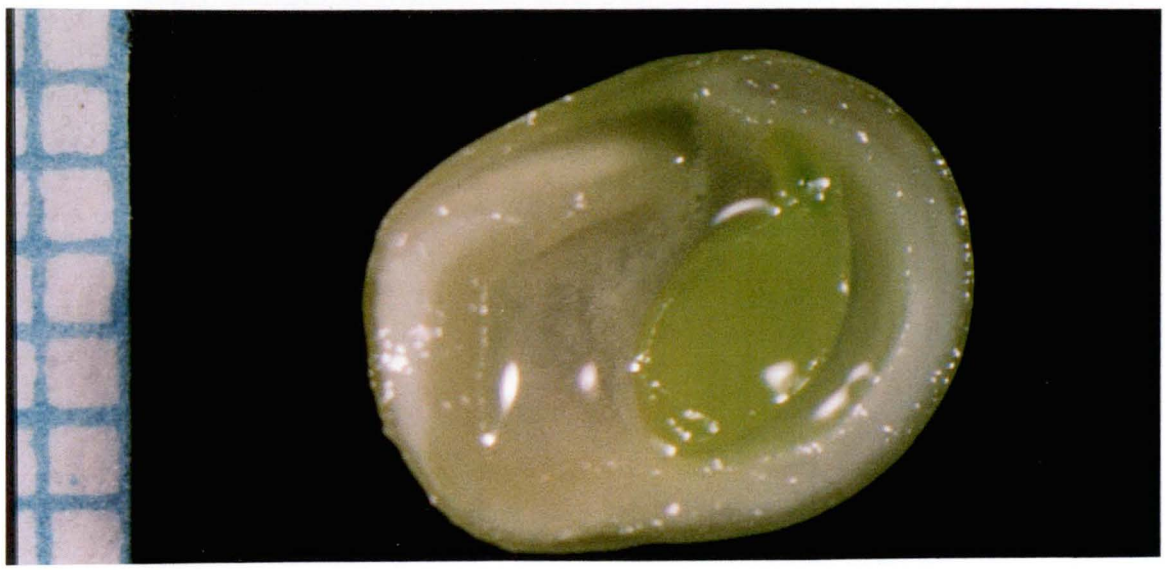
Although radicle extension is the first readily visible aspect of the *vip* mutant phenotype, it is apparent that the *vip* mutation also alters ABA sensitivity, and probably the speed of development, in the younger seed. Thus, if *vip* is deficient in a factor responsible for the prevention of precocious germination, then this factor must become active during early maturation of the seed. It cannot be ruled out that the *vip* mutation affects an earlier step which is necessary for the production of such a factor. The novel control factor missing in *vip* is sufficient for prevention of precocious germination, seems to be largely seed specific, and is probably unrelated to the major plant growth regulators. Thus, there is great potential for this *Vip* factor to be altered without negative effects on the subsequent growth of the plant.

Figure 5.1 Pod of a *vip-1* mutant plant containing germinating seed 25 days after flower opening.

Figure 5.2 *wil* seedling (right) showing wilty phenotype after exposure to hot sun, and a wild-type plant (left) exposed to the same conditions

Figure 5.3 The *lh-2* mutant shows early seed abortion (bottom pod), compared with its wild-type progenitor *Lh* (cv. Torsdag) (top pod).

Figure 5.4 Typical seeds of the *vip-1* mutants. Some seeds of the wild-type progenitor line HL107 (cv. Torsdag), and the pea ABA-deficient mutant *wil* are shown for comparison. The *vip-1* seeds shows viviparous germination 25 days after flower opening (plants grown in winter).
(Grid squares are 1 mm.)
(a) *vip-1* seed 10 days after flower opening (DAO)



(b) wild-type seed (HL107) 10 DAO; the embryo (arrow) is much smaller and less developed than the embryo of the same age (Fig 5.4a)

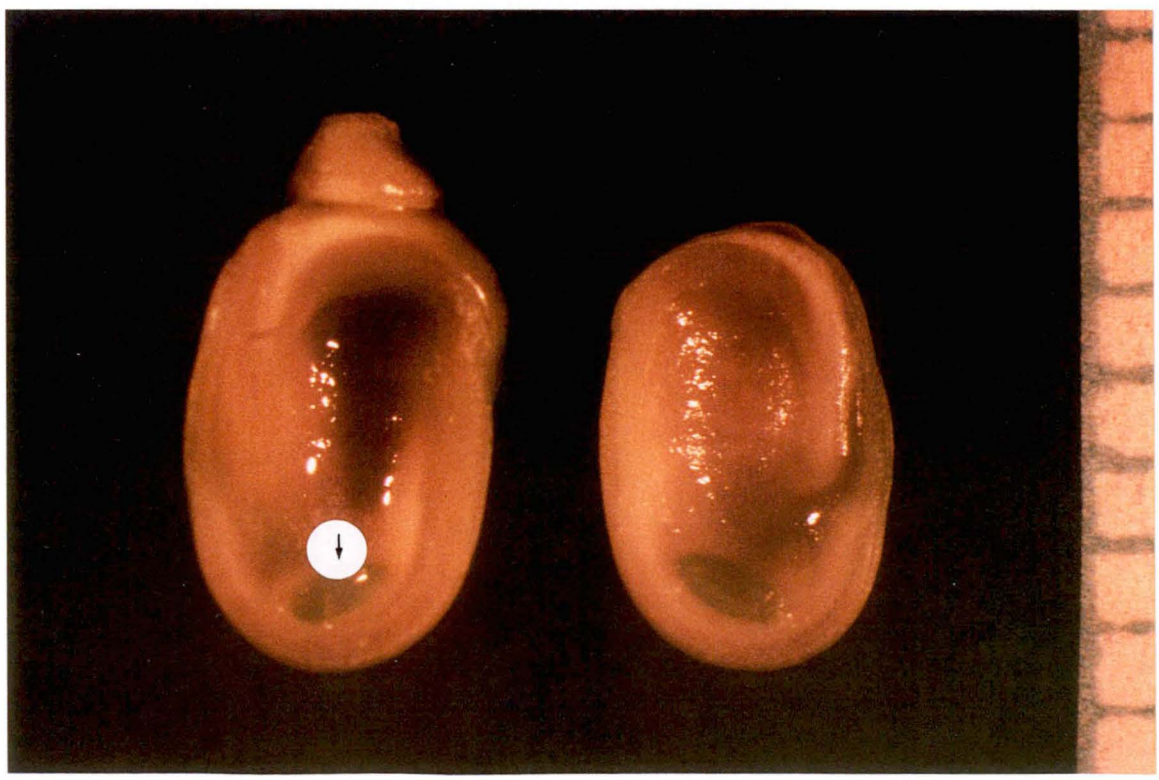
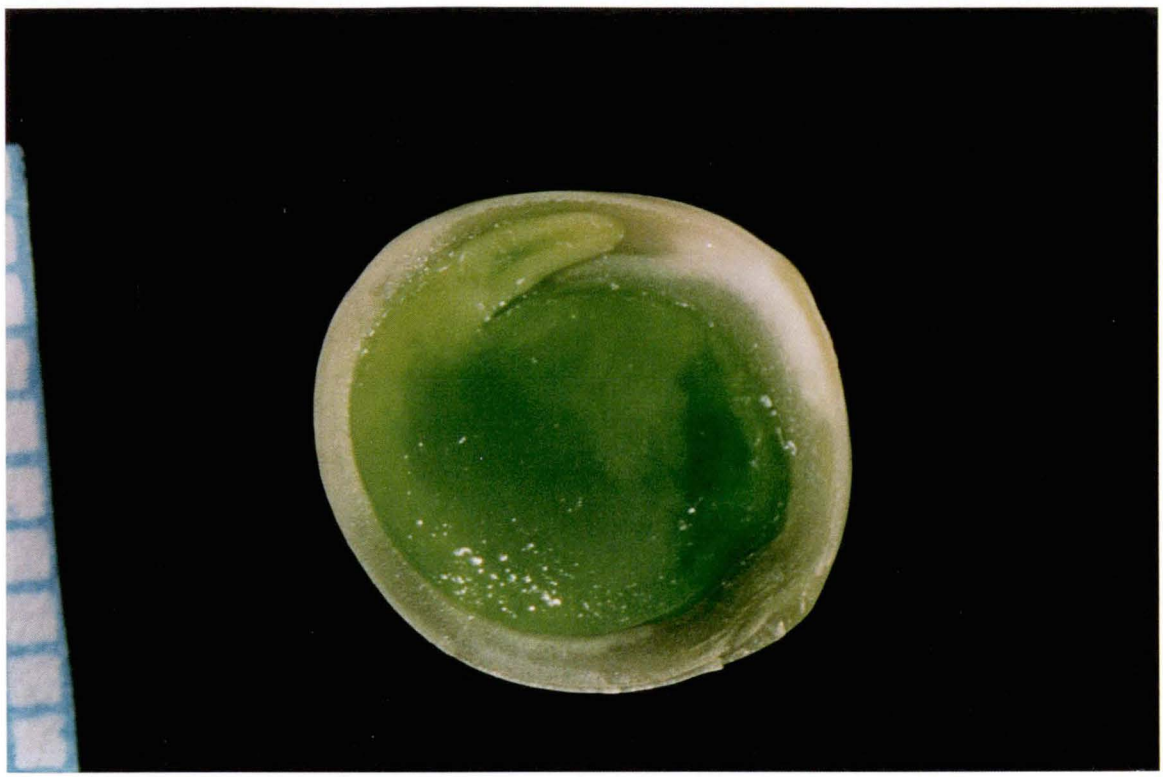


