The Physiological and Genetic Control of Vegetative Phase Change in *Pisum sativum* L.

by

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Declaration

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Abstract

Plants develop through at least three distinct phases after germination: a juvenile vegetative phase; an adult vegetative phase; and a reproductive phase. Therefore, there exist two main ontogenetic transitions, the juvenile-adult vegetative transition or vegetative phase change, and the adult vegetative-reproductive phase transition or reproductive phase change. The genetic and physiological control of the transition to flowering has been studied intensively over the past four decades but the other major transition has received much less attention. This study investigates the genetic and physiological regulation of vegetative phase change in the garden pea (*Pisum sativum* L) using a novel heterochronic mutant, accelerated phase change (*apc*), which expresses a consistent difference in the rate of vegetative phase change when compared to the wild type plants. Genetic analyses, using morphological and enzyme markers, revealed that the *apc* locus is on linkage group II (between *a* and *aat-p* genes) of the garden pea.

When the *apc* and the *WT* plants were grown under 8 h and 18 h photoperiods to determine the effect of environmental cues on vegetative phase change, the results demonstrated that not only is the timing of vegetative phase change affected by environment but the vegetative and reproductive phase changes are regulated independently.

Using isogenic lines differing only in the *apc* mutation, the nature of changes in the morphology of the shoot apical meristem (SAM) associated with the vegetative phase change was examined. The dimensions of the SAM (width, height and volume) were measured without fixation using an environmental scanning electron microscope. The materials were harvested at a critical time (11 days of age) when the vegetative phase change was being initiated in the SAM of the *apc* mutant but not the *WT*. The study found that the dimensions of SAM are larger in the *apc* mutant than in the *WT* plant suggesting

that the vegetative phase change to the more complex leaf type is controlled genetically via changes in SAM morphology.

Reciprocal grafting techniques showed that vegetative phase change was not significantly altered in the *apc* scion by the *WT* stocks and *vice versa*. This indicated that the site of action of the *apc* mutation is in the shoot system.

To determine whether vegetative phase change involved changes in endogenous hormones in the shoot apex, the levels of GA_1, GA_{20}, IAA and ABA were quantified using GC-MS-SIM. The plant materials were harvested at 11 days after sowing, at the critical time that vegetative phase change was being initiated in the *apc* SAM, but not in the *WT* plants. Differential changes in the hormone levels between the *apc* and *WT* SAM were sampled by excising only the 2mm long shoot apices. The results showed no significant difference in GA_1, GA_{20} and IAA levels between the *apc* mutant and the *WT*, which indicates that these hormones are not involved in the vegetative phase change in the garden pea.

Although, a significant difference in ABA levels was detected in the SAM of the *WT* and the *apc* plants, ABA does not appear to be a crucial hormone in vegetative phase change. Further work showed no significant differences in the rate of vegetative phase change between a prominent ABA-deficient mutant (*wil*) and the *WT* (*Wil*). The ABA-deficiency of the *apc* mutant does, however, appear to have a small effect on drought response, with the *apc* and the *WT* plants showing differences in transpiration rate and stomatal conductance.

Abbreviations

ABA	abscisic acid
BHT	botylated hydrorytoulene
CEPA	corrected endogenous peak area
CISPA	corrected internal standard peak area
cv	cultivar
DAS	day after sowing
EMS	ethyl methyl sulfonate
$F_{1,2}$	the 1^{St} and 2^{nd} generation from the crossing
FI	node of first flower initiation
FT	flowering time (days to first flowering)
FW	fresh weight
g	gram
GA_1	gibberellin 1
GA_{20}	gibbberelin 20
GC-MS-SIM	gas chromatograph-mass spectrometry-stimulation ion monitoring
^dH_2O	distilled water
HPLC	high performance liquid chromatograph
hr	hours
HR-SIM	high resolution- stimulation ion monitoring
H, W, V	height, width, volume (morphological parameters in the measurement of SAM dimension)
IAA	indole acetic acid
IS	internal standard
LD	long day
L107	wild type *cv* Torsdag
MeOH	methanol
mg	milligram
N	population number
ng	nanogram

N:P:K	nitrogen: phosphorus: potassium
P	probability
PI	plastochron index
pp	pages
RF	recombinant value
r^2	coefficient of determination
SAM	shoot apical meristem
SD	short day
SE	standard error
W	watt
WT	wild type
Wil and *wil*	the wild type and the mutant of *wilty* plants
YPO	youngest primordia order

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CHAPTER 1

General introduction

1.1 Introduction

The plant shoot passes through a series of different developmental phases during post-embryonic growth (Poethig, 1990; Bassiri *et al.,* 1992). This development can be broadly divided into: a juvenile vegetative; an adult vegetative phase; and a reproductive phase (Evans and Poethig, 1995; 1997; Lawson and Poethig, 1995). With the onset of the juvenile phase, the shoot apical meristem starts to produce a stem, true leaves and axillary buds (Poethig, 1990). The duration of this phase varies between species (Besford *et al.,* 1996). In some species, such as *Arabidopsis*, the juvenile phase lasts for a few days (Martinez-Zapater *et al.*, 1995; Medford, 1992) whereas in *Hedera* it persists for many years (Hackett, 1980). The adult vegetative phase is often characterized by different vegetative traits e.g. leaf shape, leaf trichome density, and phyllotaxy (Hackett, 1985; Martinez-Zapater *et al.,* 1994); it may also be defined by the ability of the shoot to undergo sexual reproduction (Hackett, 1985).

The transition to reproductive phase is indicated by the transformation of the shoot apex from vegetative into reproductive forms such as an inflorescence, flower, or cone. This phase is the last phase in the life of the shoot system for some plants (i.e. annual plants), but not for others (i.e. perennial plants). In annual plants, this is the final transition before senescence, whereas in perennial plants the shoot system may be perpetuated by a lateral vegetative meristem after the terminal meristem becomes reproductive or the primary meristem remains permanently vegetative and lateral shoots produce the reproductive structures (Poethig, 1990).

Thus, during post-embryonic growth the plant shoot undergoes two main transitions, namely, the transition from juvenile to adult vegetative (or vegetative phase change) phase and transition from adult vegetative to reproductive phase (reproductive phase

change). Whether the vegetative phase change is linked to reproductive phase change is still unclear (Evans and Poethig, 1995).

1.2 Vegetative phase change

Although vegetative phase change has been studied for many years, the regulation of this process remains unsolved (Poethig, 1990; Bongard-Pierce *et al.,* 1996). This may be partly because many aspects of phase change studied in woody plants differ from that in herbaceous plants (Poethig, 1990; Wiltshire *et al.*, 1994). Also vegetative phase change (i.e. leaf heteroblastic development) is affected not only by the developmental program of phase change in the shoot (Brink, 1962), but also by the factors of physiological aging (i.e. size and vigour) (Allsopp, 1967; Wareing and Frydman, 1976). However, in woody plants the features resulting from the physiological aging are distinct from those resulting from the phase change because they can be reversed by the manipulation of the growth conditions of the shoot (Wareing and Frydman, 1976).

By contrast, developmentally regulated changes resulting in leaf heteroblasty in herbaceous plants are more difficult to distinguish from changes resulting from physiological aging because leaf form tends to be more plastic in herbaceous plants (Bongard-Pierce *et al.,* 1996). The production of juvenile or adult leaf forms can be influenced by varying the level of exogenously supplied carbohydrate or minerals in such plants (Njoku, 1957; Feldman and Cutter, 1970 a, b; Steeves and Sussex, 1989). Thus it has been suggested that vegetative phase change or leaf heteroblastic development in herbaceous plants may be regulated primarily by quantitative changes in the physiology of the shoot rather than by a programmed switch in gene expression (Allsopp, 1967).

Studies have suggested that leaf heteroblastic development is controlled by morphological and physiological factors that arise both within the shoot apical meristem (SAM) (e.g. Robbins and Hervey 1970) and from outside of the shoot apical meristem (e.g. Irish and Jegla 1997). Changes in SAM size following change in the leaf heteroblastic development along the shoot has been shown in some species such

as *Lycopersicum* sp. (Whaley, 1939); *Darlingtonia* (Frank, 1976) and *Arabidopsis* (Robbelen, 1957; Medford *et al.*, 1992). In *Arabidopsis*, the SAM enlarges in size during ontogeny as leaves increase in complexity (Medford *et al.*, 1992). In many of these studies, although the results indicate that changes in the morphology of the SAM does occur, it is unclear whether this was coincidental or a causal relationship (Telfer and Poethig, 1994; Van Lijsebettens and Clarke, 1998).

If SAM size is the principal factor in leaf heteroblastic development, the question arises whether the changes to the SAM are internally directed or is there a 'signal' produced outside of the SAM that induces the change in SAM size, which, in turn affects the leaf heteroblastic development along the shoot.

It has been proposed that leaf heteroblastic development is controlled by a change in a signal produced from outside the shoot apical meristem (e.g. Irish and Jegla 1997). Irish and Nelson (1988) observed that the vegetative phase change can be delayed by removing the leaves. This indicates that the leaves are a source of the signal. Grafting studies on *Pinus radiata*, *Pinus taeda*, *Larix laricina*, and *Pseudotsuga laricine* demonstrated that phase change results in changes in the growth habit of the apical meristem that persists even when the mature meristem is re-exposed to physiological conditions associated with a young plant, including input from a juvenile rootstock (Sweet, 1973; Greenwood, 1984; Greenwood *et al.*, 1989; Ritchie and Keeley, 1994). This suggests that there is a chemical signal(s) transferred from root to the shoot that maintains juvenility. This is supported by other studies in *Hedera helix* and *Ribes nigrum* where it is suggested that the decay or decline of the signal produced and transferred from root to the shoot induces phase change, based on changes in the leaf shape (Frydman and Wareing, 1973 a, b; 1974; and Schwabe and Al-Doori, 1973).

In contrast, Moose and Sisco's (1994) study using the *gl15* mutant in *Zea mays* indicated that vegetative phase change is controlled in a cell-autonomous manner because this mutant acted directly in the epidermal juvenile-to adult phase transition but did not affect other factors involved in the process of phase change.

Regardless of where the regulator of the vegetative phase change is produced, the question remains as to the kind of signal effecting the process. Some studies in both woody and herbaceous species have suggested that the signal involves endogenous hormone(s). In *Hedera*, gibberellic and abscisic acid have been suggested as possible regulators of vegetative phase change (Frydman and Wareing 1973 a, b; 1974). The ratio of both hormones seems to affect the rate of the vegetative phase change. If the GA level in the plant was decreased but ABA increased, vegetative phase change was achieved. Evans and Poethig (1995) suggested that certain gibberellins (i.e. GA_1, GA_5, GA_3 or other GAs) are required for vegetative phase change, because the application of those hormones can accelerate the vegetative phase change in the dwarf mutant in which the process was delayed. A study on the *vp8* mutant of *Zea* indicates that a reduction in ABA levels cannot be related to a delayed vegetative phase change in that mutation.

The involvement of environmental cues such as photoperiod on the vegetative phase change is inconclusive because some studies indicated that the vegetative phase change is an independent process from environmental factors (e.g. Wiltshire *et al.*, 1994), however, some other studies have demonstrated that photoperiod seems to be a factor in vegetative phase change (Martinez-Zepater *et al.*, 1995; Telfer *et al.*, 1997). Martinez-Zepater *et al.* (1995) have shown that short days (SD) delay the vegetative phase change, using trichomes as the indicator of vegetative phase change.

Many complex morphological differences between species can be attributed to positive or negative changes in temporal parameters: time at which a process begins, the time at which it ends, and the rate at which it occurs (Alberch *et al.*, 1979; Gould, 1982). In plants, such changes can also cause morphological variation within an organism because of the prolonged, polar nature of shoot growth. All plants undergo significant changes in morphology during the shoot growth. Leaf shape is one of the most conspicuous features of the heteroblastic development in both woody and herbaceous plants (Poethig, 1990; Hall and Langdale, 1996). Leaves produced on the juvenile shoot are usually smaller and simpler than those of the adult shoot and may differ in many other respects (Poethig, 1990, 1997; Smith and Hake, 1992). For instance, in maize, leaves borne on the juvenile shoot are not only shorter and narrower than those

on the adult shoot, but their epidermal cells are of different shapes than those of adult leaves, and they lack the hairs present on the adult leaves (Poethig, 1990).

1.3 Heterochrony

Heterochrony is defined as a variation in timing of a developmental event in plants (Lord and Hill 1987). A major advance in the study of heterochrony was the formalization of the terminology by Alberch *et al.* (1979). The application of the terminology to plants has been illustrated succinctly by Wiltshire *et al.* (1994), who suggest that heterochronic variation can be categorized into *paedomorphic* and *peramorphic forms.* The first form includes: *progenesis, neoteny* and *post-displacement. Progenesis* results when the descendent reproduces precociously and, if senescence follows, the descendent can complete its life cycle while still in juvenile vegetative morphology. *Neoteny* results if the time to reproduction remains unaltered, but the rate of vegetative morphological change is slower. *Post-displacement* occurs if the onset of a particular phase of morphological development is displayed. *Peramorphy* is a form in which the descendent is vegetatively more adult than the ancestral adult, and this form can be attained by altering the same three variables in the opposite manner. If the time of reproduction is delayed but the rate of change in vegetative morphology continues, such that the final phase of the descendent is more complex than that in the ancestral adult, the resultant change is referred to as *hypermorphosis.* A similar result can be attained by a faster rate of morphological change (*acceleration*) or by the early onset of a vegetative developmental phase (*pre-displacement*). Takhtajan (1972) proposed different words such as prolongation, abbreviation and deviation to point out heterochrony in plants, but these do not appear to have gained the same widespread use.

Heterochrony appears to be an important mode of plant evolution (Poethig, 1990; Freeling *et al.*, 1992) because small changes in regulatory genes, such as those controlling the expression of phase change or temporal pattern of organ development, result in obvious changes (Itoh *et al.*, 1998) or markedly different morphological features in the descendent species (Goldschmidt 1940).

Identification of heterochronic mutants has provided important clues in the investigation of vegetative phase change. Such mutants display a prolonged juvenile phase or accelerate the expression of the adult phase (Lawson and Poethig, 1995). In maize, for instance, *Tp1*, *Tp2*, *Tp3,Cg*, *d* and *Hsf1-O* mutants delay vegetative phase change because all show a prolonged expression of the juvenile vegetative phase (Poethig 1988a,b; Betrand-Garcia and Freeling, 1991; Bassiri *et al.*, 1992). In contrast, *gl15* mutant causes a precocious expression of the adult vegetative phase (Evans *et al.*, 1994; Moose and Sisco, 1994). In both kinds of mutants, vegetative phase change seems to be affected by a change in the time and the position of the end of the juvenile vegetative traits and the onset of the expression of adult vegetative traits (Bassiri *et al.*, 1992; Evans *et al.*, 1994).

Although heterochronic mutants have been identified in species such as in *Zea* and *Arabidopsis*, these mutants do not provide an ideal model for the study of heterochrony often showing gross and dysfunctional abnormalities rather than regulating development in a manner that could conceivably be favoured under some selective regime (Wiltshire *et al.*, 1994). Furthermore, the ontogenetic changes in morphological characters such as leaf shape are not always clear and this leads an inability to further characterize the cause of heterochronic changes (Wiltshire *et al.*, 1994; Ray *et al.*, 1996). By contrast, the garden pea (*Pisum sativum*) shows obvious and discrete markers at different phases of ontogeny that lends itself to heterochronic studies (Wiltshire *et al.* 1994; Hall and Langdale, 1996).

1.4 Pea as a model of phase change

The garden pea's utility as a model system derives from the fact that the difference between juvenile and adult vegetative phase is clearly displayed in the leaf morphology (Smith and Hake, 1992, Wiltshire *et al.*, 1994; Van Lijsbettens and Clarke, 1998). In the normal development of the garden pea, the leaves at the first two nodes above the cotyledons are reduced to cataphylls but at the 3rd node the first true leaf (2-C) bears a pair of leaf-like stipules at the base of the leaf, and is a compound leaf with two leaflets and tendrils. At higher nodes, the leaf develops additional pairs of leaflets (3-

C, 4-C, 5-C, 6-C, and occasionally 7-C and 8-C) and tendrils and so that the complexity of the organ increases during heteroblastic development (Marx, 1987; Wiltshire *et al.*, 1994).

In addition, the mutagenic program conducted recently in the pea has successfully isolated a mutant showing an acceleration in the initiation of the vegetative phase change as compared with those in the wild type plants (or peramorphy). It is named *accelerated phase change* (*apc*) mutant. In this study, this mutant was used as a tool to investigate the mechanism of vegetative phase change in the pea.

1.5 The aims of the study

The general aim of this study was to investigate the genetic and physiological control of vegetative phase change in the garden pea (*Pisum sativum*). This was addressed using several approaches. The first was to determine the position of the *apc* gene in linkage groups of the pea using morphological and molecular markers (**Chapter 3**). The second was to study whether the vegetative phase change is effected by morphological changes in the shoot apical meristem (SAM) (**Chapter 4**). For this, the size of SAM was compared between the mutant *apc* and the *WT* at a critical time of growth. The third aim was to determine whether vegetative phase change also involves changes in endogenous level of hormones: GA_1, GA_{20}, IAA and ABA in the shoot apex (**Chapter 5**) using Gas Chromatograph-Mass Spectrometry-Stimulating Ion Monitoring (GC-MS-SIM). The next aim (an extension of Chapter 5) was to clarify whether the *apc* mutant is a new ABA-deficient mutant, and whether a reduction of ABA level *per se* accelerated the initiation of the vegetative phase change (**Chapter 8**).The fourth was to determine the site of action of the *apc* mutation using grafting experiments (**Chapter 6**). The fifth was to observe whether the vegetative phase change is affected by environmental cues, especially the effect of photoperiod (**Chapter 7**) in order to examine the independence of reproductive and vegetative phase changes.

CHAPTER 2

General materials and methods

2.1 Plant materials

Plant materials used in this study are line L107 (initial line *cv.* Torsdag) designated as the Wild Type (*WT*) and a novel isogenic line, Af3, derived from it in a mutagenic program using ethyl methyl sulfonate (EMS). Both lines are held in the collection of the School of Plant Science at University of Tasmania, Hobart.

Using isogenic lines or monogenic lines is an absolute requirement in the investigation of the physiological process of the plants (Koornnef *et al.*, 1982) because differences in the same genetic background between the mutant and the wild type will give incorrect attribute(s) for the process (Reid 1993). With single gene contrasts it is clear that observed physiological and biochemical differences are causally related (Koornneef *et al.*, 1982). For this reason, the backcrosses and self-fertilization were carried out repeatedly between the mutant Af3 (*apc*) plants and L107 wild type (*Apc*) plants until the isogenic *apc* line was obtained.