Fig. 5.4 (c) *vip-1* seed 15 DAO



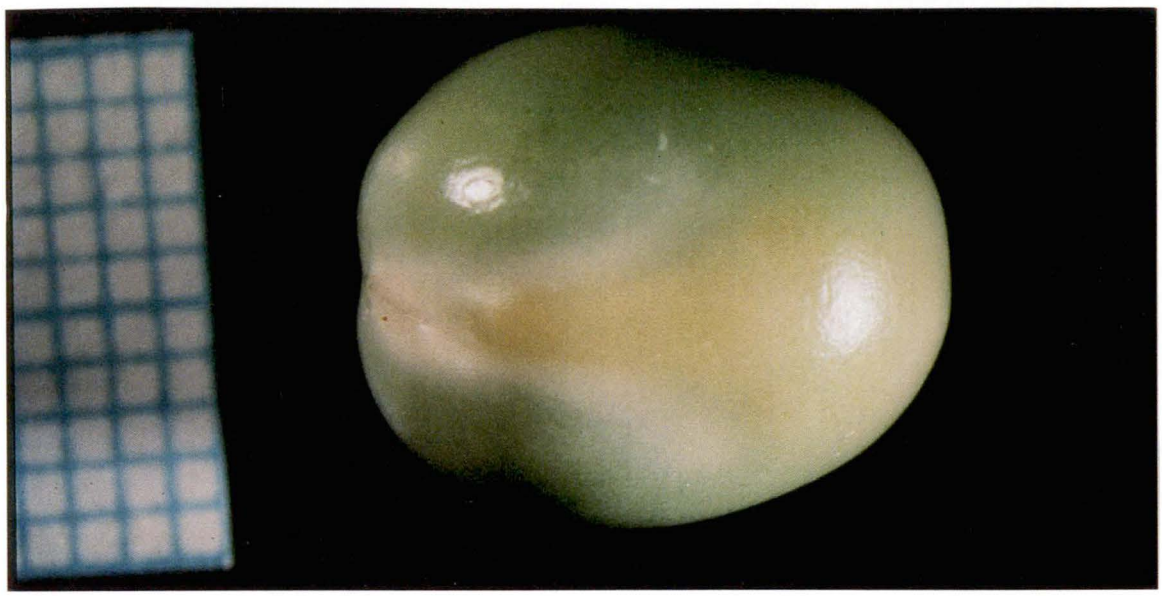
(d) *vip-1* seed 20 DAO

Fig. 5.4 (e) *vip-1* seed 25 DAO showing precocious germination with radicle emergence

Fig. 5.4 (f) *vip-1* seed 30 DAO showing precocious germination with radicle emergence

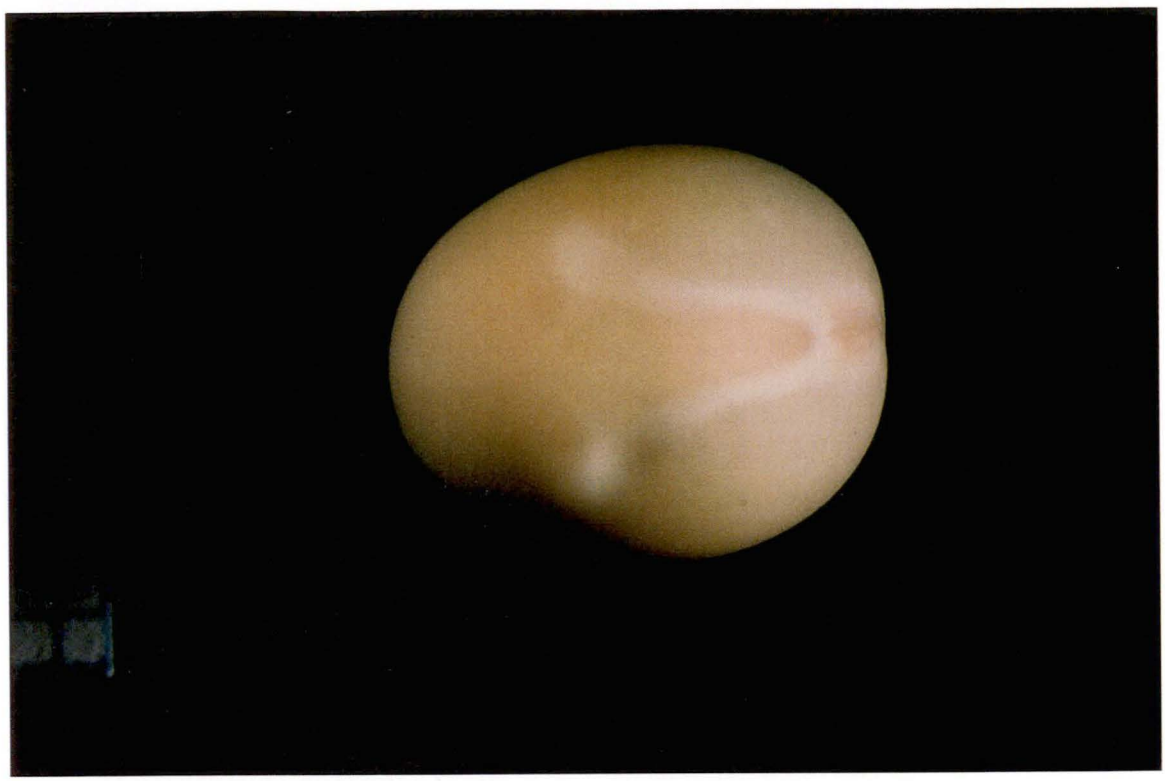
(g) *wil* seed 30 DAO showing no precocious germination despite low levels of ABA (see Figs. 5.13, 5.14)

Figure 5.5 Whole seeds of winter-grown ***vip-1*** and its wild-type progenitor (cv. Torsdag, HL107), showing precocious germination of *vip-1* seeds at 25 days after flower opening (DAO) and *vip* seeds remaining green whilst wild-type seeds become yellow (cv. Torsdag has *I*, yellow cotyledons) and begin to desiccate.
(Grid squares are 1mm)
(a) wild-type seed 25 DAO



(b) *vip-1* seed 25 DAO, showing precocious germination

Fig. 5.5 (c) wild-type seed (cv. Torsdag, HL107) 30 DAO beginning to dry and turn yellow



(d) *vip-1* seed 30 DAO remaining green and continuing to germinate

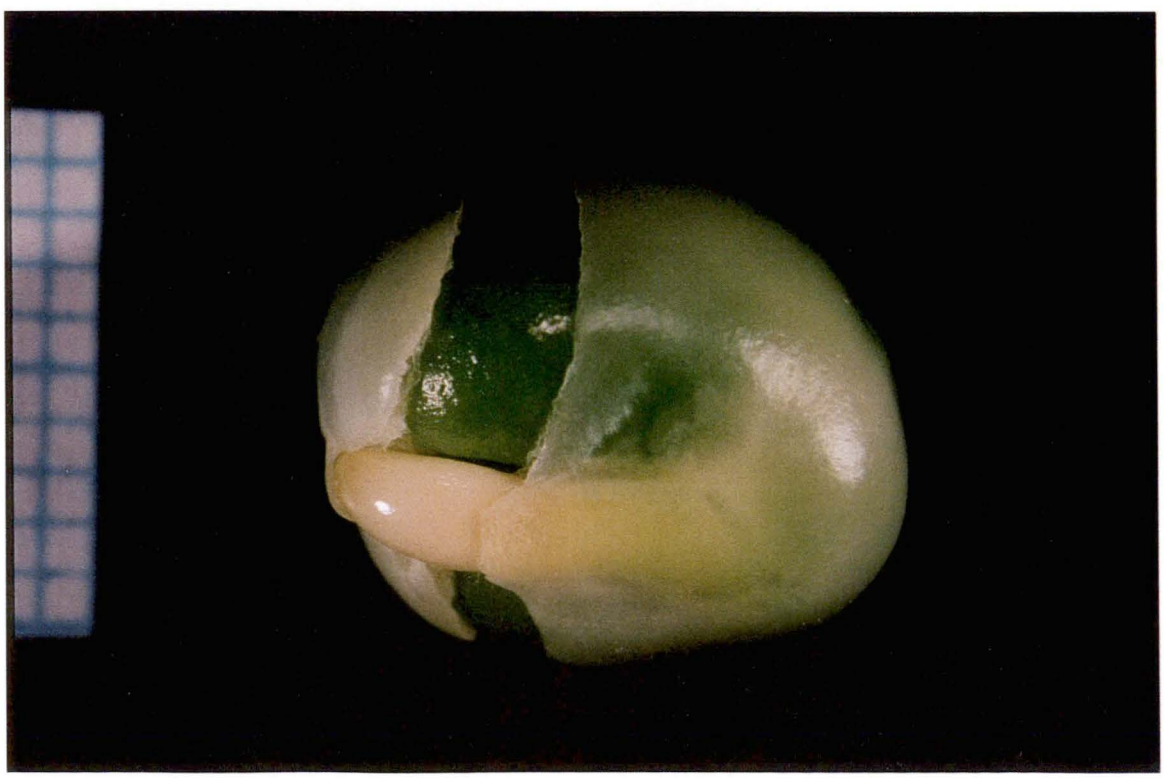


Figure 5.6 Mature dry seeds of the *vip-1* mutant (right, labelled L303), which are dead, and mature dry seeds of their wild-type progenitor cv. Torsdag (left) which are quiescent and show no signs of germination, but will subsequently germinate in a moist environment.