The known genotype background of both L107 and Af3 lines is *E, Le, Af, Sn, Ppd, Hr, Fa, Na*) but the lines differ principally in vegetative phase change. One indicator of phase change is a change in the number of leaflets from 4 to 6. This change occurs later in the L107 wild type plants than in the *apc* mutants. In the *WT* plants, the first leaf with 6 leaflets occurs at node ≥ 15, whereas in the mutants it is seen initially at node < 15 (Wiltshire *et al.*, 1994).

2.2 Growing condition

Unless otherwise noted, the *WT* and the *apc* plants were grown in either 14 cm slim line-pots or plastic tote boxes (41 x 32 cm) in a 50:50 (by volume) mixture of vermiculite and 2-3 cm dolerite chips topped with 2 cm layer of sterilized 1:1 mixture of peat moss, coarse river sand, and added macronutrients (Osmocote ® N: P: K

19:2:6 at rate of 1 kg/m^3). The testa of seeds was nicked prior to sowing to facilitate even germination, and seeds were sown at a depth of *ca.* 1.5 cm below the soil surface.

The plants were grown either in controlled environment growth cabinets or in a heated glasshouse, depending on the aim of the project. In the growth cabinet, plants were exposed to an 18 hr photoperiod with a mixed fluorescent (Thorn 40 W white tubes) and incandescent (Mazda 100 W pearl globe) illumination source (25 μmol.m^{-2}.s^{-1} at pot top). The day and night temperature in the cabinet were 20 ^{0} C. In the glasshouse, the plants were grown under long day (LD 18 hr) and short day (SD 10 hr) conditions depending on the requirement of the project. In LD treatments, natural daylight was extended using a mixture of 8 fluorescent tubes (L40W/20S cool white, Osram Germany) and 4 incandescent globes (100 W Pearl, Mazda Australia). The intensity of the supplementary lighting was 25 – 30 μmol.m^{-2}.s^{-1} at pot top. The day and night temperature fluctuation in the glasshouse varied with the season. The means of daily temperature ranged from 13 - 21 ^{0}C in winter and 17 – 27 ^{0}C in summer. In the night compartment, temperature was maintained at 16 ^{0}C throughout the year.

The plants were watered once a day until emergence through the soil surface, and then suspended until the plants had *ca.* 3 leaves expanded. The plants were then watered daily or four times a week depending on plant size and the season. Plants were fertilized weekly with nutrient solution (Aquasol ®, Hortico Australia, N:P:K 23:4:18 at a rate of 1 g/l and iron chelate at a rate of 0.05 g/l. To minimize disease and insect damage, plants were sprayed weekly with fungicide and insecticide.

2.3 Characters recorded

Description of vegetative phase change characters recorded in pea (after Wiltshire *et al.*, 1994) were the node of vegetative transition where the leaf with 3 leaflets (3-C), four leaflets (4-C), five leaflets (5-C), six leaflets (6-C), seven leaflets (7-C) and eight leaflets (8-C) are initially generated, counting from the cotyledons as zero. The node of flower initiation (FI) was number of the first node on the main shoot to bear a flower initial regardless of whether or not the bud actually develops into an open flower (Murfet, 1977).

2.4 Linkage analysis

To determine the position of *apc* in the linkage groups of *Pisum* both morphological and molecular markers were used. Morphological markers were evaluated on the phenotypic characters, for example, the presence of anthocyanin, presence of wax on the abaxial leaf surface, stipule and pod characters (see **Table 3.1** in **Chapter 3**). Molecular markers were evaluated using gel electrophoresis of isoenzymes. The characteristic states were divided into slow (S), intermediate (H) and fast (F) rate. The slow, intermediate and fast rates are identified as homozygous recessive, heterozygote and homozygous dominant (Weeden and Marx, 1984).

In this study, enzyme analysis was performed for *aspartate amino transferase,* one of the primary markers in chromosome group 1 recommended for linkage analysis (Weeden *et al.*, 1994). The protocol of this analysis was based on an outline of enzyme analyses produced by The Molecular Division of the School of Plant Science, University of Tasmania, Hobart. Healthy leaf samples were collected from the F_2 progeny and enzyme samples were extracted using a Tris-HCl buffer (pH 8.4), run on the standard gel system. Following incubation at room temperature in the dark, the gel was assayed using 50 ml of 1 M Tris-HCl buffer (8.0) containing 100 mg L-aspartic acid (Na salt), 50 mg α-ketoglutate, a trace of piridoxal phosphate, and 50 mg fast blue BB. The segregation of this enzyme was considered together with the morphological markers.

2.5 Grafting techniques

In order to examine the site of production of the effects of the *apc* mutation, two kinds of grafting techniques were used in this study. The first technique was similar to that described by Murfet (1971). Grafts were made epicotyl to epicotyl when the shoot had emerged completely from the potting mixture and had a total stem length of about 2 cm (4 - 5 days after sowing). The stem of the scion was cut below the first scale leaf and the stem of the stock was decapitated below the first scale leaf. In the second technique, the scion stem was cut below the second true leaf and the stem of stock was cut below the second true leaf. The plants used for this type of graft were around 8-9 days old after sowing. The cut end of scions was made into a wedge shape and inserted

into a slit made in the stock plants down the center of the epicotyl to approximately 12 mm. A small plastic ring was slipped over the epicotyl of the stock to hold the scion in place.

The grafted seedlings were watered, and humidity was maintained by placing a plastic bag over the seedlings and pot rim, held in place with a large rubber band. The seedlings were initially watered every second day. The bags were turned daily to allow appropriate gas exchange. After about 4 days, when the xylem connection between the scion and the stock appeared to have developed, the intact bags were replaced with bags with cut corners. At the first appearance of new growth in the scions (about one week), the bags were removed completely.

Any lateral shoots from the cotyledonary axil of the stock were removed in order to prevent the shoot competing with the scion for available nutrients.

2.6 Hormone analyses

Analysis of hormones followed the procedure in an outline of hormone analysis developed by The Physiological Hormone Division of the School of Plant Science, University of Tasmania, Hobart. The analysis of hormone levels of gibberellins (GA_1 and GA_{20}), indole acetate acid (IAA), and abscisic acid (ABA) were carried out in number of steps: extraction, addition of internal standard, purification, fractionation, and quantification.

2.6.1 Extraction

Shoot apices were harvested at *ca.* 2 mm in size from the plants (*WT* and *apc*) at 11 days old. These tissues were immediately weighed and transferred to cold (-20 ^{0}C) 100 % MeOH. The MeOH used contains a trace of the antioxidant botylated hydroxytoluene (BHT). Prior to the extraction the tissue was kept at –20 ^{0}C for 24 hours.

To begin extraction, the methanol was diluted to 80 % by adding ^dH_2O. The extracts were homogenized with a rotary cutter, and stored at 4 ^{0}C for 24 hours. Extracts were then filtered through a layer of Whatman No. 1 filter paper.

2.6.2 Addition of internal standards

Internal standards added into the extracts were [17,17-$^2H_2$] GA_1, [17,17-$^2H_2$] GA_{20}, $^2H_3$ ABA, ^5D IAA (see **Table 5.1** for more detail). [$^3H_2$] GA_{20} which was used as tracer in the extract for quantification, with approximately 360000 dpm [$^3H_2$] GA_{20} (1.11.TBq. $mmol^{-1}$). The extracts were kept at – 20 ^0C for 24 hours before the purification was performed.

2.6.3 Purification

After 24 hours, the extracts were dried under vacuum at 30 ^0C until less than 1 ml. A Sep-Pak Plus C18 cartridge (Waters Assoc. Milford, MA, USA) was preconditioned using ca. 10 ml of 100 % MeOH and *ca.* 10 ml of 0.4 % acetic acid. The dried extracts were dissolved in 1 ml of 1% acetic acid, passed through the Sep-Pak at a rate of 5 $mL.min^{-1}$. Another 1 ml and 2 ml of 0.4 % acetic acid were transferred into the Sep-Pak. The extracts were eluted with ca. 10 ml of 70 MeOH in 0.4 % acetic acid, and were dried *in vacuo*.

2.6.4 Fractionation

Fractional processes were performed using C18 High Performance Liquid Chromatography (HPLC, Waters Assoc., Milford, MA, USA) system. This consists of two M-45 Solvent Delivery System, a model 6UK Universal Liquid Chromatograph Injector fitted with a 2 ml sample loading loop, Model 660 Solvent programmer, Z-Module Radial Compression Separation System and a 10 ml Radial-Pak A cartridge C18 column 100 x 8 internal diameter. The solvent was filtered regularly through 0.5 mm type FH (MeOH) and 0.45 mm type OE 67 (^dH_2O) millipore filters.

The sample was dissolved in two successive volumes of 1.0 ml with 20 % MeOH in 0.4 % acetic acid, injected into the HPLC loading loop through a UK6 sample injection unit and 0.45 μm filter (Gelman Science, MI, USA). After allowing 3 minutes for the loaded material to equilibrate in the loading loop, the sample was injected and run on the following program: 20-70% MeOH in 0.4 acetic acid over 25 minutes, gradient curve #6 (linear), flow rate of 2 $ml.min^{-1}$.

2.6.5.Methylation

After the sample fractions were grouped based on the retention time of each hormone, and dried using the concentrator, the samples were methylated by adding 200 µm of 100 % MeOH and 750 µl of diazomethane. They were then dried under nitrogen. The sample was redissolved in 1 ml of $^{d}H_2O$ and 400 µl of ether added. When separation had occurred between the ether and the water, the ether was drawn into a Pasteur pipette, and the sample was put into a GC-MS vial. Two more washes of 400 µl ether were conducted. Any remaining water in the vial was removed by drying under nitrogen or concentrator prior to the quantification.

2.6.6 Quantification

Prior to quantifying, derivatisation was performed for the grouped fractions of GA_1, GA_{20} and IAA by adding 3 µl of pyridine and 10 µl of bis (trimethyl-silyl) trifluoroacetamide (BSTFA). Those fractions were then heated at 80 ^{0}C for 15-20 minutes. Full scan mass spectrometry and high resolution GC-SIM (HR-SIM) were performed using a Hewlett-Packard 5890 Series 11 gas chromatograph linked via direct inlet to a Kratos Concepts ISQ mass spectrometer controlled by a Mach 3 data system. A 1 µl spitless injection was made at 250 ^{0}C onto the same SGE BPI column. The carrier gas was helium with the head pressure programmed to maintain a flow rate of approximately 2 ml.min^{-1}. For the quantification of GA_1 and GA_{20}, the oven temperature was programmed to rise from 60 ^{0}C to 240 ^{0}C at 30 ^{0}C.min^{-1} then 290 ^{0}C at 3 ^{0}C.min^{-1}. For the quantification of IAA and ABA the temperature program from 60 to 150 ^{0}C at 30 ^{0}C min^{-1}, and then at 3 ^{0}C min^{-1} to a final temperature 290 ^{0}C. The ionization potential was 70 eV. The masses of the characteristic ion were calculated to 4 decimal places and the detection was achieved by voltage switching at a resolution of 10 000 (10 % valley definition) and a cycle time of 0.6 second. Perefluorekerosene was used to provide reference masses for HR-SIM.

Endogenous hormone levels were subsequently calculated on the basis of peak areas after corrections were made for the contribution of naturally occurring isotopes and for the presence of unlabelled hormones in the internal standards (Lawrence *et al.*, 1992). The formula for quantifying the level is:

The level of endogenous hormones (ng g FW^{-1}) =

$$\frac{CEPA}{CISPA} \times \text{Amount of IS added (ng g } FW^{-1})$$

where

CEPA	corrected endogenous peak area
CISPA	corrected internal standard peak area
IS	internal standard
FW	fresh weight
ng	nano gram
g	gram

Ions monitored for quantification of the endogenous hormones were 506 and 508 (GA_1), 418 and 420 (GA_{20}), 202 and 207 (IAA), 190 and 193 (ABA). Identification was confirmed based on retention time and the presence of additional ions. Those ions were 448 and 450 (GA_1), 375 and 377 (GA_{20}), 261 and 266 (IAA), 162 and 165 (ABA).

CHAPTER 3

Position of the *apc* gene in linkage groups of *Pisum sativum*

3.1 Introduction

The *a*ccelerated *p*hase *c*hange (or *apc*) gene is so named because the pea plants carrying this mutant allele display an accelerated heteroblastic transition. As described in **Chapter 2**, the vegetative phase change in leaflet number from 4 to 6 is displayed earlier in the *apc* mutant than in the wild-type (WT) plants. This transition occurs at or before node 15 in the *apc* mutant, whereas in the WT it occurs initially after node 15. Therefore, this gene can be categorized as heterochronic acting in a peramorphic manner by *acceleration* (Wiltshire *et al.*, 1994).

In a preliminary study of the position of the *apc* gene in the linkage groups of pea, RJE Wiltshire (unpublished data) suggested that this gene lies on linkage group II, as defined by Weeden *et al.* (1998) at a distance of 21 cM from the *a* locus but could not determine in which direction. This study aims to clarify the position of this gene using linkage analysis with additional markers.

3.2 Materials and methods

3.2.1 Plant materials and growing condition

The lines of the garden pea (*Pisum sativum*) that were used as the parents in this work were L31, A23, SGE80 and Af3. All lines carry the wild-type phenotype (*Apc*), except Af3 which carries the mutant phenotype (*apc*).

To determine the position of the *apc* locus in linkage groups of *P. sativum*, crosses were made first between lines (i.e. A23, L31, SGE80) carrying the *Apc* dominant allele and the line carrying the *apc* recessive allele (Af3). Seeds of the F_1 produced from the

crosses were planted, and self-fertilization between the F_1 plants was allowed to produce the F_2 progeny. The F_2 seeds were grown under the glasshouse conditions as described below.

After removing a small piece (1-2 mm^2) of the testa, the seeds were sown at a depth of 2 cm below the soil surface in 14-cm slim-line pots. The pots contained a 1:1 mixture (v/v) of vermiculite and 10-mm dolerite chips, covered by a 2-cm layer of sterilized 1:1 peat and sand mixture. The pots were watered daily until just before seedling emergence, and after the seedlings were fully emerged, they were watered every 2 days for the first 2-3 weeks. Nutrient solution (Aquasol) was supplied once weekly. The mean daily temperature in the glasshouse during this experiment ranged from 13-21 ^{o}C. Night temperature was 16 ^{o}C.

All F_2 progenies were grown under an 18-hour photoperiod (natural light supplemented with a mixed incandescent/fluorescent light), except those generated from crosses between A23 and Af3. These were grown in far-red light during the first 10 days, then in the same LD conditions as the other progenies in order to investigate the light response of plants from such a cross. The plants were grown until senescence occurred.

3.2.2 Characters scored and data analysis

The principal character recorded was the rate of vegetative phase change, with the critical transition being the first node at which the leaf expanded six (6) leaflets counting from the cotyledons as node zero. Other characters recorded were based on the morphological markers and one enzyme marker (**Table 3.1**).

To determine whether there is a linkage between the *apc* and each marker gene used in this test or not, the segregation data between the *apc* and each marker gene were collected and analyzed using the chi-squared test. If linkage was detected, the recombination value (RF) and its standard error were calculated using the Product Ratio Method outlined in Stevens (1941). The distance between *apc* and the other genes in the linkage group were then determined.

3.3 Results

All crosses between the *apc* mutant plants and the WT plants bore fully fertile F_1 plants. This generation also displayed the normal phenotype in which all leaves with six leaflets were produced initially at node > 15, indicating that the *apc* allele acts recessively in these varying genetic backgrounds. As shown in **Table 3.2**, all individual segregations were in accordance with expectation (3:1 ratio, $P > 0.05$).

The results of the segregations in the F_2 generation are shown in **Table 3.3**. The *apc* gene displayed linkage with some markers in linkage group II. Very significant ($P < 0.0001$) linkage was detected between the *apc* gene and the *a* marker for two crosses (L31 x Af3 and SGE80 x Af3, with χ^2 of 32.74 and 27.54, respectively). The combined data from the two crosses (L31 x Af3 and SGE80 x Af3) also generated a linkage $\chi^2 = 59.58$ ($P < 0.0001$ and RF ± SE at 20.3 ± 3.0%). A highly significant ($P < 0.0001$) linkage of 14.4 ± 3.6 cM was also found between the *apc* gene and the *Aatp* marker, and *apc* was also linked to *lf* ($P < 0.001$ and RF ± SE at 30.8 ± 5.1%) (**Table 3.3** and **Figure 3.1**).

Although *apc* shows linkage with the above markers in linkage group II, no significant linkages were observed between this gene and the *blb*, *fun1*, *k*, *wb*, or *s* markers located below the *a* and *lf* loci on the same linkage group (**Table 3.3**).

The RF values between *apc* and *a* (20 cM) and between *apc* and *Aat*p (14 cM) are smaller than the distance between *a* and *aat*-p (29 cM) which clearly indicates that *apc* lies between *Aatp* and *a* (**Figure 3.1**).

3.4 Discussion

This linkage analysis has clearly established the position of the new gene, *apc*. The new locus lies between *Aatp* and *a* on linkage group II, as defined by Weeden *et al.* (1998), which has been putatively assigned to Blixt's (1959, cited in Weeden *et al.* 1998) Chromosome 6 (*ibid.*). The *apc* locus appears to be 20 cM from *a*, which confirms the initial study of RJE Wiltshire (unpublished data), and is slightly closer to the *Aatp* locus (14 cM). This places the locus in very close proximity to the Sequence-Characterised Amplified Region (SCAR) V20_1100 mapped recently by Rameau *et al.* (1998).

The linkage distance between the two critical marker genes, *a* and *Aatp*, established during this study is in close accordance with those from previous studies, adding confidence to this result. Estimates of this linkage distance range from 26 cM (Weeden *et al.* 1993), 27 cM (Swiecicki and Wolko, 1987) to 38 cM (Rozov and Gorel, 1994). The linkage between *a* and *Aatp* in the present study of 29 ± 5.0 cM was in close accordance with the lower estimates.

The linkage between *a* and *blb* of 33 ± 5.2 cM is also consistent with the data obtained by Kosterin and Rozov (1993) who obtained evidence of linkage between the *a* and *blb* genes at a distance of 34 cM.

There is, however, less confidence in the linkage values obtained for *lf*. Estimates of the linkage between *a* and *lf* range from 9 cM (Murfet 1971b), 11 cM (Rozov and Gorel, 1994), 12 cM (Weeden *et al.* 1996) to 19 cM (Swiecicki and Wolko, 1987) but the present result found linkage to be 26 cM. This overestimate is most probably a result of the difficulty in discriminating between the *Lf* and *Lf-d* alleles when the plants were grown under long day conditions. Undoubtedly, results that are more accurate could be obtained using another allele such as *lf-a*, but that may confound the scoring of *apc*.

Table 3.1 Characters scored for linkage analysis of loci on linkage group II (as defined by Weeden *et al.* , 1998).

Gene	Phenotype description
Aatp-F/S	Fast/slow running of aspartate aminotransferase enzyme
Apc/apc	Accelerated phase change reaches 6-leaflets after / before node 15
A/a	Anthocyanin present/ anthocyanin absent
Lf-d/Lf	High /medium flowering node.
Fun1/fun1	Responsive/unresponsive to the far-red light
Blb/blb	Normal/narrowed leaflets, stipules. The stem is slightly thickened just above the first scale leaf.
K/k	Wing normal/adpressed to keel
Wb/wb	Wax present/absent on leaf under surface, stipules and pods.
S/s	Seeds free/seeds stuck together

Table 3.2 Monohybrid F_2 segregation for 9 characters in three crosses.

Cross*	Gene	D	R	N	χ_2(3:1)	Probability
1	*a*	85	38	123	2.28	0.2> *P* >0.1
3	*a*	93	29	122	0.10	0.8> *P* >0.7
1	*apc*	97	26	123	0.98	0.4> *P* >0.3
2	*apc*	83	33	116	0.74	0.4> *P* >0.3
3	*apc*	86	26	112	0.19	0.6> *P* >0.5
3	*Aatp*	85	37	122	1.85	0.2> *P* >0.1
2	*fun*	85	31	116	0.18	0.6> *P* >0.5
3	*blb*	85	37	122	1.85	0.2> *P* >0.1
1	*lf*	92	31	123	0.02	0.9> *P* >0.8
1	*s*	96	27	123	0.61	0.4> *P* >0.3
1	*wb*	90	33	123	0.07	0.8> *P* >0.7
1	*k*	96	27	123	0.61	0.4> *P* >0.3

*1 = cross L31 x Af3; 2 = cross A23 x Af3; 3 = cross SGE80 x Af3.
D = dominant **R** = recessive **N** = number of plants

Table 3.3 Dihybrid segregation data for *apc* and 8 markers in linkage group II (as defined by Weeden *et al.*, 1998).

Gene pairs		[1]Phase	Crosses	[2]Phenotype DD	DR	RD	RR			Total	X_2	[3]*P*	[4]RF	[5]SE
apc	*a*	C	a	79	18	6	20			123	32.74	<0.0001	19.06	4.0
apc	*lf*	C	a	79	18	13	13			123	10.77	<0.001	30.75	5.1
apc	*s*	R	a	77	20	19	7			123	0.47	ns	--	-
apc	*wb*	R	a	71	26	19	7			123	0.00	ns	--	-
apc	*k*	R	a	76	21	20	6			123	0.32	ns	--	-
a	*lf*	C	a	74	12	18	19			123	19.19	<0.0001	26.47	4.8
a	*wb*	R	a	68	20	25	13			126	1.81	ns	--	-
a	*k*	R	a	68	17	28	10			123	0.61	ns	--	-
lf	*wb*	R	a	69	23	21	10			123	0.62	ns	--	-
lf	*k*	R	a	72	20	24	7			123	0.01	ns	--	-
apc	*fun*	C	b	60	23	25	8			116	0.15	ns	--	-
apc	*a*	C	c	74	12	9	17			112	27.54	<0.0001	20.96	4.4
apc	*blb*	R	c	64	22	19	7			112	0.02	ns	--	-
a	*blb*	R	c	60	33	25	4			122	4.93	<0.05	33.08	5.2
				DF	**DH**	**DS**	**RF**	**RH**	**RS**					
apc	*Aatp*	C	c	24	48	14	0	4	22	112	43.22	<0.0001	14.35	3.6
a	*Aatp*	C	c	27	46	20	5	7	17	122	14.47	<0.001	28.94	5.0
blb	*Aatp*	R	c	30	32	23	13	10	14	122	2.68	ns	--	-

[1] C = coupling R = repulsion
[2] **D** = dominant **R** = recessive **F** = fast **H** = intermediate **S** = slow
[3] ***P*** = probability
[4] **RF** = recombination frequency
[5] **SE** = standard error
ns no significance P > 0.05
-- RF> 50 %

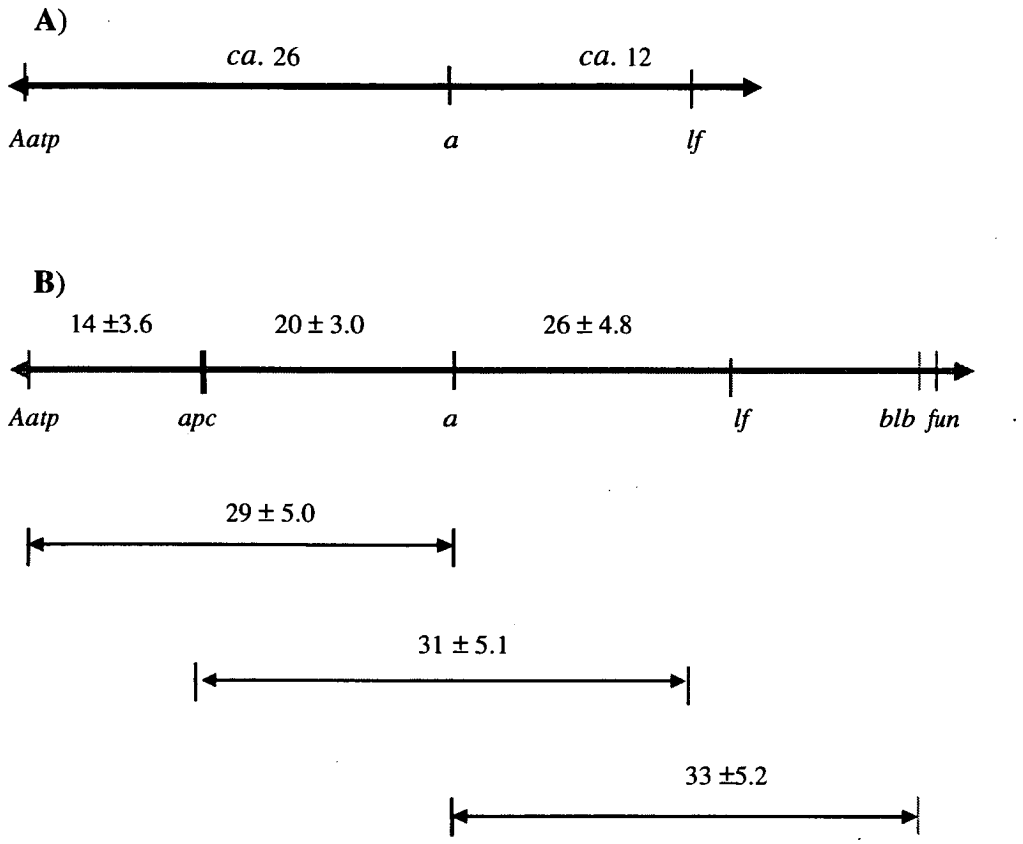

Figure 3.1. Linkage map for the mutant *apc* generated from **Table 3.3**. **A**) Linkage map of Weeden *et al.* (1996) and **B**) Linkage map of the present study

CHAPTER 4

Morphological basis of vegetative phase change within the shoot apical meristem

4.1 Introduction

The shoot apical meristem (SAM) generates the aboveground portion of the plant (Clark, 1997) and, therefore, ontogenetic changes must arise as a consequence of the changes that take place in the SAM during plant development (Medford *et al.*, 1994; Napoli and Ruehle, 1996). The most obvious changes during ontogeny are manifested in the leaves and these changes in shape and complexity are most often used as markers for assessing the process of vegetative phase change (Poethig, 1990). As products of the shoot apical meristem, the shape, size or complexity of leaves seems to be affected by changes in the SAM (Allsopp, 1954; Crotty, 1955).

The nature of the changes in the SAM has been examined in a number of studies, for example by Whaley (1939) who found that the size of leaves produced in the SAM of tomato (*Lycopersicum sp.*) plants was directly correlated with the size of the SAM itself. Popham (1960) also showed that a shoot apical meristem of larger volume was associated with the formation of larger lateral appendages such as leaves, whereas smaller meristems give rise to smaller appendages. Similar results were also found in *Darlingtonia* (Frank, 1976).

In heteroblastic plants, it might be expected that the abrupt change in form would be mirrored by an abrupt change in SAM characteristics. In ivy (*Hedera helix*) the size of the shoot apex differs between the morphologically divergent juvenile and adult growth phases (Hackett, 1985). The difference has also been found in other species *e.g. Arabidopsis* (Robbelen, 1957; Medford *et al.*, 1994), *Marsilea* (Allsopp, 1954; White, 1968), *Darlingtonia* (Frank, 1976). *Muehlenbeckia platyclados* (Bruck and Kaplan, 1980), and *Pseudopanax crassifolius* (Clearwater and Gould 1994). Medford *et al.*, (1992) observed that change in the SAM size from juvenile to adult vegetative growth phase is also followed by change in its shape.

Although the studies mentioned above concluded that there is a correlation between changes in the shoot morphology and changes in leaf heteroblastic development, the question remains whether it is a causal or coincidental relationship (Telfer and Poethig, 1994). The difficulty with these studies is that measurements performed on the shoot apex of the different growth phases of normal plants are often confounded by the effect of varying chronological or physiological ages of plants at differing stages of maturity. Furthermore, the effects of the transition to reproductive phase change may also confound the changes at the apex. Previous studies have also necessarily measured the shoot apical meristem after fixation, which may distort differences in the tissue.

A model system is required that enables a direct comparison of the SAM between plants of the same chronological and physiological age but that differ in vegetative development, without the confounding transition to reproductive phase change. The ideal system would be a comparative study between isogenic lines differing in the timing of a heteroblastic transition that occurs relatively independently of any transition to flowering, in other words a heterochronic mutant line. Likewise both the mutant and the wild type plants must be grown under controlled environmental conditions and, preferably, the measurements of the SAM should be conducted on fresh material to avoid fixation artifacts.

The garden pea *(Pisum sativum)* provides a model species in which leaf complexity increases in a clear, predictable manner with ontogeny (e.g. Gould *et al.*, 1987; Wiltshire *et al.*, 1994; Stafstrom, 1995; Lu *et al.*, 1996). However, few studies have investigated whether this vegetative transition involves a change in the SAM. The pea is also a powerful genetic tool because its preferential inbreeding characteristics and short generation time facilitates the development of pure-breeding lines.

This study uses a novel heterochronic mutant, *apc,* in a comparative study with its isogenic parental WT line, Torsdag (L107) to determine the morphological characteristics of the shoot apical meristem (especially, height, width and volume) at the critical time when the vegetative phase change from 4- to 6-leaflets is being initiated in the SAM of the *apc* plants but not in the WT. An Environmental Scanning

Electron Microscope (ESEM) was used for direct observation of the apex in a fresh (unfixed) condition.

4.2 Materials and Methods

4.2.1 Plant materials

In order to compare the shoot apical meristem at a similar chronological age, isogenic lines were used that differ in the rate of leaf development. As the wild-type, L107 plants (*Apc*) express the phenotype of a 'normal' vegetative phase change with the node of the transition from 4- to 6-leaflets occurring at node ≥ 15. By contrast, the phenotype of the recessive, mutant allele (*apc*) in the Af3 line expresses an 'accelerated' vegetative phase change with the node of the transition from 4- to 6-leaflets occurring at node <15 under the same long-day conditions (see Chapter 7). The reproductive phase change (onset of flowering) does not occur until node 16 in both lines (see Chapter 7). Therefore, as the leaf primordium of node 14 is being initiated, *apc* is undergoing the transition to 6-leaflets, whereas the WT is still at the 4-leaflet phase, and the neither is initiating flowers.

The Af3 line was derived from L107 in a mutagenesis program conducted by J. Weller at the School of Plant Science, University of Tasmania, and made near isogenic by repeated backcrossing to the parental line. The growth rates of the two lines do not differ substantially.

4.2.2 Growing conditions

The plants used in this study were grown 20 (10 wild-type and 10 mutants) per plastic tote box (41 x 32 x 12 cm) in a media consisting of a 1:1 mixture of vermiculite and dolerite chips topped with 2-3 cm of potting mix. The testa of each seed was nicked,

and then the seeds were planted 2 cm below soil level. The plants were grown in day and night temperatures of 20°C under LD (18 hours) conditions with the light provided by a combination of 8 fluorescent tubes (L40 W/20S cool white, Osram Germany) and 4 incandescent globes (100 W Pearl, Thorn Australia) in a variable height fixture delivering 200 µmols m^{-2} s^{-1} at the box surface. The plants were watered daily.

4.2.3 Determination of harvesting time.

In order to determine the critical time of harvesting of the shoot apical meristem at the initiation of the crucial 14th node, two trials were conducted. In the first trial, the WT and the *apc* plants were harvested at age 10-16 days after sowing (DAS). The shoots were dissected carefully under a binocular dissecting microscope (Wild M_3B, Heerbrugg Co., Switzerland) to record the order of the primordia, counting the first scale leaf as the first leaf to the last node in the shoot system. The youngest primordium was identified as a bulge in the shoot apical meristem (see **Figure 4.1**).

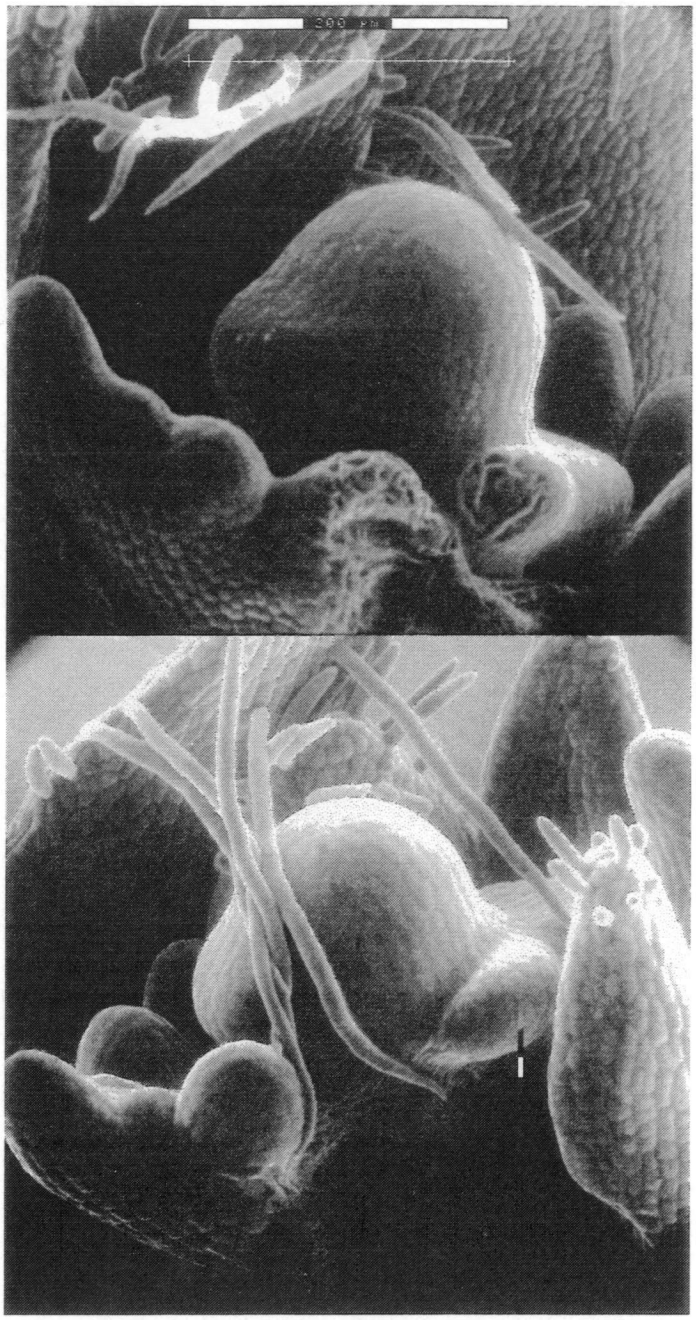

Figure 4.1 Electron scanning micrographs of shoot apical meristems of *apc* (top) and WT (bottom) dissected to measure the apical size at the initiation of the 14th leaf primordium.

Prior to the shoot dissection, the plastochron index (PI) of each plant was determined using the formula of Erickson and Michelini (as presented by Maksymowych and Erickson, 1973), where Log $_n$ = length of the smallest leaf > 10 mm, Log n+1 is the length of the next leaf.

$$PI = \frac{Log_n - Log\ 10}{Log_n - Log_{n+1}}$$

Equation 4.1

where:

PI	Plastochron index
n	The serial number, counting from node 1, of leaf which is longer than 10 mm
Log n	the logarithm of length of leaf n (= 10 mm)
Log n + 1	the logarithm of length of the next leaf (< 10 mm)

In this study, a slight modification in the formula was made to accommodate for the compound nature of the pea leaf. The reference length was taken as the length of petiole from stipules to the first leaflet pair, so that n is the serial number, counting from the base, of the leaf with a petiole longer than 10 mm.

The ages (in chronological time) of each specimen were recorded. The range of the times for both WT and *apc* plants in which the primordium 14 (P14) were initiated, were used a reference for the second trial.

In the second trial, the plants were harvested based on the range of time for production of P14 obtained from the previous experiment. The youngest primordia order was also counted using the same technique conducted in the first trial. The time and the youngest primordia order were recorded, and the ranges and averages of both parameters were compared between WT and *apc*.

4.2.4 The measurement of shoot apical meristem dimensions

The shoot apex of plants for this work was harvested at the age determined previously. Before the examination by ESEM (ElecScan 2020), the leaf primordia at node 9-12 were removed from the shoot apex with the aid of a binocular dissecting microscope to reveal the apical meristem region and youngest leaf primordia. The apical material was then quickly transferred to an aluminum stub. The shoot apical meristem was maintained in a fresh condition during the examination. To enable this the ESEM was maintained with a beam of 15 kV, the specimen changing pressure of 5 –7 T, and the wet mount or cold stage was used. The detector used during the investigation was ISD. Measurement of the SAM was by a micrometer Max-Series Electronic Digital Caliper (Japan Micrometer MFG. Co. Ltd)

The measurement of the shoot apical meristem dimensions generally followed that of Thomas and Kanchanapoom (1991). This included: height (H); diameter or width (W); and volume (V), of the meristem. The height was measured as a straight line drawn from the tip of the dome perpendicular to a line from the point of insertion of the youngest leaf primordia (H_1) and the primordia below the youngest primordia (H_2).