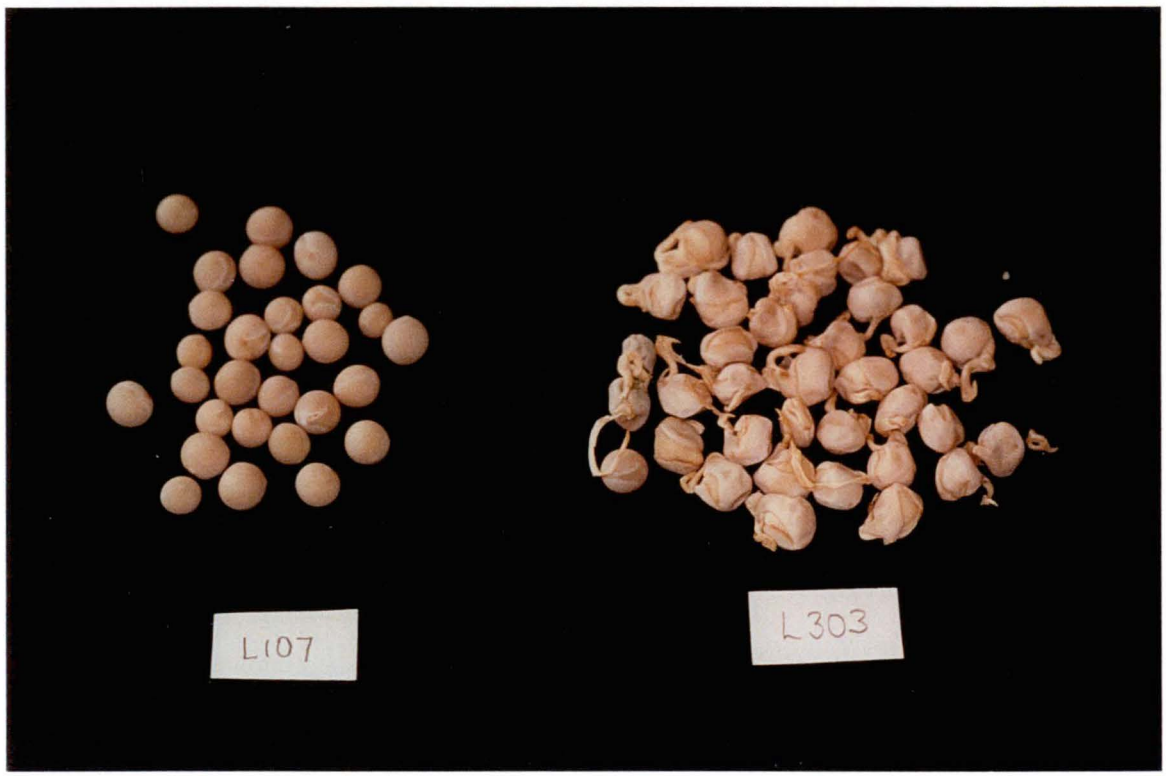


Figure 5.7 Two and a half-week-old seedlings of *vip-1* mutants (left pot) and their wild-type progenitor HL107 (right pot). The mutant plants show slower germination and higher seedling mortality.

Figure 5.8 Six-week-old plants of the *vip-1* mutant (left pot) and its wild-type progenitor HL107 (right pot). Although the mutant shows slow germination and initial growth (see Fig. 5.4), after this poor start, the growth of the mutant becomes equivalent to that of the wild-type.

Figure 5.9 Pods of the *vip-1* mutant typically have only one pod per inflorescence (middle pod), compared with the wild-type progenitor (cv. Torsdag, HL107) of this mutant, which has typically has two pods per inflorescence (left pod). Occasionally (2% occurrence) the pod of a *vip-1* mutant will have a collar of leafy tissue at the base of the pedicle (far right pod).

Figure 5.10 Changes in fresh weight and dry weight during development of summer-grown seeds of the *vip-2* mutant and its wild-type progenitor cv. Torsdag.

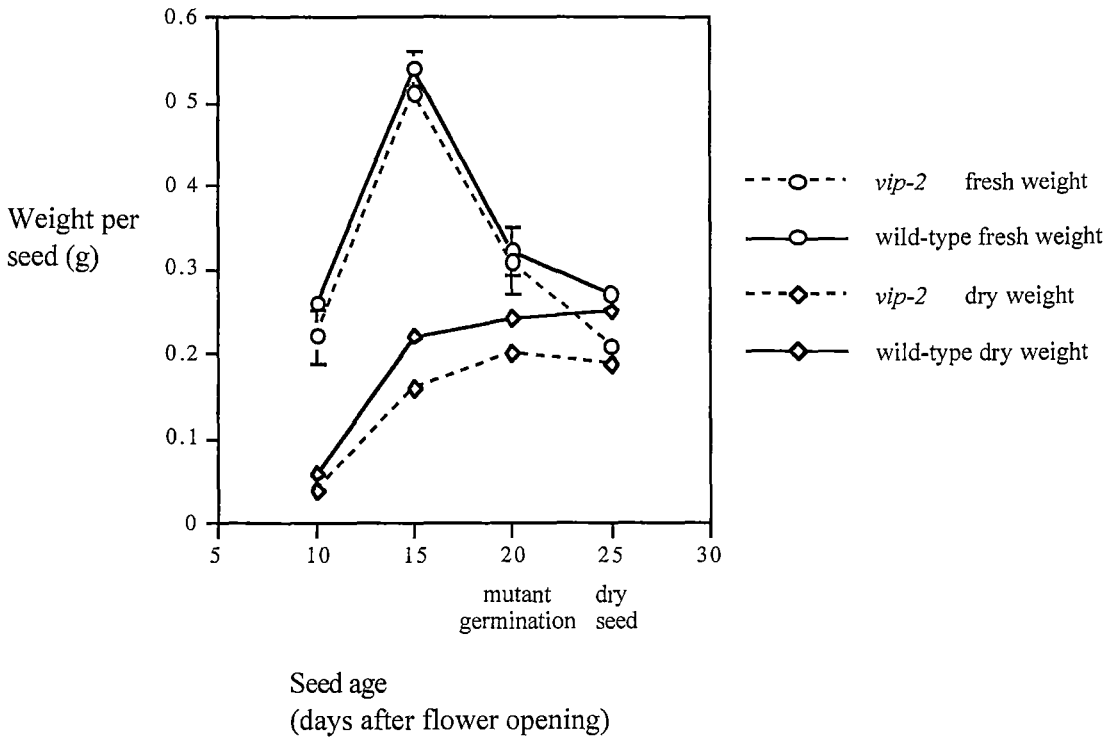


Figure 5.11 Mutant *vip* seeds (two germinating green seeds on right) segregating in an F_1 pod of the cross *vip-2* by *wil*. Thus, the testa and pod are heterozygous for the *vip* gene (*Vip-2 vip-2*). The four non-germinating, drying seeds on the right (three with yellow and one with green cotyledons) are also heterozygous for the *vip* gene (*Vip-2 vip-2).* It is the genotype of the embryo which determines its phenotype in regard to precocious germination caused by the *vip* mutation.

Figure 5.12 Position of *vip* in the lower end of the fifth linkage group of pea in relationship to four other morphological markers (distance represents RCV).

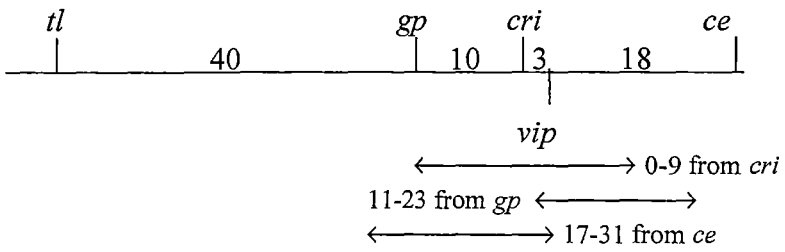


Figure 5.13 ABA levels in developing winter-grown seed of *vip-1* and *vip-2* mutants and their wild-type progenitor cv. Torsdag, and of the ABA-deficient mutant *wil*. (Each point measurement is from an aliquot of bulked seeds from several pods).

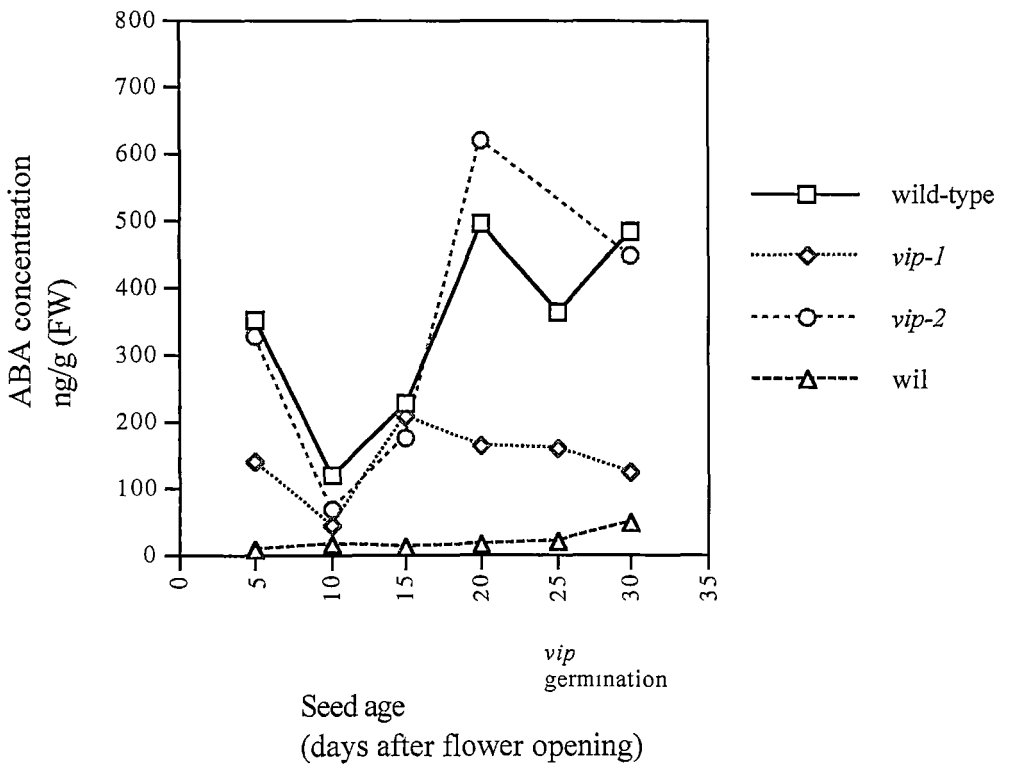


Figure 5.14 ABA levels in developing summer-grown seed of *vip-1* and *vip-2* mutants and their wild-type progenitor cv. Torsdag, and of the ABA-deficient mutant *wil*. (Each measurement is made from an aliquot of bulked seeds from several pods, standard errors are given for two or three bulked samples for seeds over and including 15 days old).

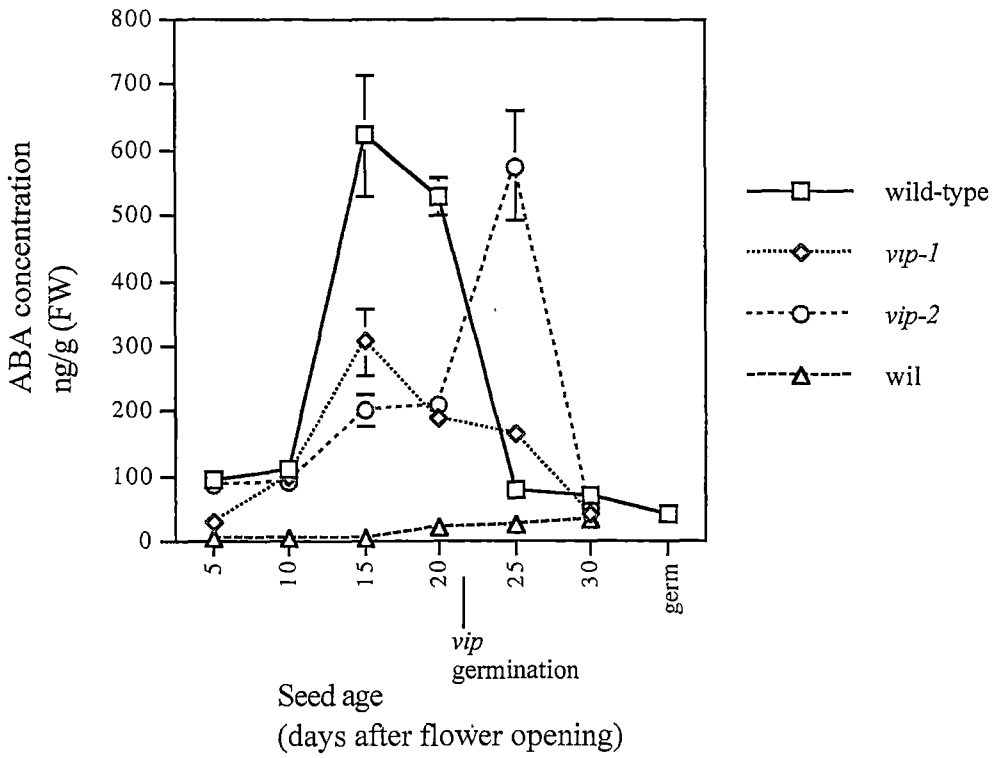


Figure 5.15 ABA levels in wild-type (HL107) and *vip-1* shoots (n=5) grown under glasshouse conditions (daily water) and after drought stress (5 days withdrawal of water). (Standard error bars for control samples are too small to be visible.)

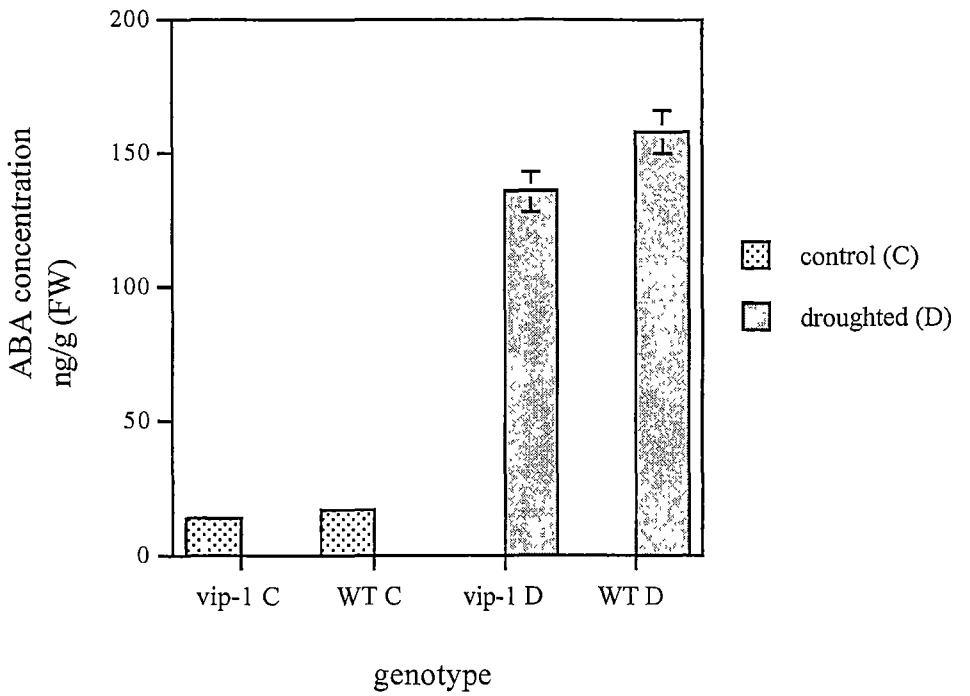


Figure 5.16 Four weeks old seedlings of the *lh-2* mutant (right pot) and the *lh-2 vip-2* double mutant (left pot). Healthy *lh-2 vip-2* double mutants (plant second from left) are approximately 60% shorter than their dwarf parent (*lh-2)* (*vip-2* is tall). Some *lh-2 vip-2* plants are very small (<10 cm) and do not set seed (far left plant).

Figure 5.17 Shoot growth of wild-type seed (cv. Torsdag, HL107) grown on media containing various ABA concentrations (n=5) (compare with Fig. 5.18).

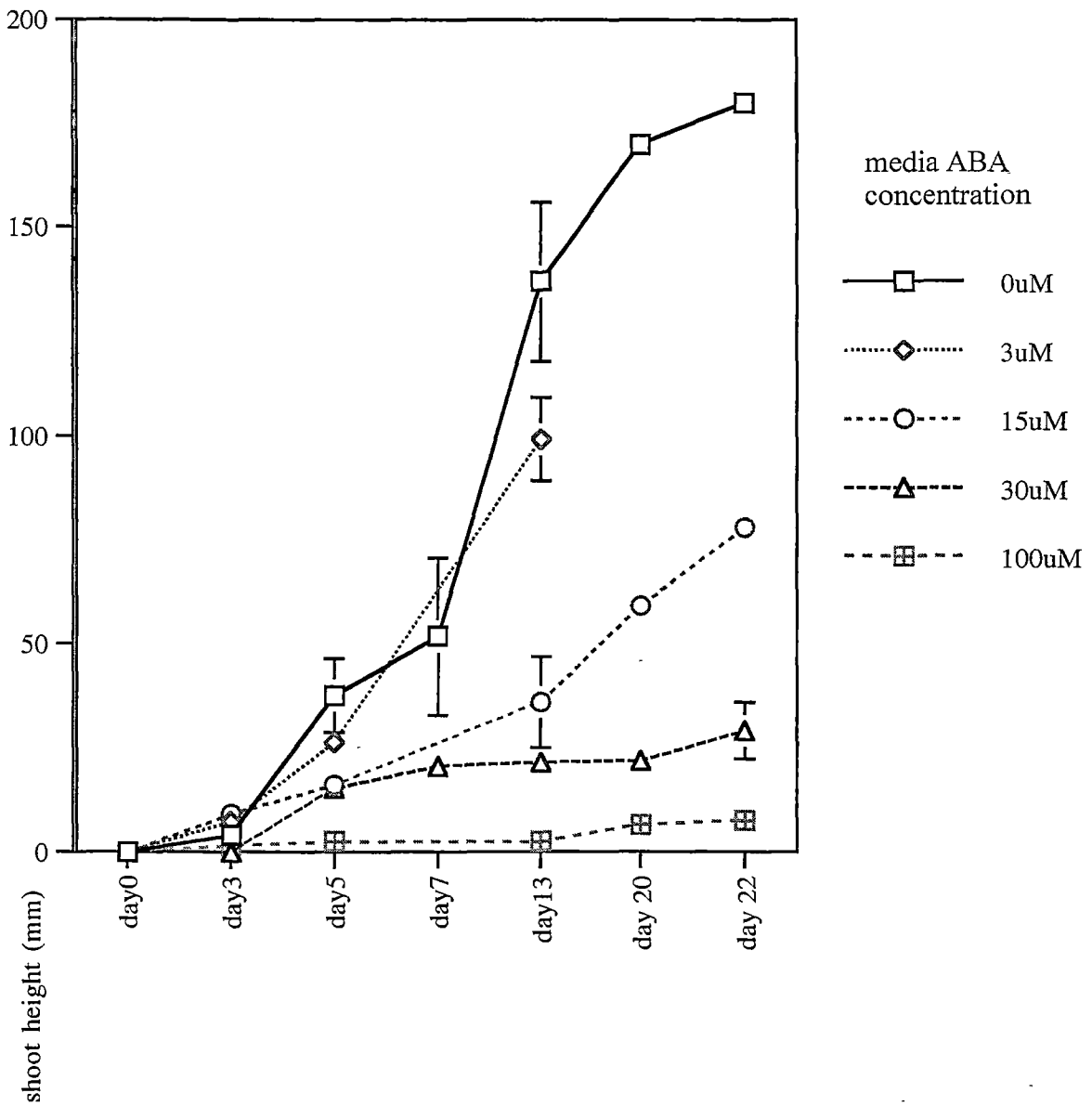


Figure 5.18 Shoot growth of germinating *vip-1* seeds on media containing various ABA concentrations (n=5) (compare with Fig 5.17).