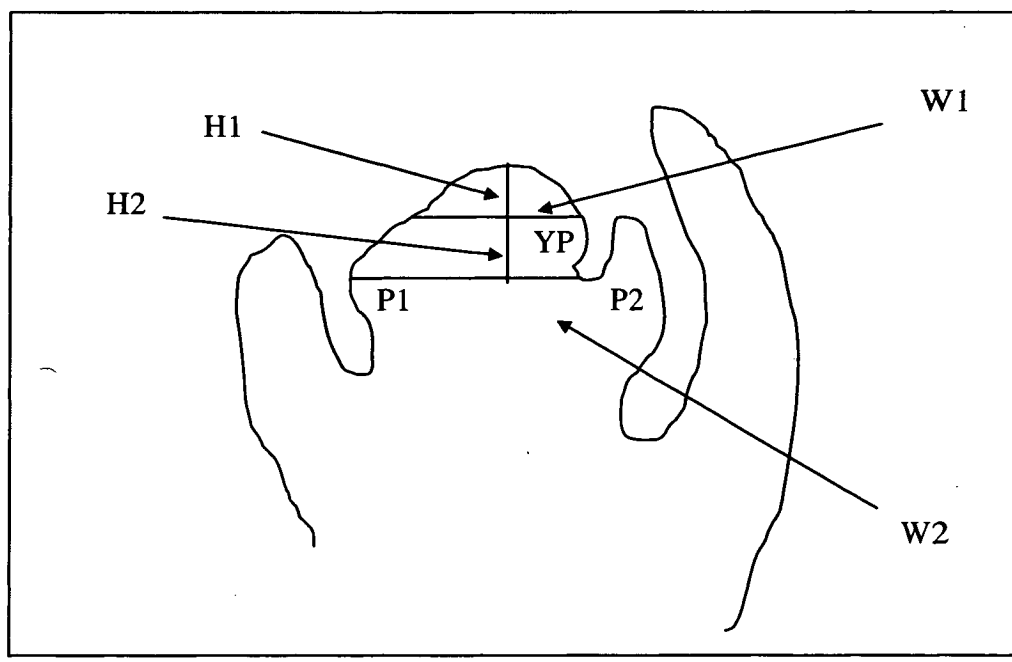

Figure 4.2 Shoot apical meristem dimension: W (width), H (height), YP (youngest primordia), and P (primordia).

The width was measured as a continuous line drawn from the point of insertion of the youngest leaf primordium perpendicular to a point on the opposite flank (W_1) and the older primordia to the point on the opposite flank (W_2).

Volume estimation was calculated by using the formula as follows,

$$V = \frac{4}{3} \times \prod \times H^2 \times W$$

Equation 4.2

where:

V	volume of shoot apical meristem
$\prod$	pi (3.14)
H	dome height
W	meristem width or diameter

Data obtained in both the harvesting determination and the measurement of the shoot apical dimension of the WT and the *apc* were analysed using the student's *t*-test (Excel Microsoft 97) and factorial ANOVA (StatView 4.4)

4.3 Results

4.3.1 Determining harvesting time

Trial 1

There is a positive correlation between 'day after sowing' (DAS) and the order of youngest primordia (YPO) in shoot apical meristem of both the WT and the *apc* plants (**Figure 4.3**) in which the coefficients of determination (r^2) are 0.81 $P < 0.0001$ (WT) and 0.76 $P < 0.0001$ (*apc*) respectively. Likewise, a close relationship is evident between the plastochron index (PI) and the YPO in both the WT ($r^2 = 0.91$, $P < 0.0001$) and the *apc* plants ($r^2 = 0.88$, $P < 0.001$). These results indicated that both chronological time (DAS) and PI could be used as an accurate indicator of developmental stage and when the plant could be harvested.

The means of the time for the production of primordia 13 – 16 by the WT and the *apc* plants (DAS) are presented in **Figure 4.5A**. A comparison of the data shows that timing of initiation of the primordia in the two genotypes was generally similar. Although the age at initiation of the 14th primordia (YPO 14) was slightly less in the WT (10.95 ± 0.08, n= 17) than that in the *apc* (11.25 ± 0.03, n= 25), these differences were not significant at the $P > 0.05$ level.

The comparison of the PI between the WT and the *apc* plants over the YPO 13 – 16 is displayed in **Figure 4.5B.** The results show no significant difference in the PI measured from the WT and the *apc* at each YPO.

Trial 2

The mean of the node (n), the plastochron index (PI), and youngest primordia order (YPO) measured at a range of plant age 10.8 – 11.5 day after sowing (DAS) are shown in **Figure 4.6**. The comparison in those parameters shows no significant differences between the WT and the *apc* ($P > 0.05$).

A comparison of the average time required by the WT and *apc* to produce P14 initials is presented in **Figure 4.7** revealing no difference ($P > 0.05$) between genotypes. A similar result is shown in the comparison of PI between both genotypes (**Figure 4.8**). Therefore, it can be assumed that the critical time of the production of the P14 in the WT and the *apc* was 11 days after sowing within 95% confidence limits (t-test).

4.3.2 Determining morphological changes

Plastochron Index

The mean plastochron index (PI) differed slightly between Trial 1 and Trial 2 (**Figure 4.8**). It indicates that the plants in the second trial were further advanced in developmental terms, but still within the critical range for 14^{th} primordia initiation. Within trials, however, there was no significant difference ($P > 0.05$) between the WT and the *apc* mutant in development.

The morphological basis

No significant alterations to the SAM dimensions were obviouswithin the measured plastochron for the WT plants because there was no significant association between differences in the SAM and PI within P14. The results of regression analysis presented in **Figure 4.9** show the values of the coefficient of determination between the PI and the shoot apical dimensions: width (W1 and W2), height (H1 and H2) and volume (V1 and V2) were generally less than 0.03. A similar result was also found in the *apc* mutant (**Figure 4.10**). Therefore the results indicated that the variation in the PI values of plants in Trial 1 and Trial does not have a significant effect on the variation in the

size of shoot apical dimension measured and so the data from the two trials could be combined.

During the initiation of the crucial 14th primordia, all measured dimensions of the SAM were smaller in the WT than in the *apc* plants (**Table 4.1**). **Table 4.2** shows that the size of the SAM dimension was significantly affected by genotype ($P < 0.0001$ *F*-test), but not by trials ($P > 0.05$) nor interaction between genotype and trials ($P > 0.1$).

4.4 Discussion

Changes in leaf heteroblastic development, or vegetative phase change, during plant ontogeny appear to be directly related to changes in the size of the shoot apical meristem in pea. Using isogenic lines of the garden pea, differing only in a heterochronic gene (*apc*) affecting the rate of the leaf development, all indices of shoot apical meristem size (width, height and volume) were larger in the *apc* mutant than in the WT at the initiation of the crucial leaf primordia 14 (P14). This clearly indicates that the transition in leaf complexity in pea is associated with a change in the morphology of the shoot apical meristem. This is consistent with the findings of Medford *et al.* (1992) who indicated that heteroblasty in leaves arrayed along the main shoot of *Arabidopsis* may be due to alteration in shoot apical meristem size. A similar result has been obtained in species such as *Lycopersicum* (Whaley, 1939), *Zea* (Abbe *et al.*, 1942), *Marsilea* (Allsopp 1954) *Darlingtonia* (Frank, 1976), *Begonia dregei* (McLellan, 1990) and *Pseudopanax crassifolius* (Clearwater and Gould, 1994).

An increase in shoot apical meristem size has also been reported just before reproductive phase change was initiated in *Sinapsis alba* (Bernier, 1971; 1997). Therefore, such an increase seems to be one of the factors required for the initiation of both vegetative and reproductive phase changes of some plants.

A number of studies have found that leaf morphology in some plants is determined when the organ is still within the shoot apical meristem. For example, the fate of the leaf margin in the *narrowsheath* mutants of *Zea* (Scanlon *et al.*, 1996), and the adaxial and abaxial leaf polarity (Evan and Barton, 1997) are determined early in the initiation of the leaf at the apex. Medford *et al.* (1992) pointed out that the leaf shape is also determined by the size of the shoot apical meristem at the time of leaf initiation. This indicates that there is a factor(s) within the SAM required for determining the identity of the leaf form produced along the shoot (Cutter 1965; Halperin, 1978; Smith and Hake, 1992). Medford *et al.* (1992) also stated that the existence of a key process controlling the vegetative shoot for generating distinct shoot form (including the leaf heteroblasty) should be localized in the shoot apical meristem itself.

Hackett (1980) and Laufs *et al.* (1998) have shown that an increase in shoot apical meristem is a consequence of an increase in an accumulation of cell divisions. Many studies, in fact, have shown a correlation between increase in cell division and increase of the shoot apical meristem size. Bernier (1971) observed that the increase in shoot apex of *Sinapsis alba* was recognized with an increase in cell division within the SAM. In *Agropyron repens*, if the cell number (cell division) increases or decreases it will be followed by an increase or decrease in shoot apex size as well (Rogan and Smith, 1974). The increase of the SAM size causing increased cell numbers from juvenile to adult phase growth has also been observed in *Arabidopsis* (Medford *et al.*, 1992; Laufs *et al.*, 1998). Other studies in the *clavata* mutant of *Arabidopsis* (Leyser and Furner, 1992) and the *fasciated* mutant of tomato (Szymkowiak and Sussex, 1992) found the mRNA representing cell division was distributed thoroughly within the region of shoot apical meristem during plant development. This indicates that cell divisions occurring within the SAM during plant development act as a regulator of shoot apex size. A study of a similar mutant in soybean has also shown that the increase of SAM is a result of a faster rate of cell division (Tang and Knap, 1998).

This leads to the question of how the changes in the morphology of the shoot apical meristem affect leaf form. As mentioned above, it has been suggested that the change in the SAM is controlled by the rate of cell division within this tissue. Because of an increase in cell division, the number of cells in the tissue increases automatically

(Laufs *et al.*, 1998). King (1983) states that more cells are needed to support the more active rate of growth in the shoot apex during the transition phase. In *Arabidopsis,* the different size of the shoot apical meristem also represents the rate of cell division in the SAM, which is followed with a difference in the shape of leaves produced between the mutant and the WT plants (Leyser and Furner, 1992). Therefore, the ability of the SAM to drive cell division within itself must be considered a requirement for the onset of leaf heteroblasty in plants (Greenwood 1984; Greenwood and Hutchison, 1993). Laufs *et al.* (1998) also proposed that a high rate of cell division which was observed in a large part of layer 1 (L1) of the SAM, supports the primordia formation. However, the expression of a specific phase in the shoot can result from an increase in size and complexity of shoot apical meristem effected by extrinsic change in the apical meristem and changed input to the shoot apical meristem (Poethig, 1990).

Based on the results of this study, and other studies mentioned above, it is proposed that the *apc* mutant accelerates vegetative phase change in pea via an increase in the rate of cell division in the shoot apical meristem. As a result of such an increase, the *apc* mutant can produce cells more rapidly than the WT plant as shown by the result in which the size of the SAM was significantly greater in the *apc* than the WT, so more cells are supplied for leaf formation. Because achievement of a certain number of cells required to induce a leaf identity occurs earlier in *apc*, an increase in leaf complexity also occurs earlier in *apc* than in the WT. The evidence from this study also indicates that the increase in the shoot apical meristem is not a coincident process, but a causal process of the increase in leaf complexity.

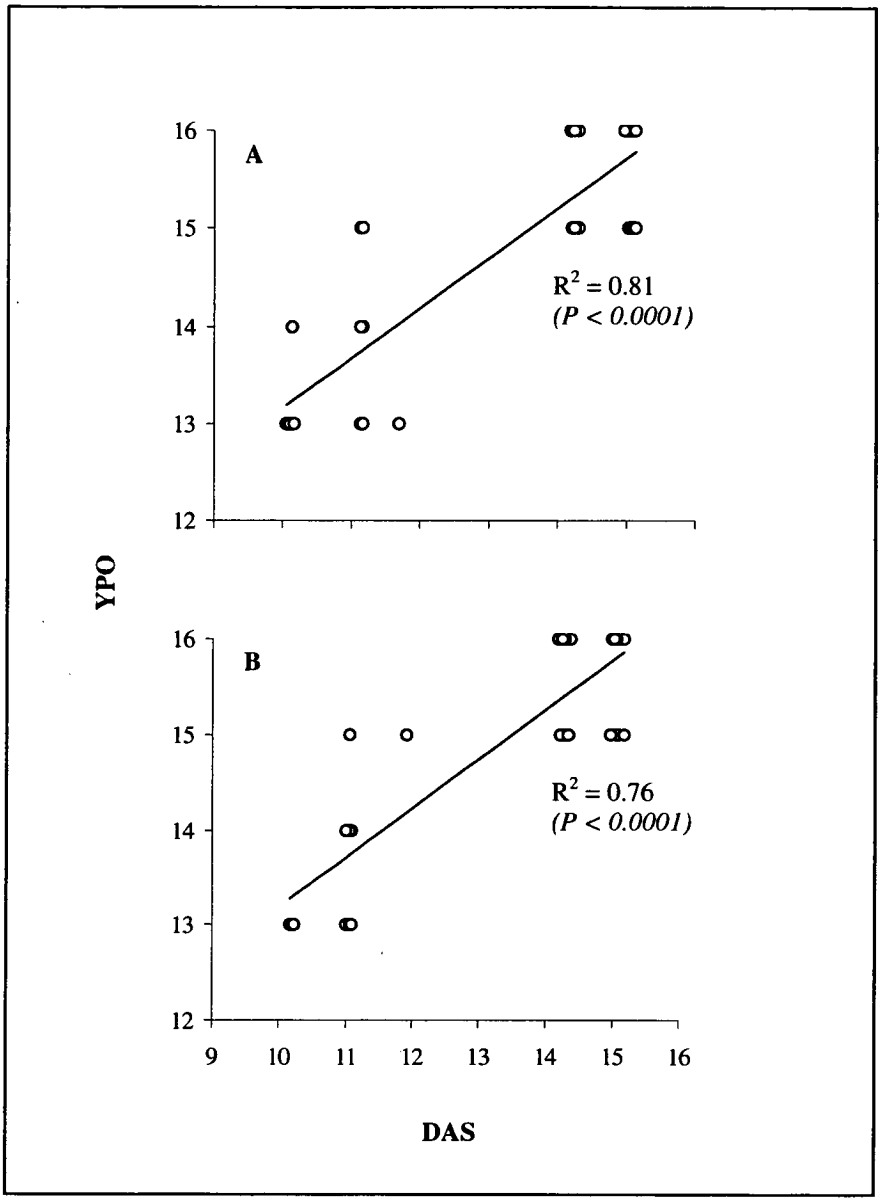

Figure 4.3. Relationship between days after sowing (DAS) and youngest primordia (YPO) in the shoot apical meristem of WT (**A**) and heterochronic *apc* (**B**) plants. N = 55-57

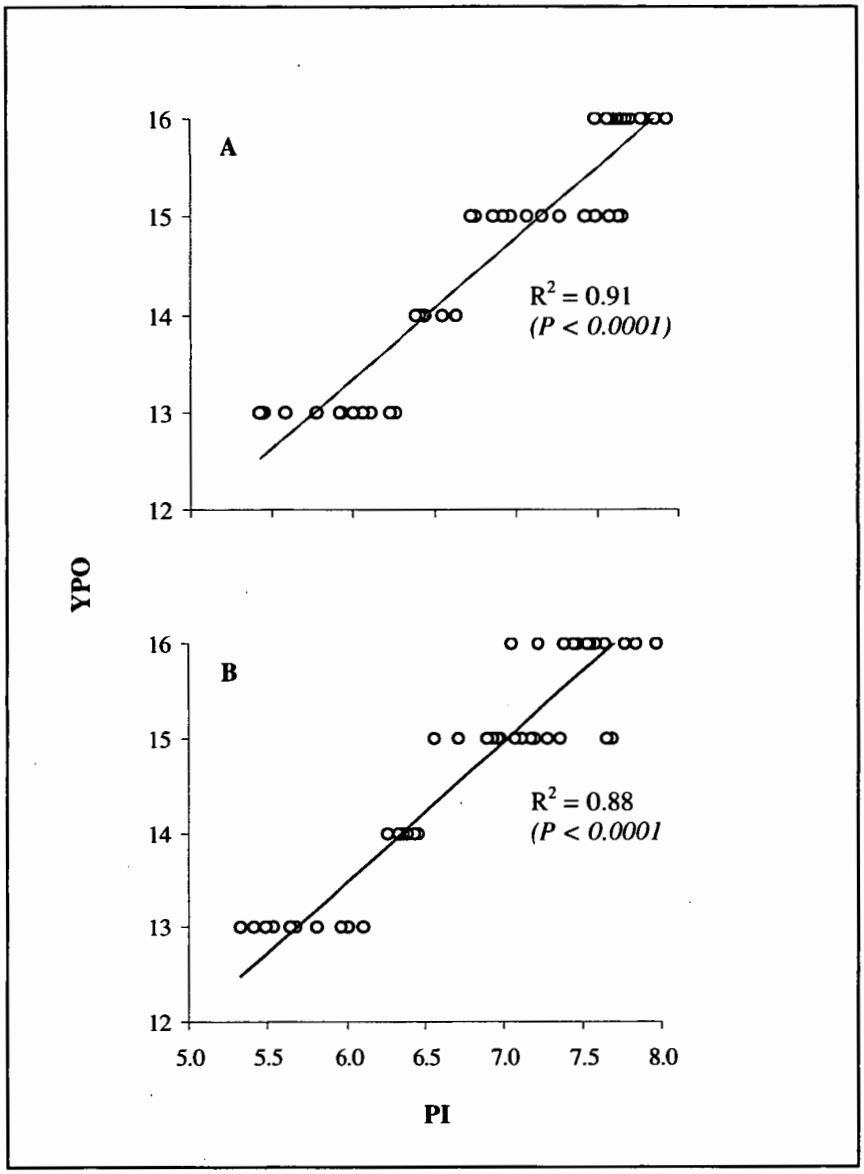

Figure 4.4. Relationship between youngest primordia (YPO) and plastochron index (PI) in the shoot apical meristem of the WT (**A**) and heterochronic *apc* (**B**) plants. N = 55 - 57.