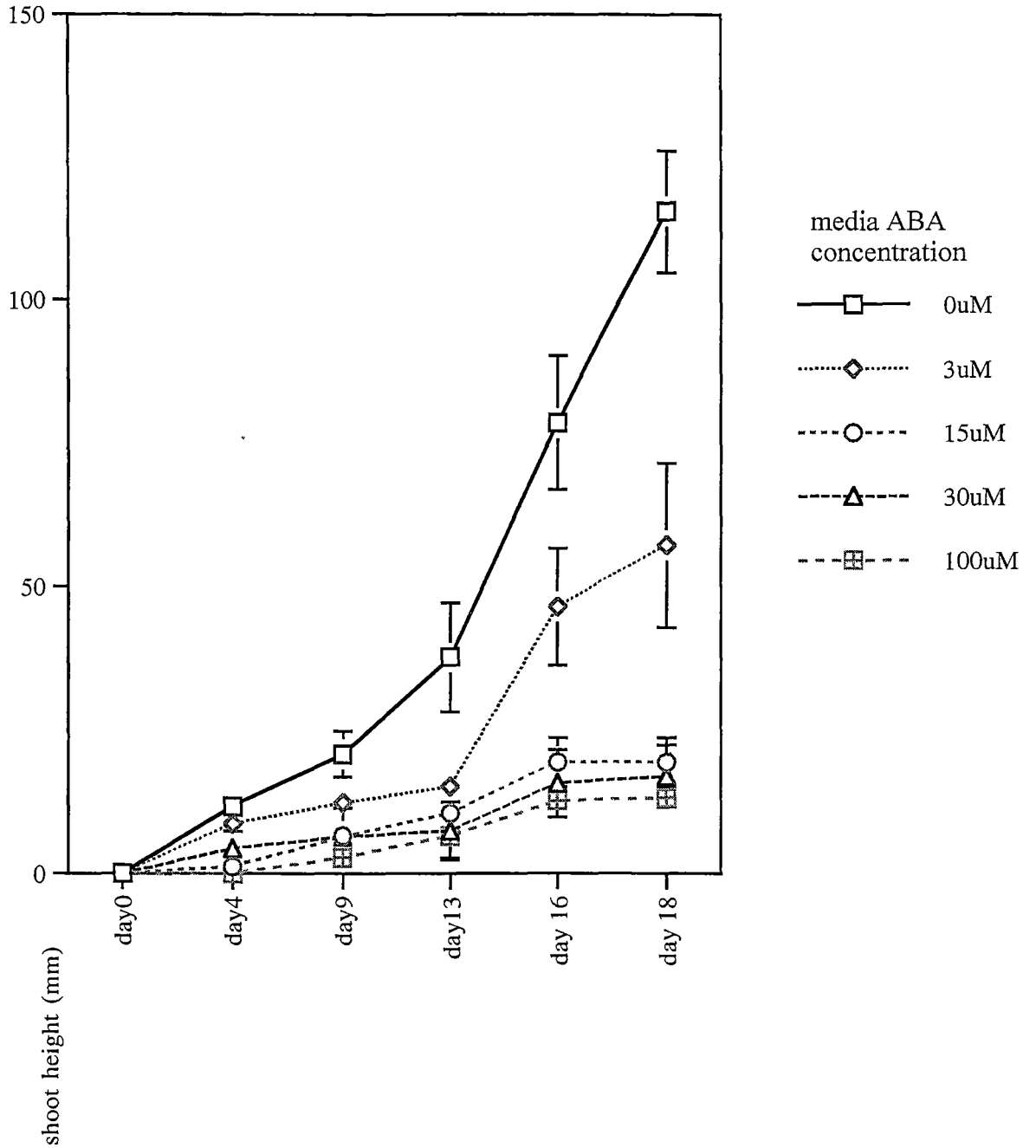


Figure 5.19 Expression of the ABA-inducible pea dehydrin gene in a northern blot of wild-type (HL107) and *vip-1* seedling shoots [S], roots [R] and cotyledons [C], grown on media with various ABA concentrations [0 to 100 µM] (see Figs. 5.17, 5.18).

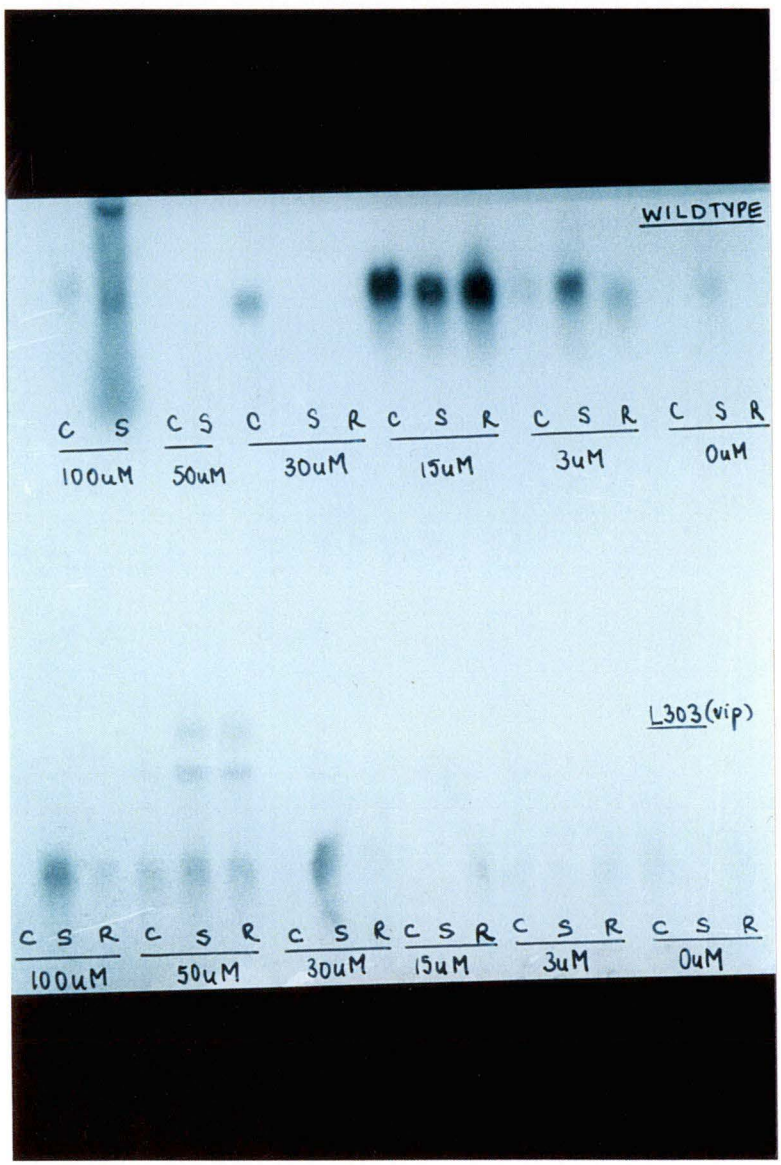
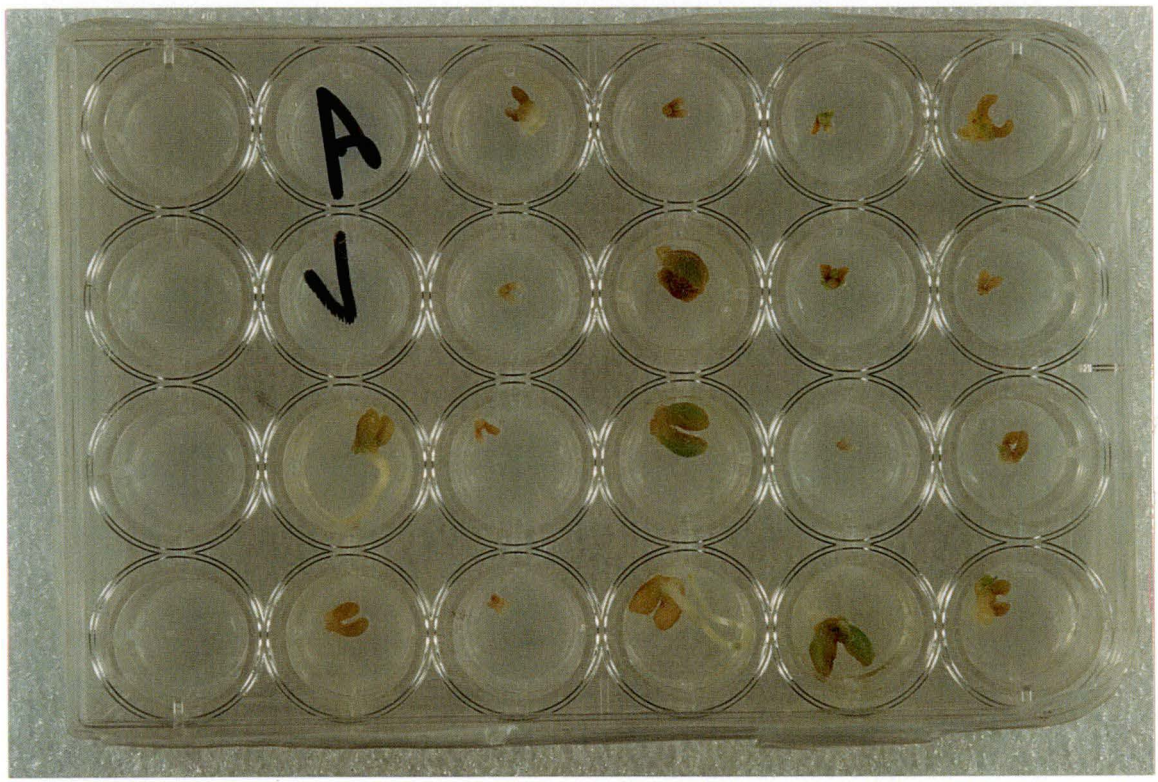
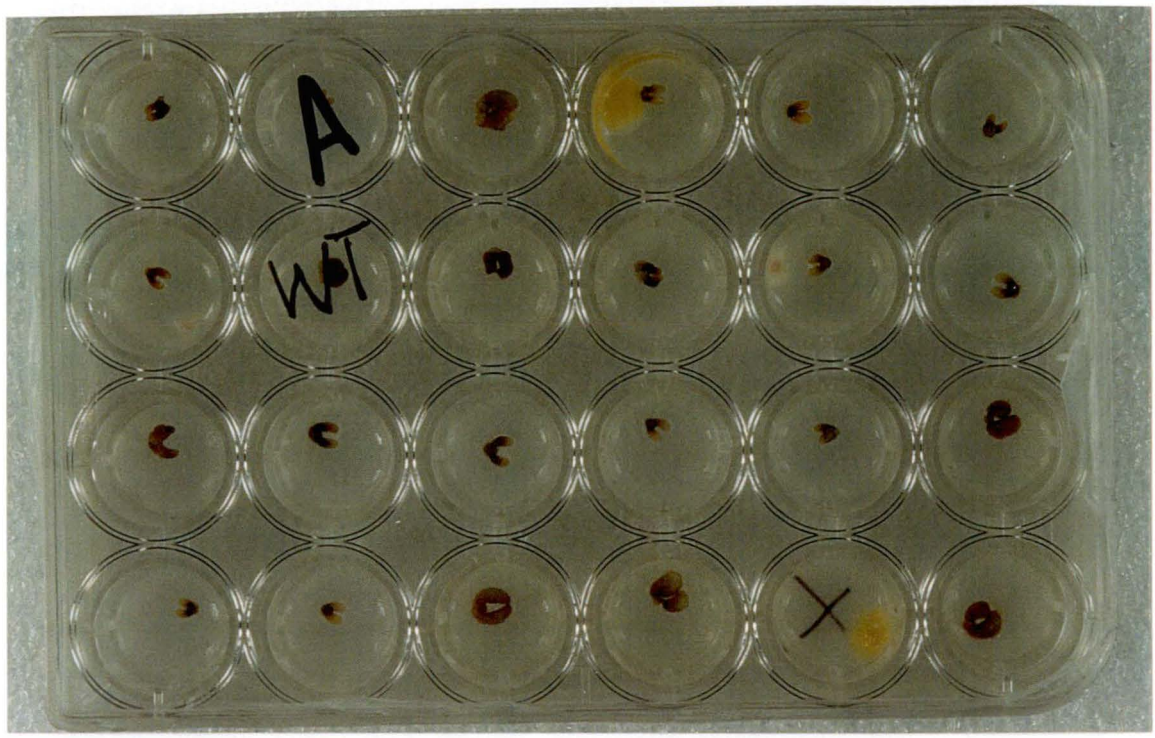


Figure 5.20 (a) Growth of twelve day old embryos of *vip-1* seeds after 10 days on media containing 200µM ABA (compare with Fig 5.21a).