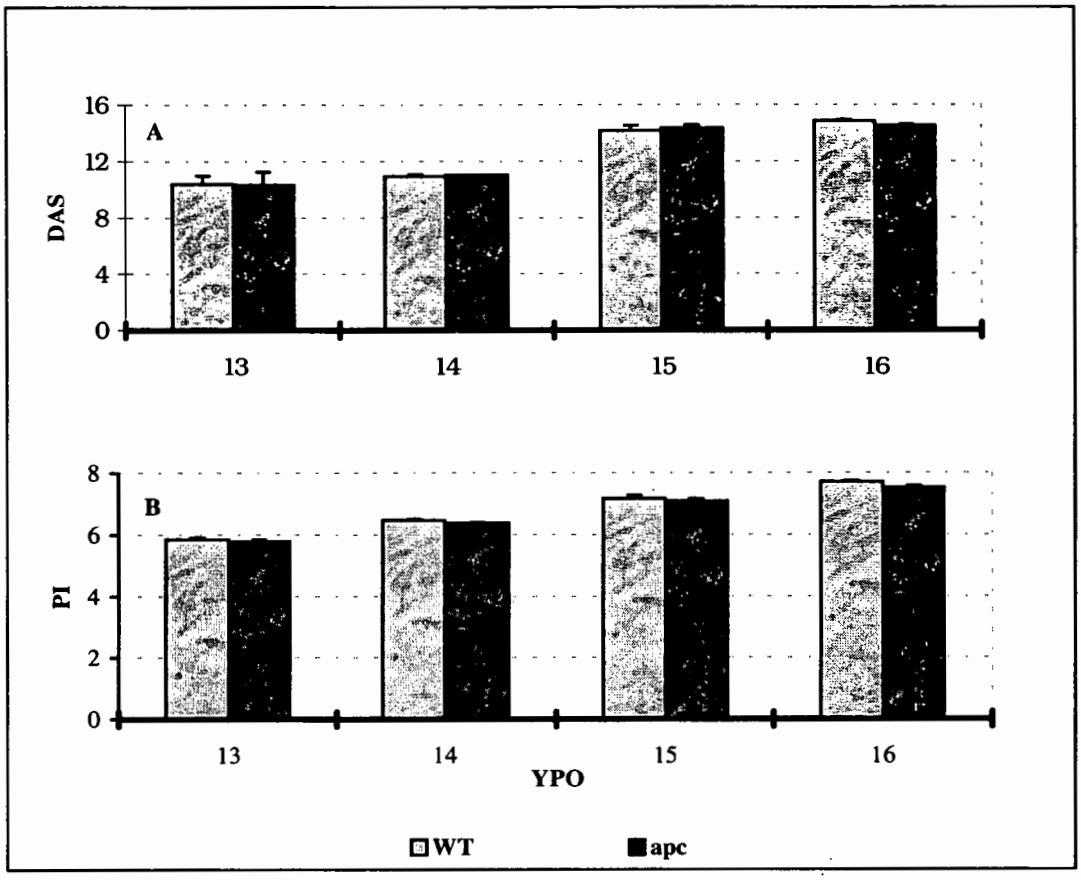

Figure 4.5. Time represented as days after sowing (DAS) needed by the WT and heterochronic *apc* plants for producing youngest primordia (YPO) from 13 to16 (**A**). Plastochron index (PI) is measured from the plant with the different YPO (**B**). The bar lines represent the mean value and standard error. The population number of WT and the *apc* plants was 9 - 18.

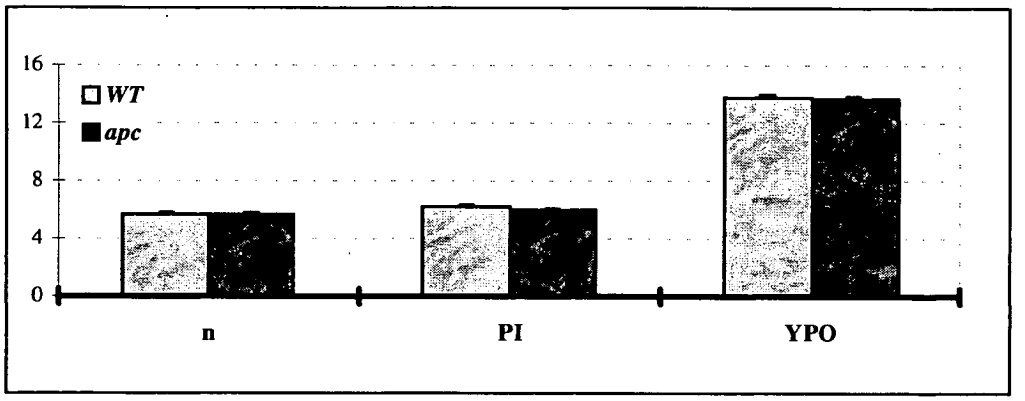

Figure 4.6. Leaf development indices, including number of leaves with a petiole longer than 10mm (n), plastochron index (PI) and youngest primordium (YPO) measured from WT and the heterochronic mutant *apc* 10.8–11.5 days after sowing (DAS). The bar lines represent the mean and standard error. N = 14 – 20.

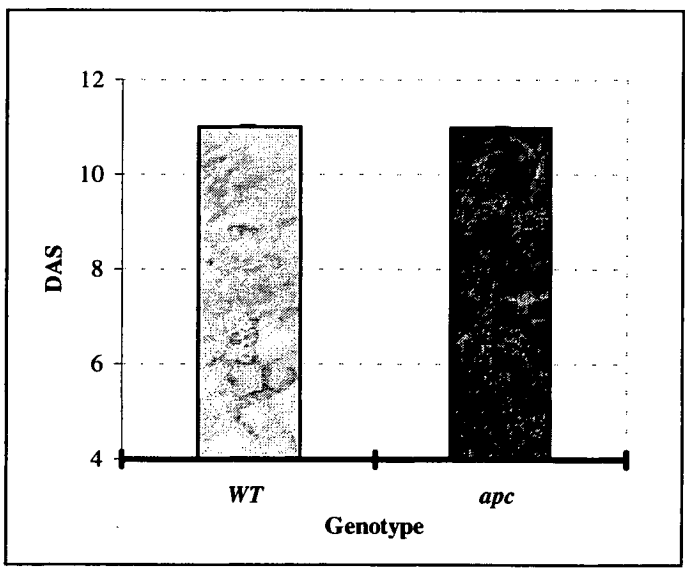

Figure 4.7 Age at harvesting of WT and heterochronic *apc* plants in days after sowing (DAS) for shoot apical meristem measurements. The bar lines represent the mean value and standard error. N = 14 – 20.

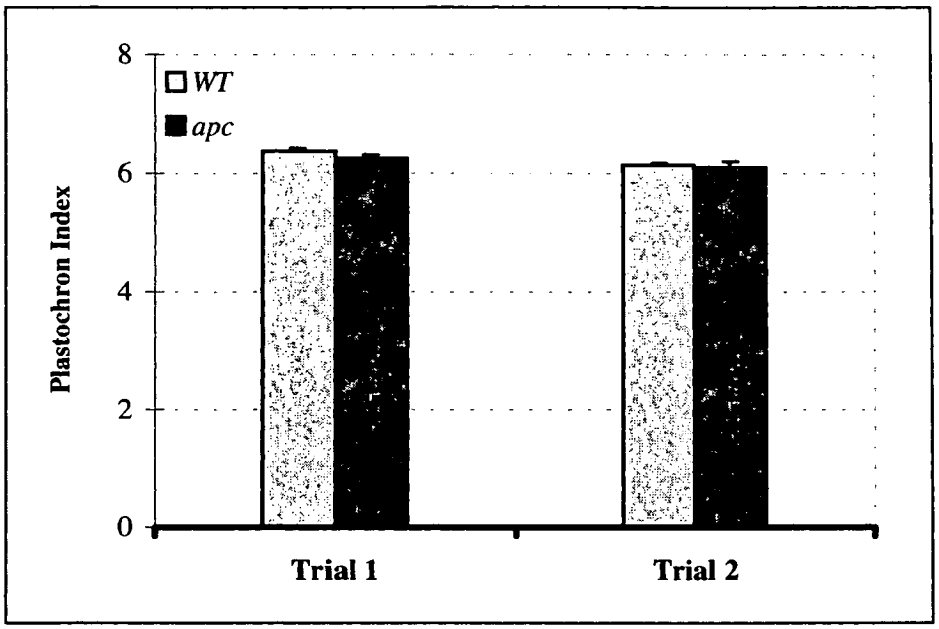

Figure 4.8 Plastochron index (PI) measured from Trial 1 and Trial 2 of the WT and the *apc* plants. The bar lines represent the mean value and standard error respectively. N = 16 – 26.

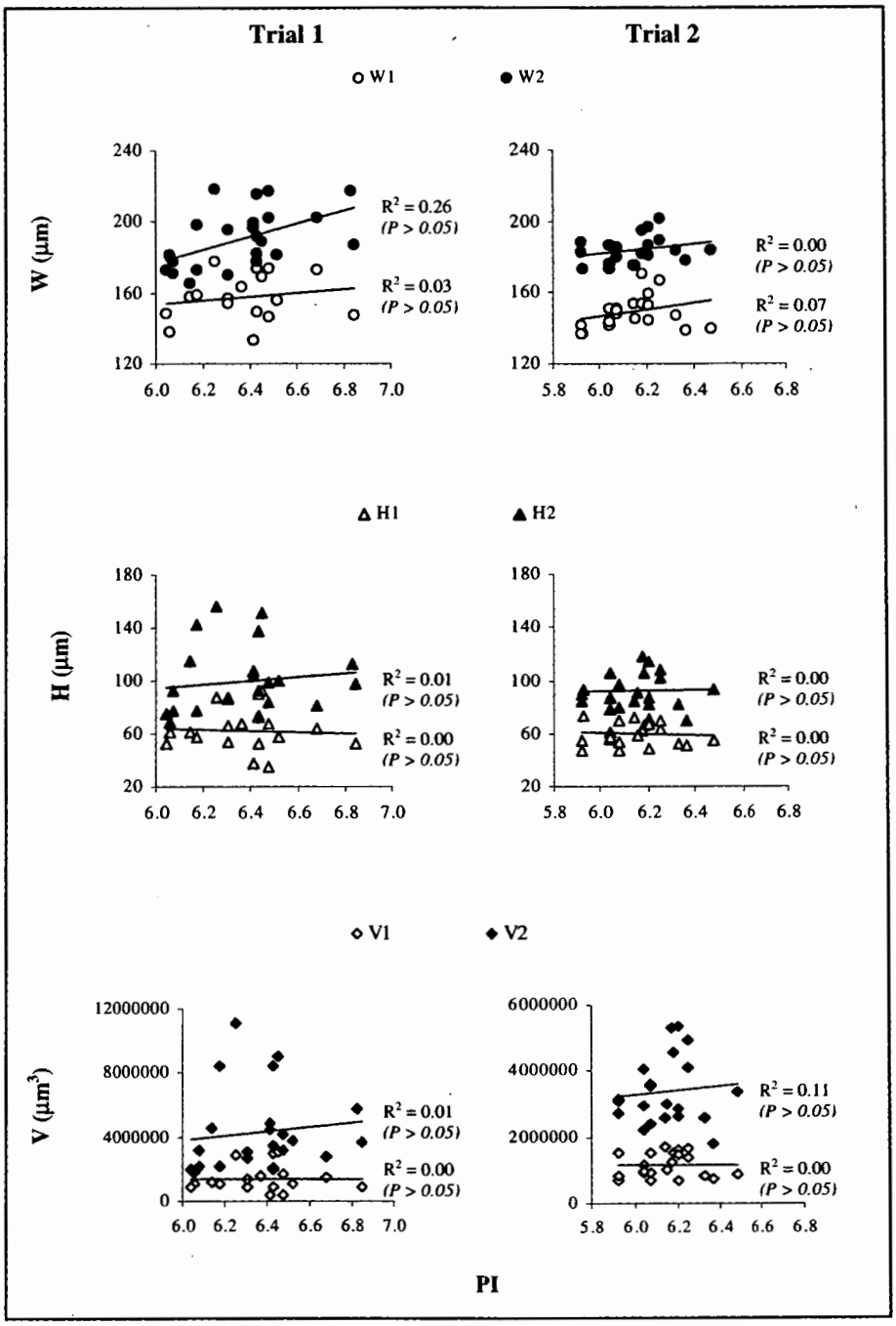

Figure 4.9 Relationship between PI and width (W1, W2), height (H1, H2) and volume (V1, V2) in the SAM of the WT plants. N = 17 – 23.

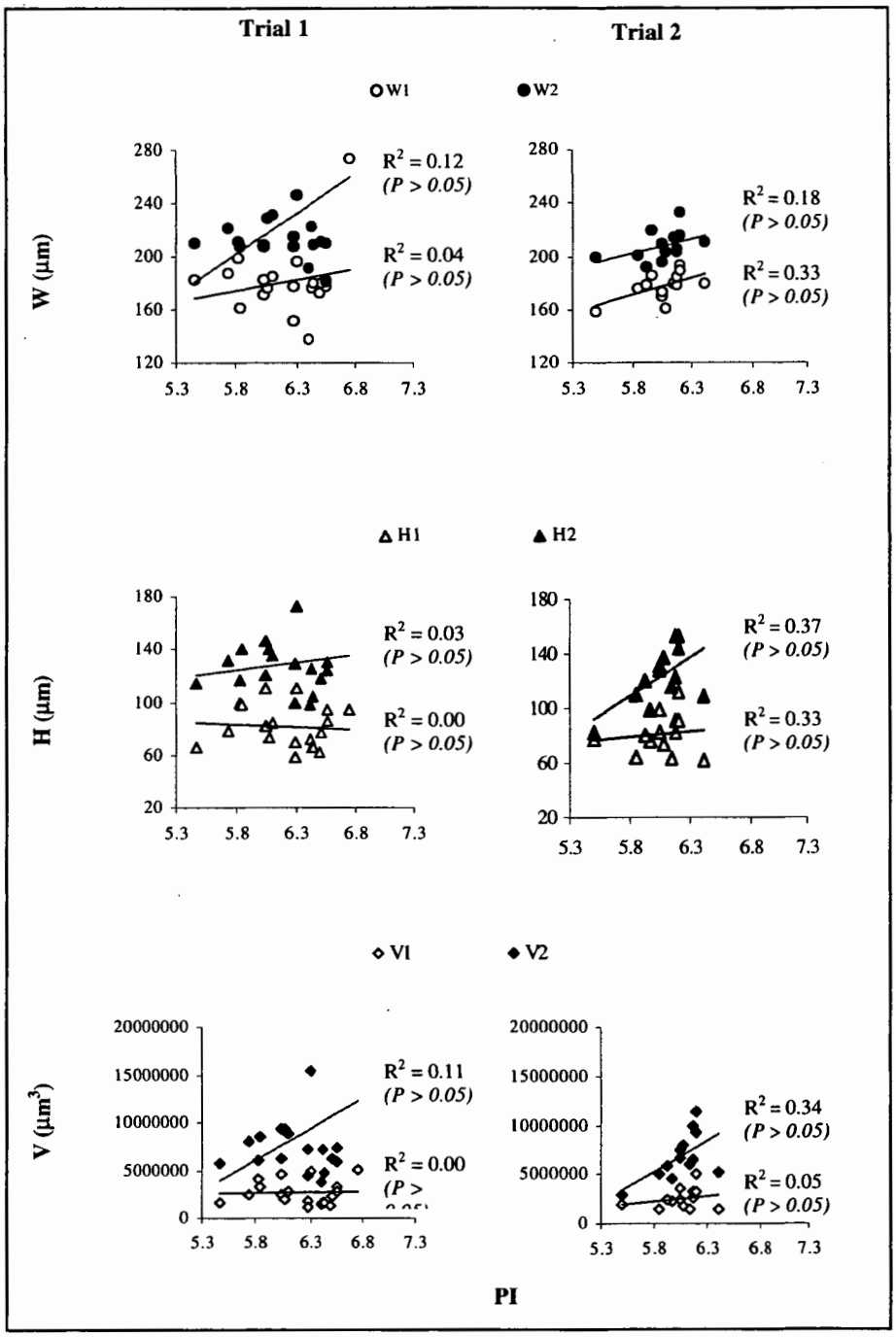

Figure 4.10 Relationship between plastochron index (PI) and width (W1, W2), height (H1, H2) and volume (V1, V2) in the SAM of the *apc* plants. N = 16 – 26.

Table 4.1 The mean and standard error values of the width (W1, W2), height (H1, H2) and volume (V1, V2) of the SAM of the WT and heterochronic *apc* plants during the initiation of the 14th leaf primordium, which will progress to 6-leaflets in the *apc* but will remain in the 4-leaflet form in the WT.

Traits measured	**Genotype**					
	WT			***apc***		
	Mean	**SE**	**N**	**Mean**	**SE**	**N**
Trial 1						
W1 (μm)	157	3.2	17	180	4.6	26
H1 (μm)	62	3.9	17	82	3.5	26
V1 (μm^3)	1401981	200789.7	17	2720274	269203.9	26
W2 (μm)	191	3.4	23	222	10.1	25
H2 (μm)	100	5.4	23	128	4.4	25
V2 (μm^3)	4599790	539257.7	23	8330165	1144115.9	25
Trial 2						
W1 (μm)	150	2.2	21	179	2.4	16
H1 (μm)	60	1.9	21	81	3.3	16
V1 (μm^3)	1148307	78503.4	21	25202003	231090.8	16
W2 (μm)	184	1.7	21	209	2.8	16
H2 (μm)	97	2.7	21	126	4.8	16
V2 (μm^3)	3379043	219986.0	21	7103840	563993.1	16

Table 4.2 The effect of genotype (WT vs. *apc*), trial and genotype-trial interaction on the width (W1, W2), height (H1, H2) and volume (V1, V2) of the SAM at the initiation of the 14th leaf primordia (6-C in *apc*, 4-C in WT).

Traits	Effect of Genotype (G)		Effect of Trial (T)		Effect of Interaction between G X T	
	F-test	*p*	*F-test*	*p*	*F- test*	*p*
W1	40.01	0.0001	2.12	0.15	0.21	0.65
H1	36.10	0.0001	0.17	0.66	0.11	0.74
V1	36.28	0.0001	0.74	0.39	0.07	0.77
W2	16.74	0.0001	2.99	0.09	0.66	0.42
H2	36.99	0.0001	1.69	0.20	0.01	0.92
V2	20.43	0.0001	2.62	0.11	0.32	0.57

CHAPTER 5

Quantification of the endogenous level of GA_1, GA_{20}, IAA, and ABA in the shoot apex of pea at vegetative phase change

5.1 Introduction

Growth hormones play a crucial role in developmental processes of plants e.g. stem elongation, flowering and senescence (Pharis and King, 1985; Poethig, 1990; Ross *et al.,* 1993). Hormones have also been considered as a major determinant of vegetative phase change (Allsopp, 1965), largely because of quantification studies that have demonstrated differences in the levels of endogenous hormones between phases of plant growth. In ivy (*Hedera helix*), for example, the gibberellin (GA) levels are lower in adult shoots than in juvenile shoots (Frydman and Wareing, 1973a,b; Rogler and Hackett, 1975a) and also in black currant (*Ribes nigrum*) (Schwabe and Al-Doori, 1973). These finding have been interpreted that a reduction in GA level may be a promoter of the initiation of the phase change. This view was reinforced by application experiments where gibberellins induced a reversion of the shoot from adult to juvenile characteristics (Robbins, 1960; Marc and Hackett, 1991).

Another indication of the involvement of GA in vegetative phase change has been shown in a study of maize mutants, such as *dwarf1* (*d1*), *dwarf3* (*d3*), *dwarf5* (*d5*), and *anther ear1* (*an1*) (Evans and Poethig, 1995). These mutants showed a dwarf habit in response to a significant decrease in the GA levels compared with the WT. This was caused by blocks in the GA synthesis pathway e.g. from GA_{20} to GA_1 (Phinney 1984; Spray *et al.,* 1984), from GA_{20} to GA_5 (Fujioka *et al.,* 1988), and GA $_{20}$ to GA_3 (Fujioka *et al.,* 1990). Because of the effect on height, these mutants showed a delay in the transition from juvenile, vegetative to adult, vegetative phase change, but when exogenous GA were applied, the rate of transition in the mutants was the same as the WT (Evans and Poethig, 1995).

In addition to changes in GA levels, endogenous ABA levels also alter during plant growth. In ivy, the ABA level is higher in the adult shoot than in the juvenile shoot (Frydman and Wareing 1973 a). In heterophyllic aquatic plants, e.g. *Hippuris vulgaris,* it was found that this hormone increased from juvenile to adult phases (Goliber and Feldman, 1989). These results suggest that an increase in ABA level may be involved in the phase change. This is supported by some studies that found that production of adult leaves in the juvenile shoot (or submerged shoot) can be promoted by application of exogenous ABA hormone (Anderson, 1978; Mohan Ram and Rao, 1982; Deschamp and Cooke, 1984).

Many of these studies of the roles of hormones in plant growth and development follow two approaches. Firstly, correlating change in endogenous levels with physiological effect and /or, secondly, applying the pertinent hormone and investigating the resulting response (Finkelstein and Zeevaart, 1994).

An alternative method is the identification of single gene mutants that display phenotypic differences suggestive of an altered hormone level or response (Reid and Ross, 1993). This approach has successfully elucidated many physiological processes in the garden pea relating to hormone function, from the first hormone mutants with dwarf and wilty phenotypes attributable to GA and ABA, respectively (Phinney, 1956, 1961; Tal and Nevo, 1973) to more recent studies examining the interaction of hormones, such as GA and auxins (Ross *et al.* 2000). The garden pea has been used extensively in these types of studies investigating the effect of changes in endogenous hormone levels on a range of growth and developmental processes (e.g. Ross *et al.,* 1993; Zhu and Davies, 1997), but none have addressed the possible role of hormones in the vegetative phase change.

The site of action of hormones involved in the juvenile to adult phase change is clearly at the shoot apex. However, past studies of hormone levels in other species may have been confounded by the size of the tissue samples assayed, swamping small differences in levels in the apex, or by dramatic differences in the size or age of plants from which the material was harvested for comparison. In this study, the role of hormones on the vegetative phase is examined in precise assays of the shoot apical

tissue. The endogenous level of GA_1, GA_{20}, IAA and ABA hormones were quantified from material harvested at a critical time when the phase change to the 6-leaflet form was initiated in the apex of the *apc* mutant but the isogenic control WT plants of the same size and age were still initiating leaves with 4-leaflets.

5.2 Materials and methods

5.2.1 Plant materials

The plant materials used were the L107 *cv* Torsdag (*WT*) and the isogenic line Af3 (*apc)* as described in Chapters 2 and 4.

5.2.2 Growing conditions

Plants were grown in plastic tote boxes (41 x 32 x 12 cm) in a 50:50 (by volume) mixture of vermiculite and 2-3 cm dolerite chips topped with 2 cm layer of sterilized 1:1 mixture of peat moss and coarse river sand in the growth cabinet. Plants were exposed to an 18 hour photoperiod with a mixed fluorescent (Thorn 40 W white tube) and incandescent (Mazda 100 W pearl globe) illumination source (25 $\mu mol.m^{-2}.s^{-1}$ at a pot top). The day and night temperature in the cabinet was 20 ^{0}C.

5.2.3 Experimental design

The experiment compared the endogenous levels of GA_1, GA_2, IAA and ABA in the shoot apices of WT and *apc* plants at the critical time (11 days after sowing, see **Chapter 4**) when the vegetative phase change from 4- to 6-leaflets takes place in the shoot apex of *apc* but not WT plants. The size of the shoot apices dissected from the plants were *ca.* 1- 2 mm (Figure 5.1). The shoot apices were immediately put into cold MeOH (- 20 °C) with added BHT. The bulked samples were divided and analyzed

separately. The harvesting data, including the age, the fresh weight of the shoot apices and the amount of the internal standard added are presented in **Table 5.1**.

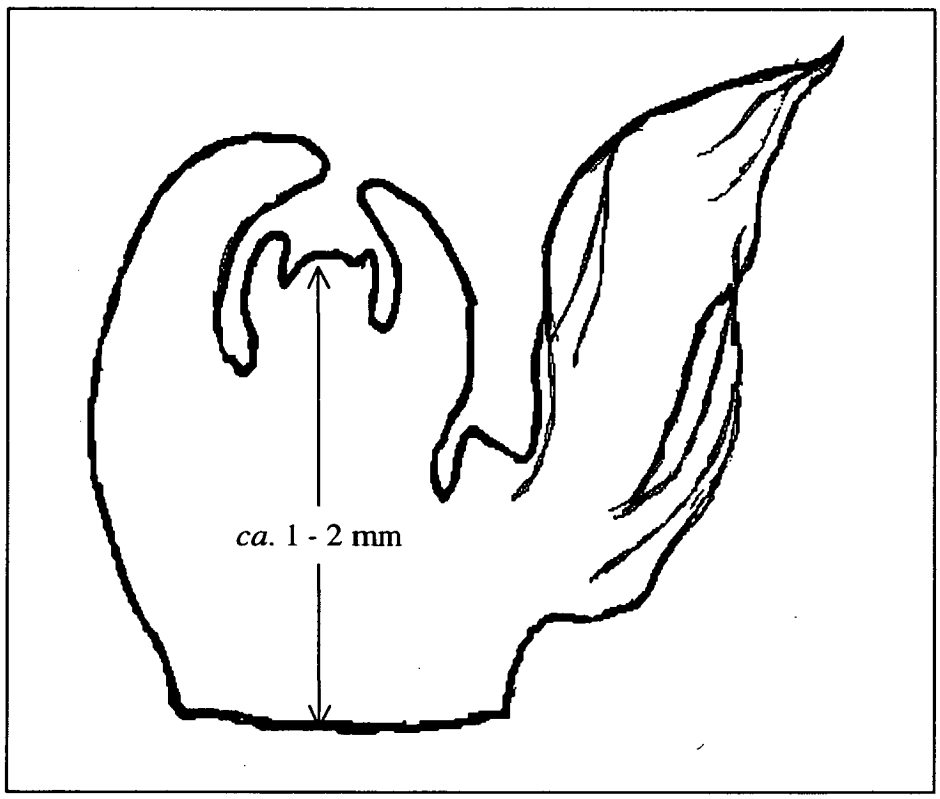

Figure. 5.1 The size (in height) of the pea shoot apex harvested for hormone analysis.

The analyses of the GA_1, GA_2, IAA and ABA levels were based on the protocols developed by the Plant Hormone Division of the School of Plant Science at the University of Tasmania, Hobart. The analysis of hormone levels was carried out in following steps: extraction, addition of internal standard, purification, fractionation, and quantification (for more details see **Chapter 2**).

5.4.4 Data analysis

Means of the level of each hormone in the shoot apex of the *WT* plants were compared with those in the *apc* mutant, using Student's the *t*-test, performed in Excel 97 on PC computer.

5.3 Results

The results of the quantification of endogenous levels of the hormones in this study using GC-SIM-MS are presented in **Table 5.2** and **Figure 5.2**. Generally, the shoot apex of the *apc* plant contained higher levels of GA_1, GA_{20} and IAA, but less ABA compared to the shoot apex of WT (**Figure 5.2**).

5.3.1 GA_1 and GA_{20} levels

The results show that the mean GA_1 level in the shoot apex of the *apc* mutant initiating 6-leaflet leaves is higher than the shoot apex of the WT apex initiating 4-leaflet leaves. However, the comparison of the levels between both genotypes shows no significant difference at $P > 0.05$ using the *t*-test. The means of GA_1 level in the WT and the *apc* plants are 7.9 and 12.5 ng/g FW^{-1} , respectively. The level of GA_{20} is also slightly higher in the *apc* than in the WT, but again this difference was not significant. The means of GA_{20} level were 18.8 ng/g FW^{-1} in the apc and 12.5 ng/g FW^{-1} in the WT. In general, the shoot apex of both the WT and the *apc* has a higher level in GA_{20} than GA_1.

5.3.2 IAA level

The shoot apex of the *apc* plants had 16% more IAA than the *WT* shoot apex (**Table 5.2**), but the difference is not significant ($P > 0.05$) (**Figure 5.2**). The means of IAA levels of the shoot apex in the *apc* and in the WT were 51.9 and 44.4 ng/g FW^{-1} , respectively.

5.3.3 ABA level

The level of ABA in the shoot apex was 36 % lower in the *apc* plant than in the WT plant ($P < 0.05$). The means of the ABA level quantified by GC-SIM-MS were 9.8 ng/g FW^{-1} in the WT and 6.6 ng/g FW^{-1} in the *apc*.

5.4 Discussion

The involvement of GA in vegetative phase change was suggested by studies of woody plants in which significant differences were found in GA content in the shoot of different growth phases. The levels of GAs in juvenile shoots tended to be higher than in the adult shoot (Fryman and Wareing, 1973a,b; Rogler and Hackett, 1975a). This is not supported by the present finding in pea, with no dramatic difference found in the GA_1 and GA_{20} levels in the shoot apices of the 4-leaflet WT and 6-leaflet *apc* plants, using GC-MS SIM. The results suggest that large differences in GA_1 or GA_{20} levels in the shoot apex are not crucial to the regulation of leaflet number in pea.

As discussed in **Chapter 4,** the vegetative phase change appears to be related to the rate of cell division in the SAM. The present results indicate that differences in GA levels are not required to effect changes in cell division. Other studies have demonstrated that the rate of cell division of plants was not affected by GA application (Barrat and Davies 1997; Daykin *et al.,* 1997). A study on a *dwarf* mutant of pea *cv* Meteor showed that the GA treatment did not accelerate the cell division rate of the SAM (Daykin *et al.,* 1997), with no difference in cell doubling times, mitotic indices and percentage labelled mitosis between the GA-treated plants and the control plants. The same result was also found in the pea stem (Barrat and Davies, 1997). A more recent study in rice (*Oryza sativa*) plants also showed that GA promoted cell elongation rather than cell division (Matsukura *et al.,* 1998). Evans and Poethig (1995) also suggested that GAs were not actually required for the vegetative phase change. Although, the delay of the initiation of the vegetative phase in the *dwarf* mutant, restored by GA application in the heterochronic phenotype of the *dwarf* mutant, was relatively minor compared to other heterochronic mutants such as *Teopod* mutants, and the GA treatment of the WT had only a small effect on the timing of the process. Therefore, it can be concluded that the vegetative phase change may depend on several factors, including GA (Evans and Poethig, 1995). Similar conclusions were also obtained from studies in ivy (Wareing and Frydman, 1976) and in the heteroblastic plant, *Pseudopanax crassifolius* (Horell *et al.,* 1990).

No consistent difference was found between the levels of IAA in the shoot apices of the WT and the *apc* plants, suggesting that a large difference in IAA is not involved in vegetative phase change in pea. This is supported by an observation that the application of IAA to ivy did not induce morphological change from juvenile to adult form (Robbins, 1960; Hackett, 1975).

The level of ABA detected in the shoot apex of the *apc* mutant was, however, less than in the WT in all three replicates. This indicates that there is a decrease in endogenous ABA levels during the vegetative phase change in the pea from the 4- to the 6-leaflet form. Changes in ABA levels during plant ontogeny have also been demonstrated in woody plants, for example, in ivy, the ABA level was higher in the adult shoot than in the juvenile (Frydman and Wareing 1973b; Rogler and Hackett 1975b). These studies suggest that phase change may be promoted by an increase in the ABA level. In another study, the main factor in phase change was suggested to be an increase in size and complexity of the plant, rather than an increase in ABA levels during plant development, which were a consequence of an increase in plant stress (competition for water) caused by the increase in size and complexity (Walton 1988).

The mutation *vp8* in maize causes a delay in vegetative phase change, with a higher node of transition from juvenile to adult traits in *vp8* than in the *WT* (Evans and Poethig 1997). A previous study found that the *vp8* mutant had a significantly lower level of ABA than the WT (Neil *et al.*, 1986). The reduction in ABA level in the mutant was investigated only in the seed at a single point in time (Evans and Poethig, 1997), so the levels operating in the shoot apex at phase change are unknown.

Therefore, although, a reduction in ABA level has been found in the pea mutant *apc*, there is no conclusive evidence that it is the controlling factor in the acceleration of vegetative phase change. The effect of the ABA in the vegetative phase change is examined in more detail in **Chapter 8**.

Table 5.1 Harvest details and internal standards added for the quantification of the endogenous GA_1, GA_{20}, IAA and ABA levels in the shoot apex of the *WT* and the *apc* plants.

Genotype	Age (days)	FW of shoot apex (g)	Internal standard added (ng)			
			$^2H_2GA_1$	$^2H_2$ GA_{20}	$^2H_3$ IAA	$^2H_3$ ABA
Replication 1						
WT	11	0.33	4	4	10	3
apc	11	0.42	4	4	10	3
Replication 2						
WT	11	0.61	4	5	25	5
apc	11	0.72	4	5	25	5
Replication 3						
WT	11	0.44	4	5	25	5
apc	11	0.41	4	5	25	5

Table 5.2 The endogenous levels of GA_1, GA_{20}, IAA and ABA in the shoot apex of the *WT* and the *apc*.

Genotype	Endogenous levels (ng.g FW^{-1})			
	GA_1	GA_{20}	IAA	ABA
Replication 1				
WT	6.2	5.5	36.6	10.7
apc	4.7	10.2	68.3	3.9
Replication 2				
WT	10.7	12.3	66.3	10.3
apc	11.4	12.9	45.8	6.6
Replication 3				
WT	6.8	19.6	30.2	8.5
apc	11.4	33.4	41.5	7.5

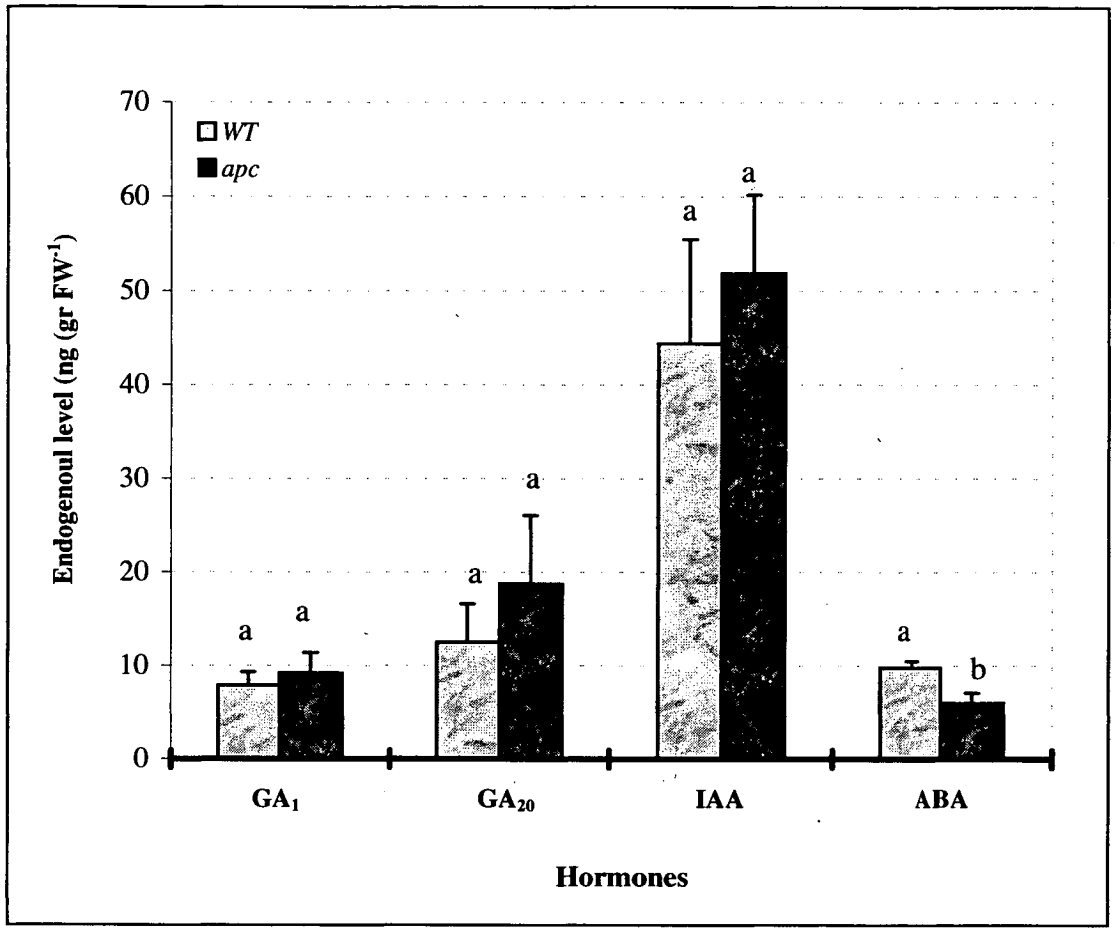

Figure 5.2 Comparison of the endogenous levels of GA_1, GA_{20}, IAA, and ABA in the shoot apex of the *WT* and the *apc*. The bar lines represent the mean value and standard error. The different letter indicated a significant different in the level of the hormone at $p < 0.05$ using *t*-test. N= 3 replications. The data were derived from **Table 5.2**

CHAPTER 6

Revealing the site of action of the *apc* mutation

6.1 Introduction

Grafting experiments have proved useful tools for determining the site of action of developmental mutations in a number of species. For example in the control of branching, Tucker (1979) showed that the site of action of *lateral suppressor* (*ls)* in tomato was in the shoot system because the mutant was unable to branch, despite being grafted to the *cv.* Craigella plant (with weak apical dominance). In the pea, by contrast, the site of action of the *rms-2* mutation appears to be in the root and shoot because the branching pattern was controlled by the *rms-2* via a graft-transmissible substance produced in the root system and supplied to the shoot system (Beveridge *et al.*, 1994).

Grafting studies have widely been performed to determine the site of action of genes controlling other aspects of development, particularly flowering. In the pea, grafting studies indicated that the site of action of the *Sn* gene in the control of flowering is by the production of a flower inhibitor in both shoot and cotyledons (Murfet, 1971). A grafting study in *Arabidopsis* found that the *acaulis1* (*acl1-1*) mutation, which caused cessation of development of inflorescence meristems, does not affect diffusible substances because the grafted *acl1-1* inflorescence was not affected by grafting onto the WT plant, nor was the WT inflorescence affected by grafting on the *acl1-1* (Tsukaya *et al.*, 1993). Satoh (1996) also found that the root was the site of production of a graft-transmissible substance that promoted vegetative to reproductive transition in *Cucurbita.*

Grafting experiments have demonstrated that the site of production of a substance(s) controlling vegetative phase change in the woody species, English ivy, appears to be in the root system (Fryman and Wareing, 1973 b). In many model systems, however,

the control of vegetative phase change is difficult to disengage from the reproductive phase change. The *apc* mutation is particularly useful in this context for it has been shown to accelerate vegetative phase change in pea without dramatically affecting reproductive phase change (Chapter 7). When grown under short day (SD) conditions *apc* plants attain the six-leaflet form of leaf (6-C) approximately seven nodes earlier than in the *WT*.