(b) Best growth seen of a twelve day old *vip-1* embryo after 10 days on media containing 200µM ABA (compare with Fig 5.21b).

Figure 5.21 (a) Growth of twelve day old embryos of wild-type seeds (cv. Torsdag, HL107) after 10 days on media containing 200µM ABA (compare with Fig 5.20a).



(b) Best growth seen of a twelve day old wild-type embryo after 10 days on media containing 200µM ABA (compare with Fig 5.20b).

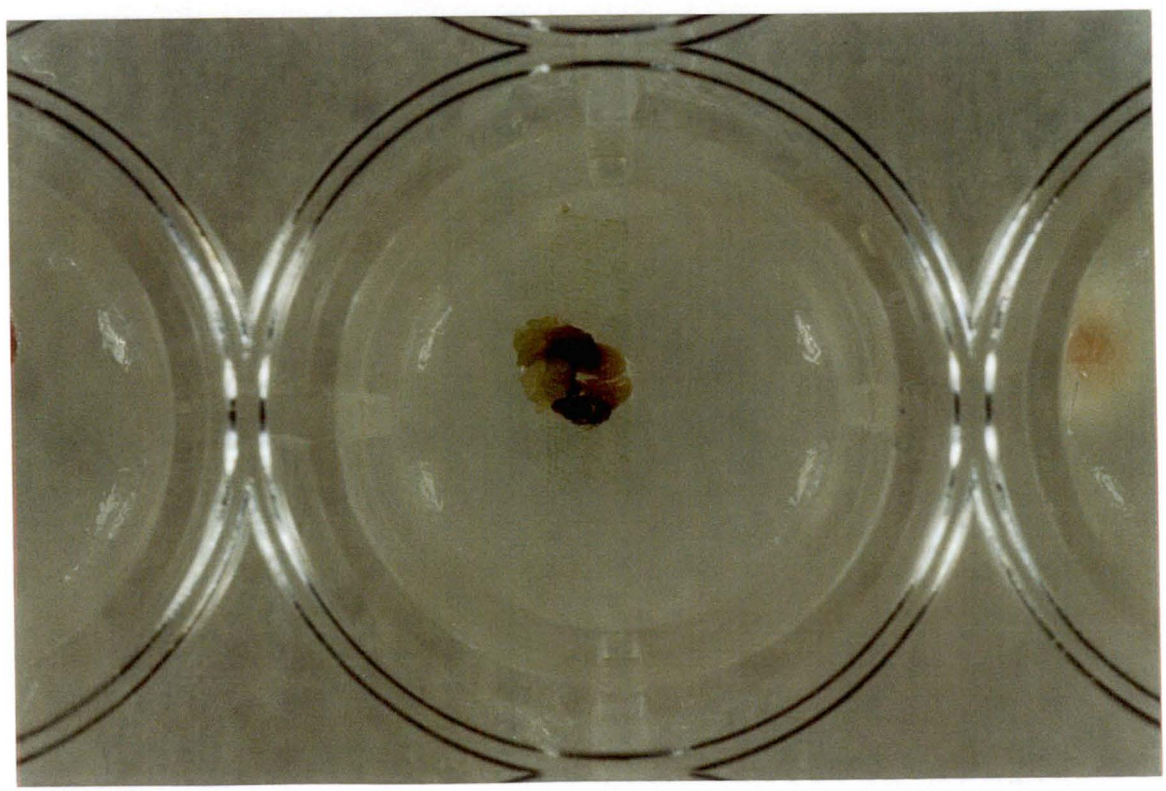
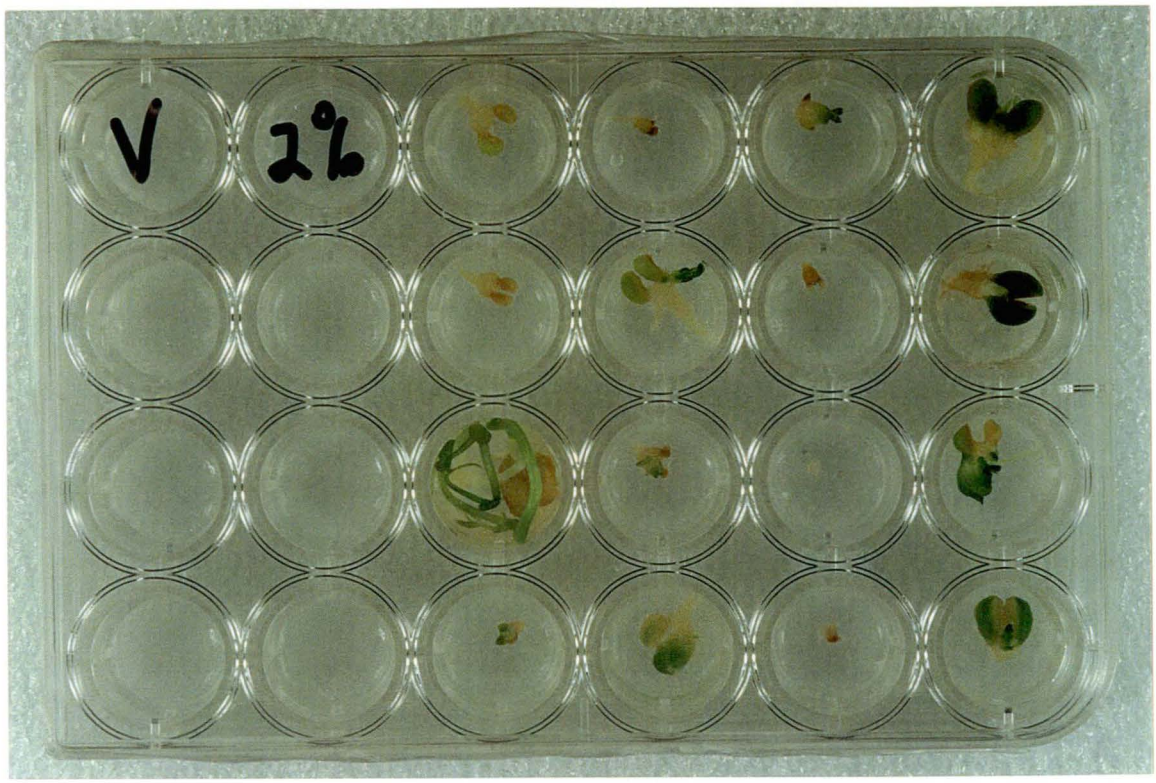
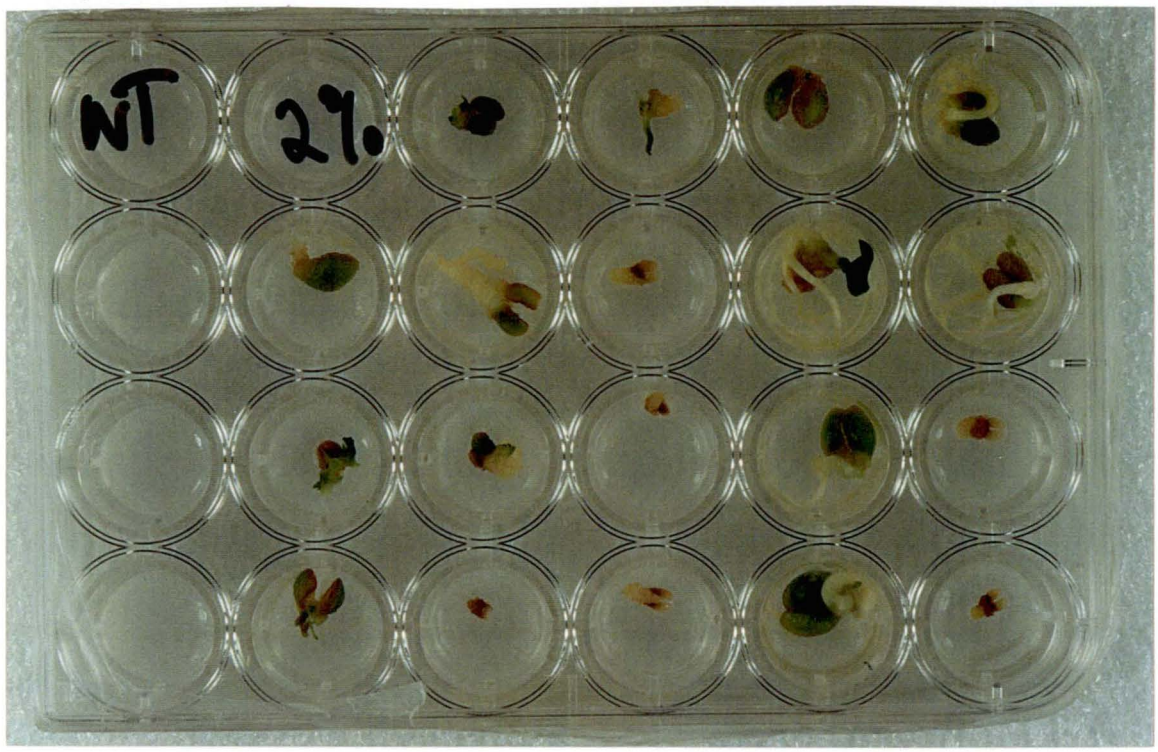


Figure 5.22 (a) Growth of twelve day old embryos of *vip-1* seeds after 10 days on media containing 2% sucrose (compare with Fig 5.23a).