In this study, we use near isogenic lines of the *apc* mutant and *WT* (L107 *Torsdag*) in a combination of grafts (epicotyl to epicotyl and 4th node to 4th node) to reveal the site of action of the *apc* mutation on vegetative phase change in pea.

6.2 Materials and Methods

6.2.1 Plant materials

Plant materials used in this study were the L107 *cv* Torsdag as *WT* plants and the isogenic lines derived from Af3 as mutant *apc*. Both line have the same genotype background (*E, Le, Af, Sn, Ppd, hr, Fa, Na*) except that the *Apc* gene controls the rate of vegetative phase change. L107 line carries the dominant gene (*Apc*), while the Af3 carries the *apc* recessive gene. Gene *apc* accelerates vegetative phase change in pea (see **Chapters 2** and **7** for further details).

6.2.2 Growing condition

Plants used as stocks were grown one per pot in 14 slim-line pots, while plants to be used as scions were planted in tote boxes at a density of 50 plants per tote box. Plants were watered daily, and nutrient solution (aquasol) was supplied once weekly after active growth had resumed. The plants were grown in the glasshouse under LD (18 hr) and SD (10 hr) conditions. Source illumination provided is described in **Chapter 2**.

6.2.3 Experimental design

The grafts used to determine the site of action of the *apc* mutation, included grafts between genotypes (test-graft) and grafts within genotypes (self-grafts) as controls. In addition, intact plants of both *apc* and *WT* were grown under SD to determine the effect of grafting on plant growth. The rates of vegetative and reproductive transitions in the various combinations were compared.

Two types of grafts were used (**Figure 6.1**). The first graft type was made between the first internode (epicotyl) above the cotyledons on both stock and scion (**graft A**). The second type was made between both stock and scion at internode 4 (four) described as **graft B**.

The plants used as stocks and scions were of a similar age for each graft type. In **graft A** they were performed 4-5 days after sowing, and **graft B** 8-9 days after sowing. The critical vegetative phase change at the leaf primordia level takes place 11 days after sowing (see **Chapter 4**).

The cut end of each scion was made into a wedge shape and inserted into a slit made in the stock plants down the center of the epicotyl to approximately 12 mm. A small plastic ring was slipped over the epicotyl of the stock to hold the scion in place.

The grafted seedlings were watered and humidity was maintained by placing a plastic bag over the seedlings and pot rim, held in place with a large rubber band. The seedlings were initially watered every second day. The bags were turned daily to allow appropriate gas exchange. After about 4 days, when the xylem connection between the scion and the stock appeared to have developed, the intact bags were replaced with bags with cut corners. At the first appearance of new growth in the scions (about one week), the bags were removed completely.

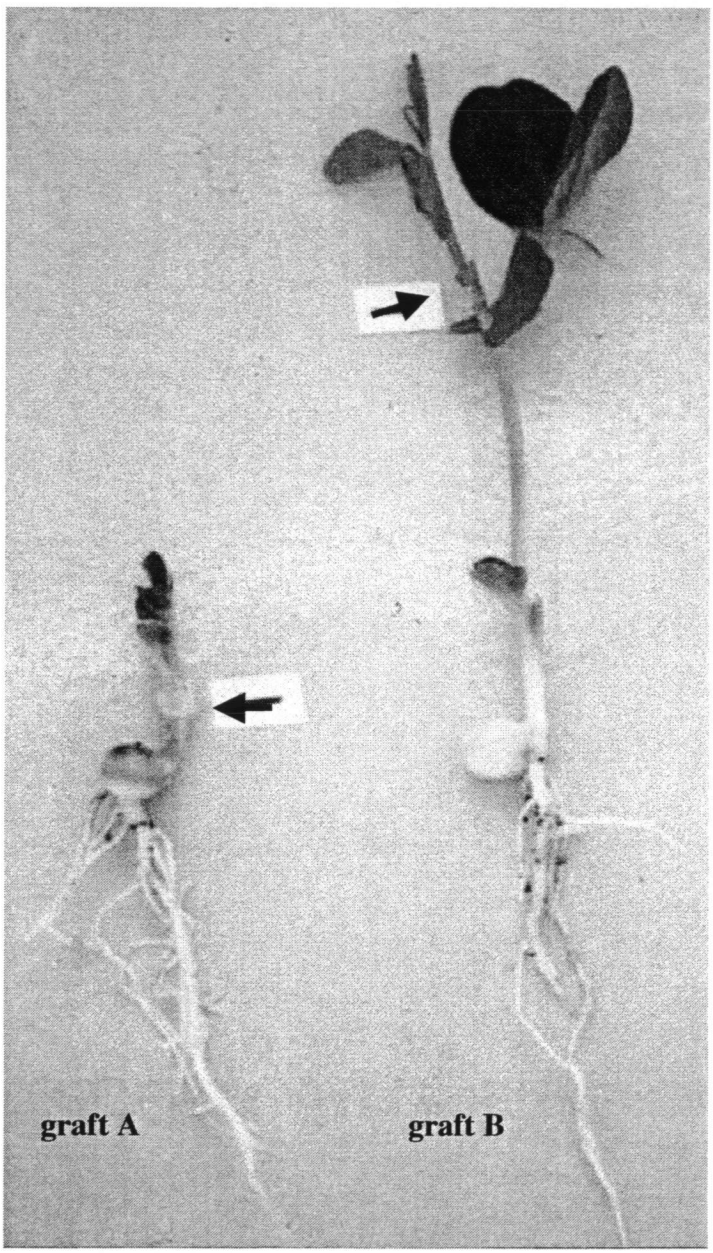

Figure 6.1 Types of grafts: Graft made between the first node internode above the cotyledon on both stock and scion (**graft A**) and between the sion and stock at internode four (**graft B**)

6.2.3 Characters scored

Characters scored included the node of vegetative transition to the first leaf with three-leaflets (3-C), four-leaflets (4-C), five-leaflets (5-C), six-leaflets (6-C), seven-leaflets (7-C) and eight-leaflets (8-C), counting from the cotyledons as node zero. The node of flower initiation (FI) was the first node on the main shoot to bear an initial flower, regardless of whether or not the bud actually developed into an open flower. Slow and weak grafts were excluded.

Data from grafts grown under LD and SD conditions were analyzed separately. A comparison was performed between self-graft and test-graft which possessed a scion of similar genotype. In order to determine the effect of the grafting experiment on the plant, growth comparisons were made between the intact plant and the self-graft. The means of treatment were considered to be significantly different at $P \leq 0.05$ and were separated by the Scheffe F-test. Analyses were performed using StatView 5.0.

6.3 Results

6.3.1 Comparison between self-graft and test-graft in graft A

The apc/apc vs the apc/WT under long day and short day conditions

WT stocks did not significantly affect ($P > 0.05$) the initiation of vegetative phase change (3-C, 4-C and 6-C) in the *apc* scions in the epicotyl to epicotyl grafts (**graft A**) under either LD or SD conditions (**Table 6.1** and **6.3**). Under LDs, the vegetative phase change to six-leaflets (6-C) occurred at a mean of 16.8 (±0.28) nodes in the *apc* scion of the *apc/apc* self-graft and 15.7 (±0.37) in the *apc* scion of the *apc/WT* test-graft.

The *apc* scions grafted onto the *WT* and *apc* stocks displayed similar maximum leaf complexity. All scions in both *apc/WT* test-grafts and *apc/apc* self-grafts reached a leaf complexity with six leaflets (6-C) in LD (**Table 6.1**) and eight leaflets (8-C) in SD (**Table 6.3**).

No significant difference ($P > 0.05$) was found in reproductive phase change (FI) between the *apc* scions of the test-graft and the self-graft of **graft A** under either LD or SD conditions.

The WT/WT vs the WT/apc under long day and short day conditions

The *apc* stocks were unable to induce *WT* scions to the six-leaflet form (6-C) under LD conditions. The *apc* stocks were also unable to accelerate the vegetative phase in the *WT* scions It was evident that there was no significant difference ($P > 0.05$) in response in terms of 4-C between the *WT* scion of the *WT/apc* test-graft and the *WT* stock of *WT/WT* self-graft. The means (±SE) of the first node of 4-C in the test-graft and the self-graft were 13.4 (±0.29) and 13.3 (±0.29), respectively. Under SD conditions, there was no significant difference ($p > 0.05$) in the 6-C of the *WT* scion grafted to the different stocks (**Table 6.4**).

The maximum leaf complexity in the *WT* scions of the *WT/apc* test-graft was similar to the leaf complexity in the *WT* of *WT/WT* self-graft: 4-C under LD and 6-C under SD.

The *WT* scion was not affected significantly by the *apc* stocks in respect of FI. The means (±se) of the first node of FI were 15 8 (±0.22) in the *WT* scion of the *WT/apc* test-graft, and 15.9 (±0.20) in the *WT* scion of the *WT/WT* self-graft under LD, and. 20.1 (±0.27) in the *WT* scion of the test-graft, and 20.2 (±0.30) in the *WT* scion of the self-graft under LD. As found in vegetative transition, FI was also not significantly different ($P > 0.05$) in the *WT* scion of the test-graft and in the *WT* scion in the self-graft (23.4 (±0.27) in the test-graft and 23.6 (±0.29) in the self-graft).

6.3.2 Graft A vs graft B

The apc/apc vs the apc/WT under long day and short day conditions

As described above, the *WT* stock without true leaf (**graft A**) cannot inhibit the rate of vegetative phase change in the *apc* scion. In **graft B,** the *WT* stocks complete with true leaf were also unable to delay the rate of the vegetative phase change in the *apc* scions under either LD (**Table 6.1**) or SD (**Table 6.3**). The means of 6-C in the *apc* scions of the *apc/apc* self-graft (14.4±0.22) in **graft B** were not significantly different ($P > 0.05$) to that of the *apc/WT* test-graft (14.9±0.25).

The maximum leaf complexities were also unaffected by **graft B**, for, in both test-grafts and self-grafts, the *apc* scion reached six-leaflets under LD and eight-leaflets under SD.

The WT/WT vs the WT/apc under long day and short day conditions

The *apc* stock with a true leaf did not affect significantly the *WT* scion regarding the rate of the vegetative phase. A similar response was shown in **graft A** in which there is no significant difference in the rate of phase change in both the *WT* scion of the self graft and of test graft under LD and SD (**Table 6.2 and 6.4**).

In **graft B** (under SD) the means (±se) of the vegetative phase change (6-C) of the *WT* scions grafted to the *apc* stock and the *WT* stock were 19.93 (0.22) and 20.75 (0.22) respectively. The *apc* stock did not affect the maximum leaf complexity of the WT scions; either, each scion of both self-grafts and test-grafts reached six-leaflets.

As shown in **Figure 6.2,** the vegetative phase change in the graft plant was slower than in the intact plant. This may be because the stocks of **graft B** have produced a true leaf, which provided more energy for recovery, whereas in **graft A** an epicotyl used as stock was unable to photosynthesize, so the recovery occurred more slowly.

6.4 Discussion

The present study indicates that the vegetative phase change (or leaf heteroblasty) in the garden pea does not involve a graft-transmissible substance(s) from root system to shoot system since the *apc* stock did not accelerate the vegetative transition in the *WT* scions and the *WT* stocks were unable to inhibit the vegetative transition in the *apc* scions. Furthermore, that the marked difference in maximum leaf complexity of the *apc* and *WT* scions was unaffected by the opposite stock. Therefore this study demonstrates that site of action of the *apc* mutation is confined to the shoot system.

The present study also indicates that the vegetative phase change or the leaf heteroblasty seems to be controlled by the shoot system itself. This is consistent with a number of previous studies. Robbin and Harvey (1970) assumed that the heteroblastic characteristics shown along the shoot from one phase to another are regulated by physiological control derived from the shoot system.

As described in the result section, the *apc* and *WT* stocks with a true leaf did not significantly affect the rate of vegetative phase change of the opposite scions. It indicates that there is no graft-transmissible substance from the leaf to the shoot apical meristem capable of inducing vegetative phase changes. A previous study in *Zea* (Irish and Jugle, 1997) indicated that the leaf primordia may be the site of production of a transmissible substance required to promote vegetative phase change since the rate of vegetative phase change depended on the number of primordial leaves in the shoot system. Therefore, there are two possible sites of action of the *apc* mutation, namely, shoot apical meristem itself and/or leaf primordia. To verify this a further study is required using Y grafts in which two scions with different genotypes are grafted to a stock with same genotype, to determine if the growing point of *apc* can accelerate the vegetative phase change in connected *WT* plants.

Table 6.1 The response of vegetative and reproductive transition in the test-graft (*apc/WT*) and the self-graft (*apc/apc*) grown under LD (18 hr).

Graft [1]	Characters	Graft (scion/stock)				*F* - value	*p*
		apc/WT [2]	n [3]	*apc/apc* [2]	n		
A	3-C	9.67 ± 0.33	3	9.33 ± 0.33	3	0.17	ns
	4-C	10.44 ± 0.24	9	10.11 ± 0.26	9	0.29	ns
	5-C	16.00 ± 0.37	6	15.33± 0.33	3	0.54	ns
	6-C	16.78 ± 0.28	9	15.67 ± 0.37	9	2.39	ns
	7-C	-	-	-	-	-	-
	8-C	-	-	-	-	-	-
	FI	15.33 ± 0.17	9	15.11 ± 0.20	9	0.76	ns
B	3-C	9.33 ± 0.33	3	10	1	0.33	ns
	4-C	10.45 ± 0.25	11	9.75 ± 0.25	8	1.31	ns
	5-C	18.25 ± 0.25	4	15.83± 0.31	6	8.49	0.05
	6-C	18.55 ± 0.49	11	17.12 ± 0.40	8	2.11	ns
	7-C	-	-	-	-	-	-
	8-C	-	-	-	-	-	-
	FI	14.73 ± 0.24	11	14.25 ± 0.16	8	0.92	ns

1 Graft performed between epicotyl and epicotyl (A) and between scion cut below node 4 and stock cut above node 4 (B)
2 Values represent mean and standard error
3 Number of plants displaying the characteristic scored
ns: no significance at p = 0.05

Table 6.2 The response of vegetative and reproductive transition in the test-graft (*WT/apc*) and the self-graft (*WT/WT*) grown under LD (18 hr).

Graft[1]	Characters	Graft (scion/stock)				*F* - Value	*p*
		WT/apc[2]	n[3]	*WT/WT*[2]	n		
A	3-C	12.67 ± 0.67	3	12.25 ± 0.25	4	0.81	ns
	4-C	13.44 ± 0.29	9	13.33 ± 0.29	9	0.10	ns
	5-C	-	-	-	-	-	-
	6-C	-	-	-	-	-	-
	7-C	-	-	-	-	-	-
	8-C	-	-	-	-	-	-
	FI	15.78 ± 0.22	9	15.89 ± 0.20	9	0.05	ns
B	3-C	12.00 ± 0.58	3	12.00 ± 0.63	5	0	ns
	4-C	12.57 ± 0.53	7	13.68 ± 0.60	9	0.95	ns
	5-C	-	-	-	-	-	-
	6-C	-	-	-	-	-	-
	7-C	-	-	-	-	-	-
	8-C	-	-	-	-	-	-
	FI	14.43 0.20	7	15.00 0.17	9	1.26	ns

*1 Graft performed between epicotyl and epicotyl (**A**) and between scion cut below node 4 and stock cut above node 4 (**B**)*
2 Values represent mean and standard error
3 Number of plants displaying the characteristic scored
ns: no significance at p = 0.05

Table 6.3 The response of vegetative and reproductive transitions in the test-graft (*apc/WT*) and the self-graft (*apc/apc*) grown under SD (10 hr).

Graft[1]	Characters	Graft (scion/stock)				*F- value*	*p*
		apc/WT[2]	n[3]	*apc/apc*[2]	n		
A	3-C	10.67 ± 0.33	3	10.50 ± 0.57	8	0.01	ns
	4-C	11.60 ± 0.21	14	11.21 ± 0.30	14	0.40	ns
	5-C	16.33 ± 0.88	3	16.00 ± 0.32	5	0.09	ns
	6-C	17.14 ± 0.25	14	17.00 ±.0.18	14	0.05	ns
	7-C	21.20 ± 0.20	5	21.14 ± 0.14	7	0.01	ns
	8-C	22.13 ± 0.17	14	22.14 ± 0.36	14	0.00	ns
	FI	21.80 ± 0.14	14	21.93 ± 0.16	14	0.03	ns
B	3-C	9.50 ± 0.50	4	9.00	4	0.10	ns
	4-C	9.60 ± 0.13	14	10.07 ± 0.25	15	0.60	ns
	5-C	13.85 ± 0.34	7	12.50 ± 0.22	6	2.59	ns
	6-C	14.86 ± 0.25	14	14.40 ± 0.21	15	0.57	ns
	7-C	19.86 ± 0.34	7	19.25 ± 0.25	3	0.57	ns
	8-C	20.79 ± 0.26	14	19.93 ± 0.30	15	1.65	ns
	FI	20.64 ± 0.32	14	19.47 ± 0.32	15	2.72	0.05

1 Graft performed between epicotyl and epicotyl (A) and between scion cut below node 4 and stock cut above node 4 (B)
2 Values represent mean and standard error
3 Number of plants displaying the characteristic scored
ns: no significance at p > 0.05

Table 6.4 The response of vegetative and reproductive transitions in the test-graft (*WT/apc*) and the self-graft (*WT/WT*) grown under SD (10 hr).