(b) Best growth seen of a twelve day old *vip-1* embryo after 10 days on media containing 2% sucrose (compare with Fig 5.23b).

Figure 5.23 (a) Growth of twelve day old embryos of wild-type (cv. Torsdag, HL107) seeds after 10 days on media containing 2% sucrose (compare with Fig 5.22a).



(b) Best growth seen of a twelve day old wild-type embryo after 10 days on media containing 2% sucrose (compare with Fig 5.22b).

Figure 5.24 (a) Growth of twelve day old embryos of *vip-1* seeds after 10 days on media containing 13% sucrose (compare with Fig 5.25a).

(b) Best growth seen of a twelve day old *vip-1* embryo after 10 days on media containing 13% sucrose (compare with Fig 5.25b).

Figure 5.25 (a) Growth of twelve day old embryos of wild-type (cv. Torsdag, HL107) seeds after 10 days on media containing 13% sucrose (compare with Fig 5.24a).

(b) Best growth seen of a twelve day old *vip-1* embryo after 10 days on media containing 13% sucrose (compare with Fig 5.24b).

Figure 5.26 Northern blot showing similar expression of the pea *ABI3* homologue in wild-type (WT) (HL107) seed 25 days after flower opening (DAO) and germinating *vip-1* seeds 25 DAO. *Arabidopsis* RNA from whole flowering plants is included for comparison.

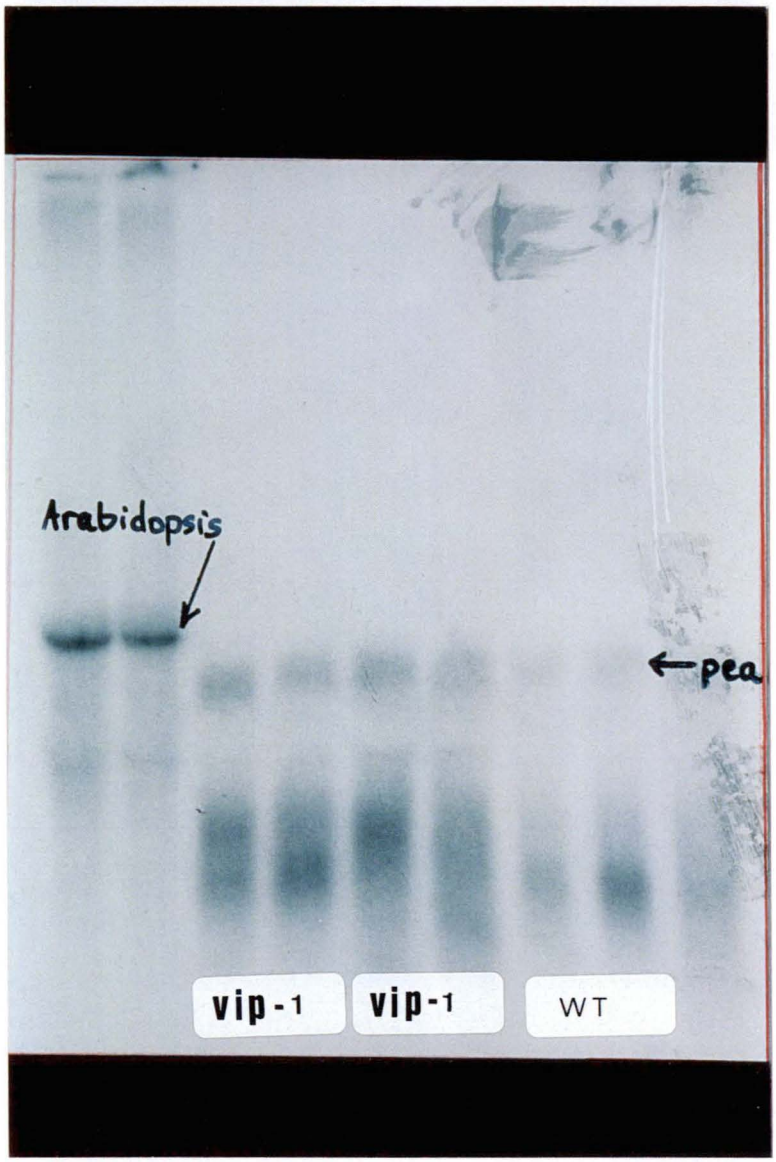


Figure 5.27 Northern blot showing expression of the ABA-responsive *ABR-17* gene, in wild-type, *wil* and *vip* seeds of different ages. (Numbers = age of seeds in days after flower opening, G = germinating, L303 = *vip-1*, L353 = *vip-2*)

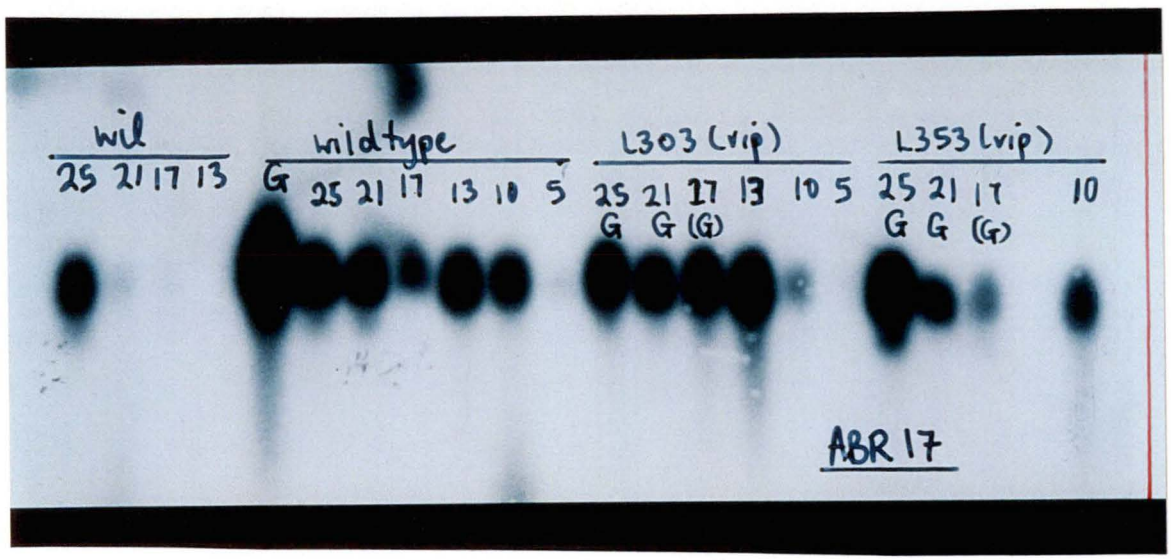


Figure 5.28 Northern blot showing expression of the late abundant albumin (PA2) gene, in wild-type, *wil* and *vip* seeds of different ages. (Numbers = age of seeds in days after flower opening, G = germinating, L303 = *vip-1*, L353 = *vip-2*)

Figure 5.29 Northern blot showing expression of the dehydrin (pSB12) gene, in wild-type, *wil* and *vip* seeds of different ages. (Numbers = age of seeds in days after flower opening, G = germinating, L303 = *vip-1*, L353 = *vip-2*)

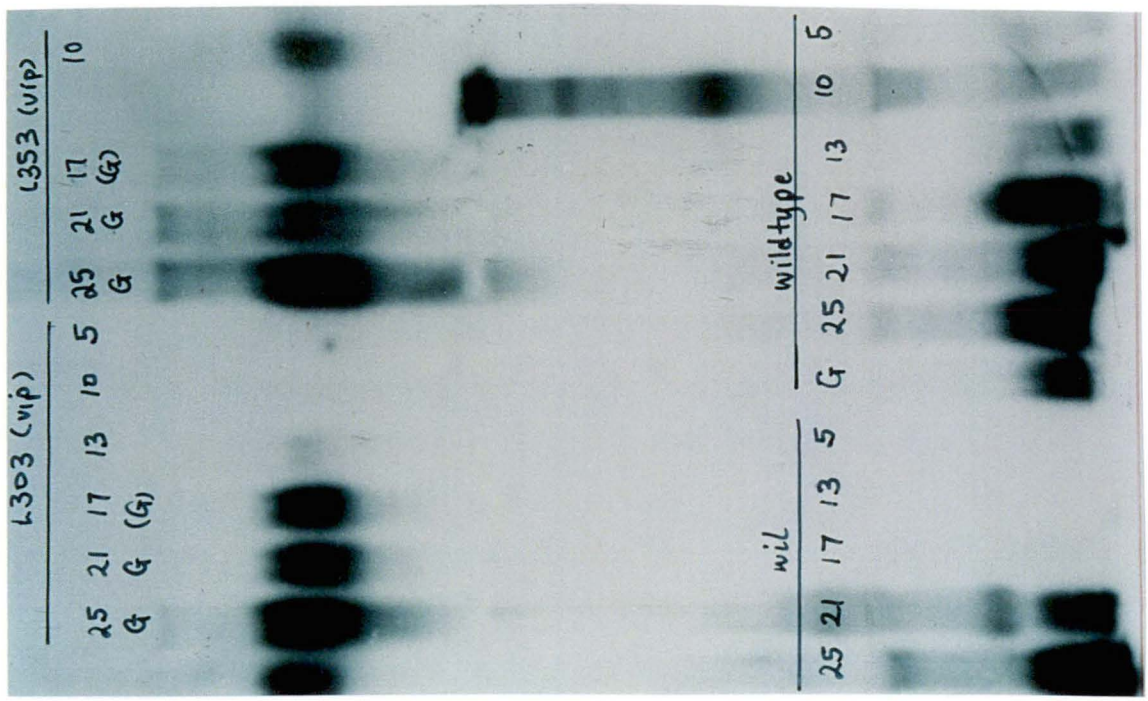


Figure 5.30 Northern blot showing expression of the dehydrin (pSB12) gene, in wild-type (WT) and *vip-2* germinating seedlings of different ages. (Numbers = number of days germinating seeds were grown in moist vermiculite)

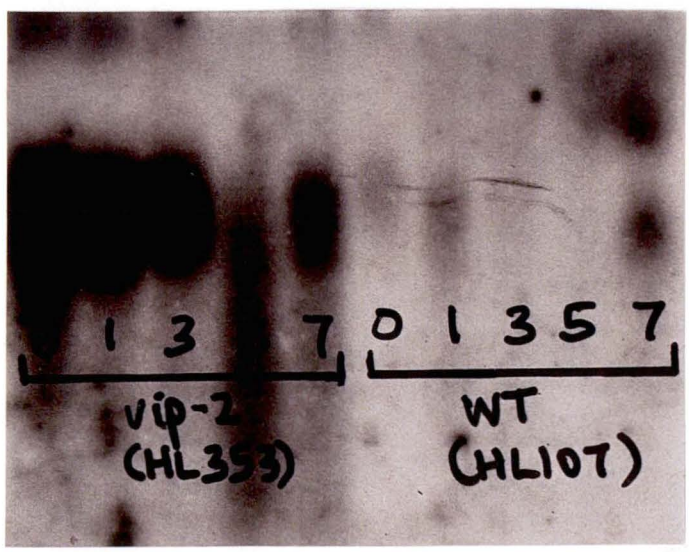


Figure 5.31 Northern blot showing expression of the lectin (pS15-50) gene, in wild-type, *wil* and *vip* seeds of different ages. (Numbers = age of seeds in days after flower opening, G = germinating, L303 = *vip-1*, L353 = *vip-2*)

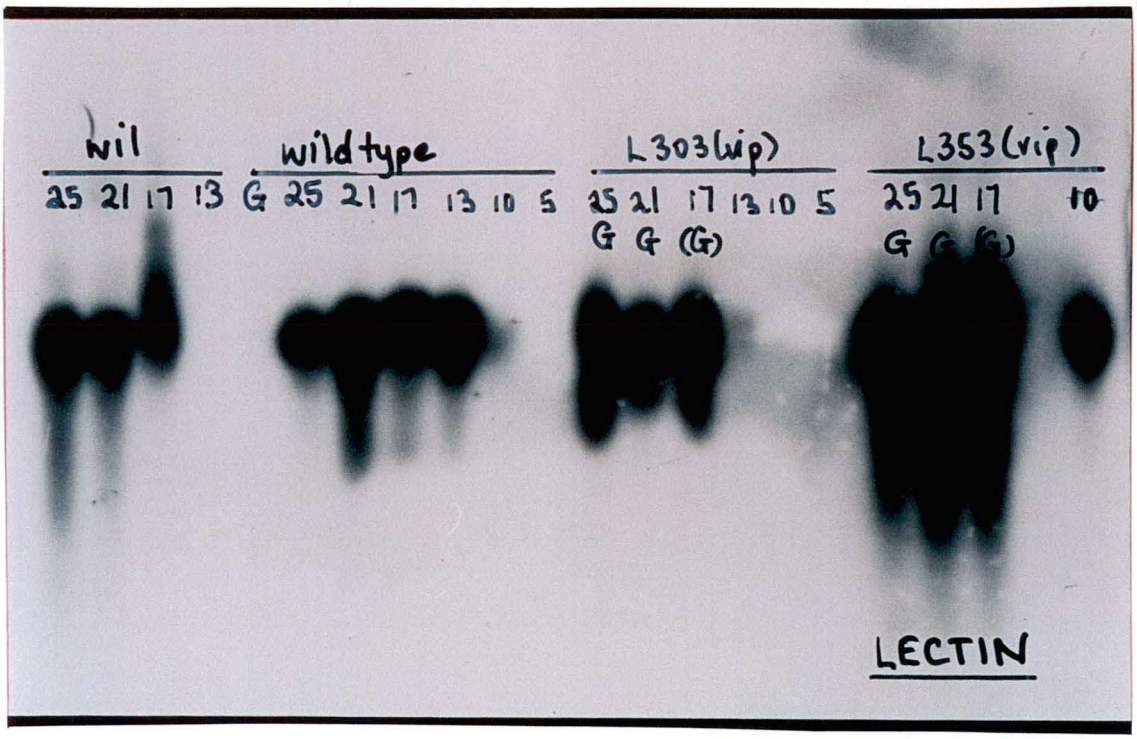


Figure 5.32 Northern blot showing expression of the major pea seed storage gene legumin (pCD43), in wild-type, *wil* and *vip* seeds of different ages. (Numbers = age of seeds in days after flower opening, G = germinating, L303 = *vip-1*, L353 = *vip-2*)

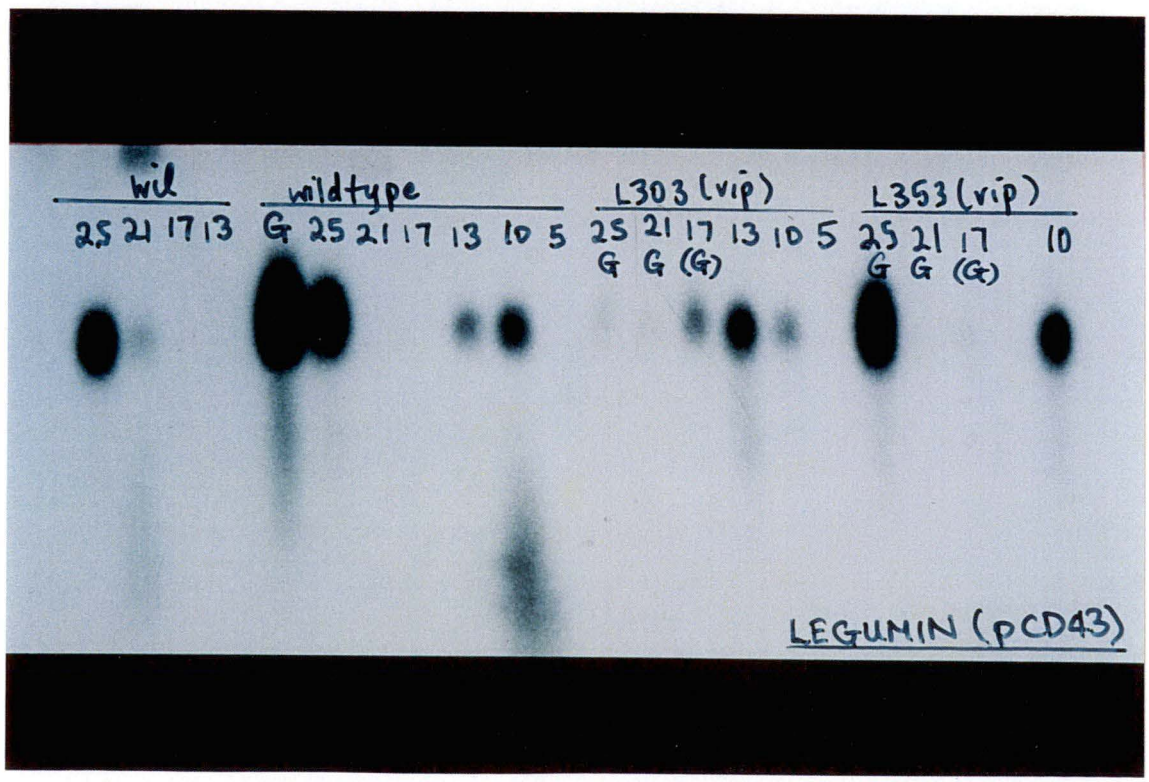


Table 5.1 Growth habits of *vip* and wild-type plants; there is no significant difference between the genotypes for the first node with greater than two leaflets, node of first flower initiation and total nodes.

	first node with > 2 leaflets	node of first flower initiation	total nodes
vip-1 (n=9)	10.75 ± 0.4	16.6 ± 0.3	19.0 ± 0.3
wild-type (n=9) (for *vip-1*)	11.0 ± 0.2	16.0 ± 0.2	19.3 ± 0.1
vip-2 (n=10)	11.3 ± 0.2	17.8 ± 0.2	19.8 ± 0.2
wild-type (n=10) (for *vip-2*)	11.6 ± 0.1	17.6 ± 0.2	20.4 ± 0.2

Table 5.2 Combined data from the F_2 of several crosses for segregation of wild-type and *vip* seeds.

Segre-gating alleles	**Total wild-type seeds**	**Total mutant seeds**	**Total seeds**	**% of mutant seeds**	**Segre-gation χ^2 (3:1)**	**Prob.**	**Hetero-geneity χ^2 (df=12)**	**Prob.**
Vip/vip-1	253	46	299	15.4	14.74	<0.001	2.82	>0.9
Vip/vip-2	231	43	274	15.7	12.66	<0.001	4.88	>0.9

Table 5.3 A comparison of the number of pods per plant, and the number of seeds per pod, between *vip-1* and *vip-2* mutants and their wild-type progenitor (HL107) cv. Torsdag

Genotype	**Seeds per pod**				**Pods per plant**			
	n	**Average**	**t (cf. WT)**	**Prob.**	**n**	**Average**	**t (cf. WT)**	**Prob.**
vip-1	10	3.5	1.55	0.2<p<0.1	10	3.1	4.78	<0.001
wild-type (HL107)	9	3.0	—	—	9	5.0	—	—
vip-2	8	2.9	1.00	0.3<p<0.2	8	3.3	7.71	<0.001
wild-type (HL107)	10	3.3	—	—	10	6.1	—	—

Table 5.4 Joint F_2 segregation data for *vip* and various morphological loci from the fifth linkage group of pea. All crosses are in repulsion.
(A/a first gene, B/b second gene)

Gene Pair	Number of Progeny				Total Plants	Joint $\chi 2$	Prob.	RCV	SE
	AB	Ab	aB	ab					
Vip-2—Tl	120	39	26	9	194	0.02	0.9>p>0.8	—	—
Vip-2—Gp	129	68	29	1	227	11.92	<0.001	17	6
Vip-2—Cri	97	80	68	0	245	45.61	<0.0001	3	6
Vip-1—Ce	69	67	38	5	179	19.25	<0.0001	24	7

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