Graft[1]	Characters	Graft (scion/stock)				*F* - Value	*p*
		WT/apc[2]	n[3]	*WT/WT*[2]	n		
A	3-C	-	-	-	-	-	-
	4-C	16.64 ± 0.34	14	15.60 ± 0.35	15	1.80	0.05
	5-C	-	-	-	-	-	-
	6-C	23.07± 0.16	14	23.73 ± 0.23	15	1.30	ns
	7-C	-	-	-	-	-	-
	8-C	-	-	-	-	-	-
	FI	23.36 ± 0.27	14	23.60 ± 0.29	15		ns
B	3-C	-	-	-	-	-	-
	4-C	13.36 ± 0.20	14	14.17 ± 0.27	12	0.97	0.05
	5-C	-	-	-	-	-	-
	6-C	19.93 ± 0.22	14	20.75 ± 0.22	12	1.30	ns
	7-C	-	-	-	-	-	-
	8-C	-	-	-	-	-	-
	FI	20.14 ± 0.27	14	20.17 ± 0.30	12	0.09	ns

1 Graft performed between epicotyl and epicotyl (A) and between scion cut below node 4 and stock cut above node 4 (B)
2 Values represent mean and standard error
3 Number of plants displaying the characteristic scored
ns: no significance at p = 0.05

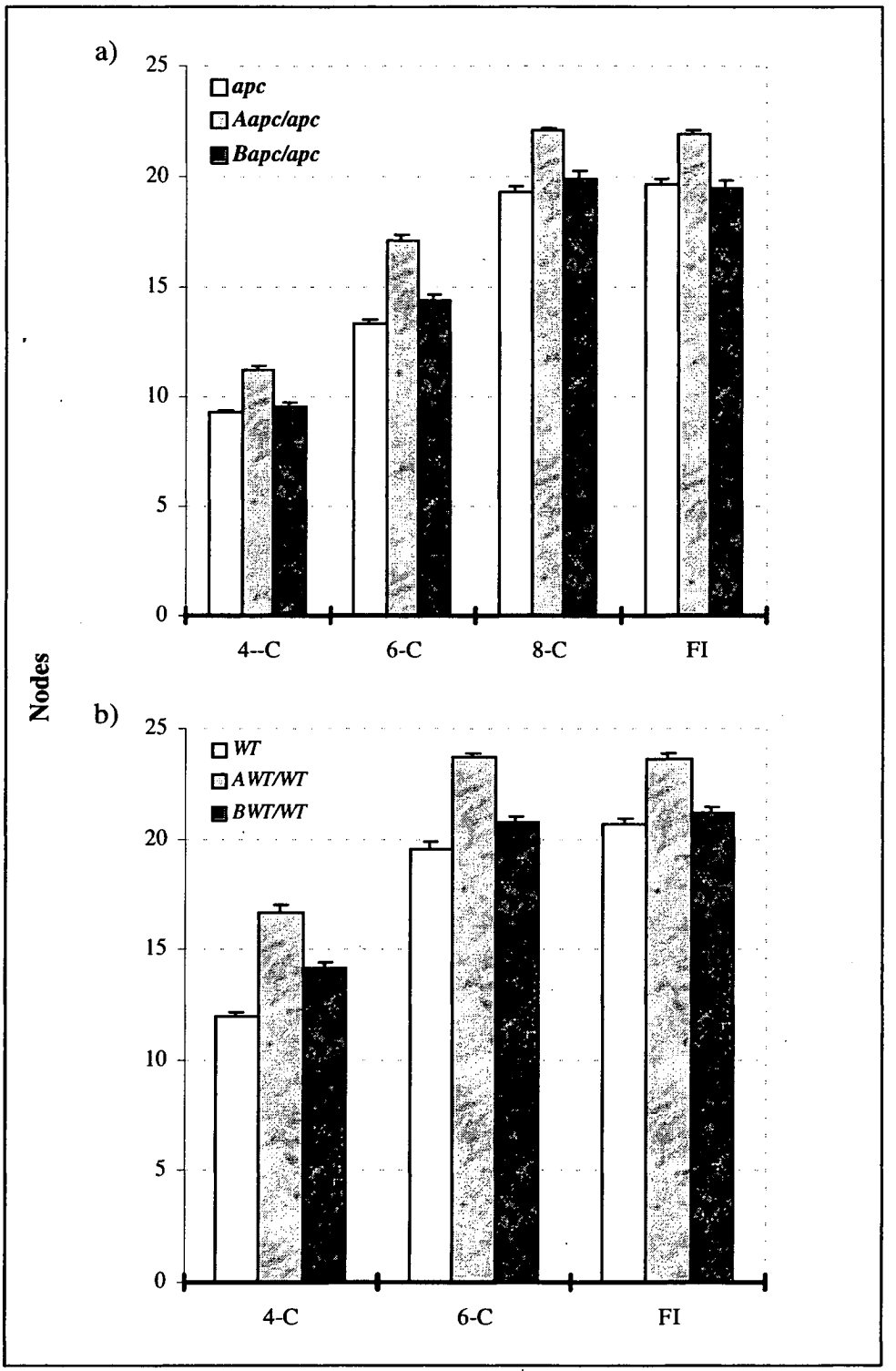

Figure 6.2 Grafting effect on the graft growth in SD: a) *WT* scion, b) the *apc* scion. The bar lines represent the mean and standard error values.

CHAPTER 7

Effect of photoperiod on vegetative phase change in pea

7.1 Introduction

Many studies have reported the effect of photoperiod on developmental events in plants, e.g. the germination of seeds (Vince-Prue, 1975), root and tuber formation (Bhella and Robberts, 1974; Machackova *et al.*, 1998), and senescence (Proesbting *et al.*, 1978). The control of photoperiod on the transition from vegetative phase to reproductive phase has also been demonstrated in many species, including: *Arabidopsis* (e.g. Telfer *et al.*, 1997; Xu *et al.*, 1997); *Pisum sativum* (e.g. Weller *et al.*, 1997); *Hardenbergia violacea* (King, 1998), and *Sorghum bicolor* (Lee *et al.*, 1998).

Photoperiod has also been shown to affect the rate of vegetative phase change, or leaf heteroblastic development, in a number of species. A study on *Ulex europaeus* conducted by Millener (1961) found that the longer photoperiod induced the development of the adult phase, and that this was not a function of the total amount of light available for photosynthesis. In *Ipomoea caerulea,* there is pronounced differences in heteroblastic development between plants grown under 8 hr and 16 hr photoperiods (Ashby, 1950). However, Njoku (1956) pointed out that such differences (e.g. leaf shape) were not caused by a direct effect of photoperiod, but by the onset of the flowering process.

The onset of vegetative phase change in *Arabidopsis,* recognizable by the formation of trichomes on the abaxial surface of the leaf, occurred earlier in plants grown under LD than under SD (Chien and Sussex, 1996). However, this study also suggested that the vegetative and reproductive phase changes were associated. The independent examination of the control of vegetative and reproductive phase changes is often difficult, with vegetative phase change often being the precursor to, or perhaps the

product of, the transition to flowering. Although there is often an association between the phase changes in the pea, the two processes can be separated (Wiltshire *et al.*, 1994) so that the photoperiodic control of vegetative and reproductive phase changes can be examined independently.

The garden pea's utility as a model system derives from the fact that the difference between juvenile and adult vegetative phase is clearly displayed in the leaf morphology (Smith and Hake, 1992, Wiltshire *et al.*, 1994; Van Lijsbettens and Clarke, 1998). The normal development of the garden pea is described in **Chapter 1**, but, in summary, the leaves at the first two nodes above the cotyledons are reduced to cataphylls and the 3rd node the first true leaf (2-C) with two leaflets. At higher nodes, the leaf develops additional pairs of leaflets (3-C, 4-C, 5-C, 6-C, and occasionally 7-C and 8-C), so that the complexity of the organ increases during heteroblastic development (Marx, 1987; Wiltshire *et al.*, 1994).

In this study, a mutant *a*ccelerated *p*hase *c*hange (*apc*), showing an acceleration in the initiation of the vegetative phase change compared to the wild type plants is used to investigate the effect of photoperiod on the vegetative phase change, and the association between the vegetative and reproductive phase changes.

7.2 Materials and Methods

7.2.1 Plant material

The plant materials used in this study were the L107 *cv* Torsdag, and the isogenic line Af3. They posses similar genetic background (e.g. *Lf, E Sn, Dne, hr, Ppd, Af*), except in a gene controlling vegetative phase change. L07 carries the dominant allele *Apc*, whereas Af3 carries the recessive allele *apc*.

7.2.2 Growing conditions

Plants were grown in 14 cm slim-line pots filled with a 1:1 mixture of vermiculite and dolerite chips topped with 3-4 cm of peat-sand potting mixture in a phytotron in which

daily temperatures were 20 - 23 ^{0}C in the day and 13-18 ^{0}C in at night. The plants were watered daily and nutrient solution (Aquasol) was provided once a week.

In order to determine photoperiodic effects on the vegetative and reproductive phase changes, two photoperiods were used: SD (8 h daylight) and LD 18 h (natural day extended by light from fluorescent tubes [Wotan 40 W cool white] and incandescent globes [Sylvania 100 W] providing 25 mmol $m^{-2}s^{-1}$ at pot top). The temperature of dark compartments was maintained at 16 ^{0}C.

7.2.3 Characters scored

Characters scored included several stages of vegetative and reproductive transitions. In the vegetative transition they included the first node at which a leaf bore 4-leaflets (4-C), 5-leaflets (5-C), 6-leaflets (6-C), 7-leaflets (7-C), or 8-leaflets (8-C). The character scored in the reproductive transition was flower initiation (FI) taken as the first node to bear a flower initial regardless of whether or not the initial developed. The nodes were counted starting from the first scale leaf as node 1.

The time of vegetative and reproductive transitions was also scored. The time was measured from day after sowing (day zero) until the 4-C, 5-C, 6-C, 7-C and 8-C leaf emerged from the enclosing stipules (for the vegetative transitions), or until the flower initially opened (for the reproductive transition, FT). All characters were scored from the primary shoots, not laterals.

7.3 Results

The *apc* mutant differed from the WT in both vegetative and reproductive phase changes under both photoperiods (**Table 7.1**), with significant genotype x photoperiod interactions (**Table 7.2**). The vegetative transitions to the 4-leaflet (4-C), 5-leaflet (5-C) and 6-leaflet (6-C) conditions were initiated more than one, three and four nodes earlier in *apc* than the *WT* under LD photoperiod (**Table 7.1**). Under short days, the differences in the initiation of 4-C, 5-C and 6-C between *apc* and the *WT* were magnified, with the transition to the six-leaflet form occurring seven nodes earlier in

the mutant line (**Table 7.1**). This acceleration also occurred in the chronological time of vegetative transitions under each photoperiod (**Table 7.1**). The 4-C, 5-C, 6-C transitions were accelerated by about 2, 8, 8 days (in LD) and 2, 16, 16 days (in SD) in *apc* by comparison with *WT* plants.

The main difference between the two genotypes, however, was in the final vegetative phase change to the eight-leaflet form (8-C). None of the WT plants progressed past six leaflets, whereas all 20 replicates of the *apc* mutants under each photoperiod attained the eight-leaflet leaf (**Table 7.1**).

The effect of *apc* on reproductive phase change was less pronounced than the effect on timing of vegetative phase change. Under LD photoperiod, there was no significant difference between the *apc* and the *WT* plants in the rate of reproductive transition, whether measured in nodes or days (FI and FT, **Table 7.1**). There was a small, but significant, difference between genotypes when grown under SD conditions, with flowers initiated (FI) two nodes earlier in *apc* than in the *WT* and flowering (FI) a corresponding 3 days earlier (**Table 7.1**)

The effect of photoperiod on vegetative and reproductive phase changes also differed between genotypes. The transition to four-leaflets was not affected by photoperiod in either *apc* or *WT* plants. The means (±se) of 4-C under LD vs. SD were 9.85 (0.11) vs. 9.65 (0.11) in *apc* plants, and 11.55 (0.11) vs. 11.59 (0.12) in the *WT*. There were no significant differences in the number of days to 4-C, either (**Tables 7.1, 7.2**). The transition to the six leaflet (6-C) form was delayed under SD in the WT by 3.5 nodes or 10 days, but by less than one node and only 2 days in *apc* (**Table 7.1, Figures 7.1 and 7.2**). The reproductive phase change also occurred later under SD conditions in both genotypes, but the difference was less marked (5.9 nodes, 16 days in WT, and 4.4 nodes, 13 days in *apc*).

7.4 Discussion

Vegetative phase change is controlled by photoperiod in garden pea lines derived from Torsdag (L107). In this study, WT plants grown under short day conditions (SD) showed a delay in their vegetative phase change to the six-leaflet form of 3.5 nodes (21%) compared with plants grown under long day conditions (LD). The *apc* mutant reached all vegetative developmental transitions (4-C, 5-C, 6-C) at an accelerated rate, compared with the WT. Furthermore, the *apc* plants under both photoperiods attained a greater degree of complexity (8-C) than the WT (6-C), although this transition to the most complex leaf form was also later under SD than LD (2.9 nodes, 18%). This is consistent with the finding on *Arabidopsis* in which the vegetative phase change was postponed in SD (Martinez-Zapater *et al.*, 1995), using the appearance of the trichomes on the abaxial leaf epidermis as a marker of juvenile-adult vegetative transition (Chien and Sussex 1996; Telfer *et al.*, 1997). Studies on *Solanum aviculare* also concluded that vegetative phase change was delayed by shorter photoperiod (James and Mantell, 1994).

Some early studies (e.g. Milliner, 1961) have suggested that the rate of heteroblastic development is affected by photoperiodic conditions via the production of carbohydrate. In theory, plants grown in LD can be assumed to receive more light than those in SD. This increases photosynthetic activity in the plants, with a consequent increase in carbohydrate that could supply more energy for the developmental processes in the plant. Investigations on the effect of carbohydrates on vegetative phase change in *Marsilea* (Allsopp 1954; 1955) found that increased carbohydrate (i.e. glucose) concentration induced an increase in the rate heteroblastic development.

In this process, photoperiod may act as the second factor by changing nutrient status as mentioned previously, or metabolic processes e.g. the production of endogenous hormone (s) as demonstrated in some studies on senescence process (Xu *et al.*, 1997). In this process, photoperiod may also produce certain endogenous substance(s) that could affect the rate of phase change. Yet the plant response to changes in nutrient status, or the levels of endogenous substance, is under tight genetic control. Therefore, photoperiod affects vegetative phase change through a modification of carbohydrate

level or metabolism and the change may form part of a signal of the vegetative phase change transition.

The results of this study indicate that vegetative phase change and reproductive phase change are separable but appear to be coordinated in garden pea, as suggested for *Arabidopsis* by Martinez-Zapater *et al.* (1994) and Telfer *et al.* (1997). In both the WT (L107) and the mutant (*apc*) plants, the final transition in vegetative phase change to the most complex leaf form occurs very close to the node of reproductive phase change. This is despite the dramatic difference in flowering produced by different photoperiods (six nodes difference in the WT and four nodes in *apc*) and the dramatic acceleration of the rate of vegetative phase change in the *apc* mutant.

Growing the WT L107 plants under SD photoperiod delayed flowering compared to the plants under LDs, and also delayed the change to the six-leaflet leaf, although by only 3.6 nodes, so that the plants attained their most complex leaf near flowering under both photoperiods. In the *apc* mutants, the transition to the six-leaflet leaf was markedly accelerated and occurred well before flowering. In *apc* the most complex leaf was the eight-leaflet leaf rather than the six-leaflet form, but it too was attained at the same node as the first flower under LDs and one node before flowering under SDs.

The independent regulation of vegetative and reproductive phases has been shown in previous studies (Lawson and Poethig, 1995; Evans and Poethig, 1997; Itoh *et al.*, 1998). *Tp* and *Vp8* mutants of *Zea* prolonged the juvenile phase but did not affect the initiation of reproductive phase change (Lawson and Poethig 1995; Evans and Poethig, 1997). Abedon *et al.* (1996) found that flower initiation was negatively correlated with the first leaf with adult wax but correlated with the last leaf with juvenile wax, suggesting independence of some aspects of vegetative phase change from reproductive phase change. In woody plants, too, such as *Eucalyptus risdonii,* it has been demonstrated that vegetative and reproductive phase changes were highly heritable and under independent genetic control between populations (Wiltshire *et al.*, 1998). Therefore the two developmental transitions in pea may be under separable genetic and physiological control, however, in this particular background (Torsdag, L107), reproductive phase change coincides with the vegetative phase change to the

most complex leaf form. This may be due to a shared control mechanism or due to inadvertent selection for maximal leaf area at the time of greatest photosynthetic load, the onset of reproduction.

Table 7.1 Means and standard errors of parameters measured from the *WT* and *apc* plants grown under 8 h (SD) and 18 h (LD) photoperiodic conditions.

Parameters	Photoperiod									
	SD					LD				
	WT		*apc*		*p*	*WT*		*apx*		*p*
	X + SE	n	X + SE	n		X + SE	n	X + SE	n	
Nodes										
4-C	11.59 ± 0.12	17	9.65 ±0.11	20	0.01	11.55 ±0.11	20	9.85 ±0.11	20	0.01
5-C	20.00 ±0.41	4	12.58 ±0.26	20	0.01	16.25 ±0.25	4	12.57 ±0.20	20	0.01
6-C	20.76 ±0.20	17	13.75 ±0.14	20	0.01	17.20 ±0.12	20	13.05 ±0.18	20	0.01
7-C	-		18.20 ± 0.36	10		-		15.00 ± 0.00	4	
8-C	-		19.40 ± 0.13	20		-		16.50 ± 0.11	20	
FI	22.70 ± 0.24	17	20.80 ± 0.22	20	0.01	16.80 ± 0.12	20	16.40 ± 0.17	20	ns
Time										
4-C	28.94 ± 0.31	17	26.95 ± 0.15	20	0.01	28.50 ± 0.32	20	26.55 ± 0.13	20	0.01
5-C	48.75 ± 0.85	17	32.33 ± 0.51	20	0.01	39.25 ± 0.48	20	31.17 ± 0.31	20	0.01
6-C	50.76 ± 0.50	17	34.75 ± 0.41	20	0.01	40.90 ± 0.18	20	32.55 ± 0.41	20	0.01
7-C	-		44.50 ± 0.79	10		-		40.00 ± 0.00	4	
8-C	-		48.60 ± 0.29	20		-		41.60 ± 0.23	20	
FT	60.06 ± 0.81	17	57.20 ± 0.59	20	0.01	43.90 ± 0.22	20	44.55 ± 0.13	20	ns

ns no significance at $p > 0.05$

Table 7.2 Effect of genotype, photoperiod, genotype x photoperiod interaction on vegetative and reproductive phase changes in the *WT* and *apc* plants

Parameters	Effect of Genotype (G)		Effect of Photoperiod (P)		Effect of Interaction between G and P	
	F-test	*p*	*F-test*	*p*	*F-test*	*p*
Nodes						
4-C	254.69	0.0001	0.50	ns	1.09	0.30
5-C	291.38	0.0001	33.50	0.0001	33.07	0.0001
6-C	1179.02	0.0001	172.03	0.0001	77.62	0.0001
7-C	-	-	30.27	0.0001	-	-
8-C	-	-	270.83	0.0001	-	-
FI	36.64	0.0001	731.9	0.0001	15.63	0.0002
Time						
4-C	66.35	ns	3.02	ns	0.01	0.93
5-C	359.33	0.0001	68.11	0.0001	41.57	0.0001
6-C	990.28	0.0001	242.81	0.0001	77.62	0.0001
7-C	-	-	12.74	0.004	-	-
8-C	-	-	347.39	0.0001	-	-
FT	4.83	0.03	822.15	0.0001	12.2	0.0008

ns no significance at $P > 0.05$

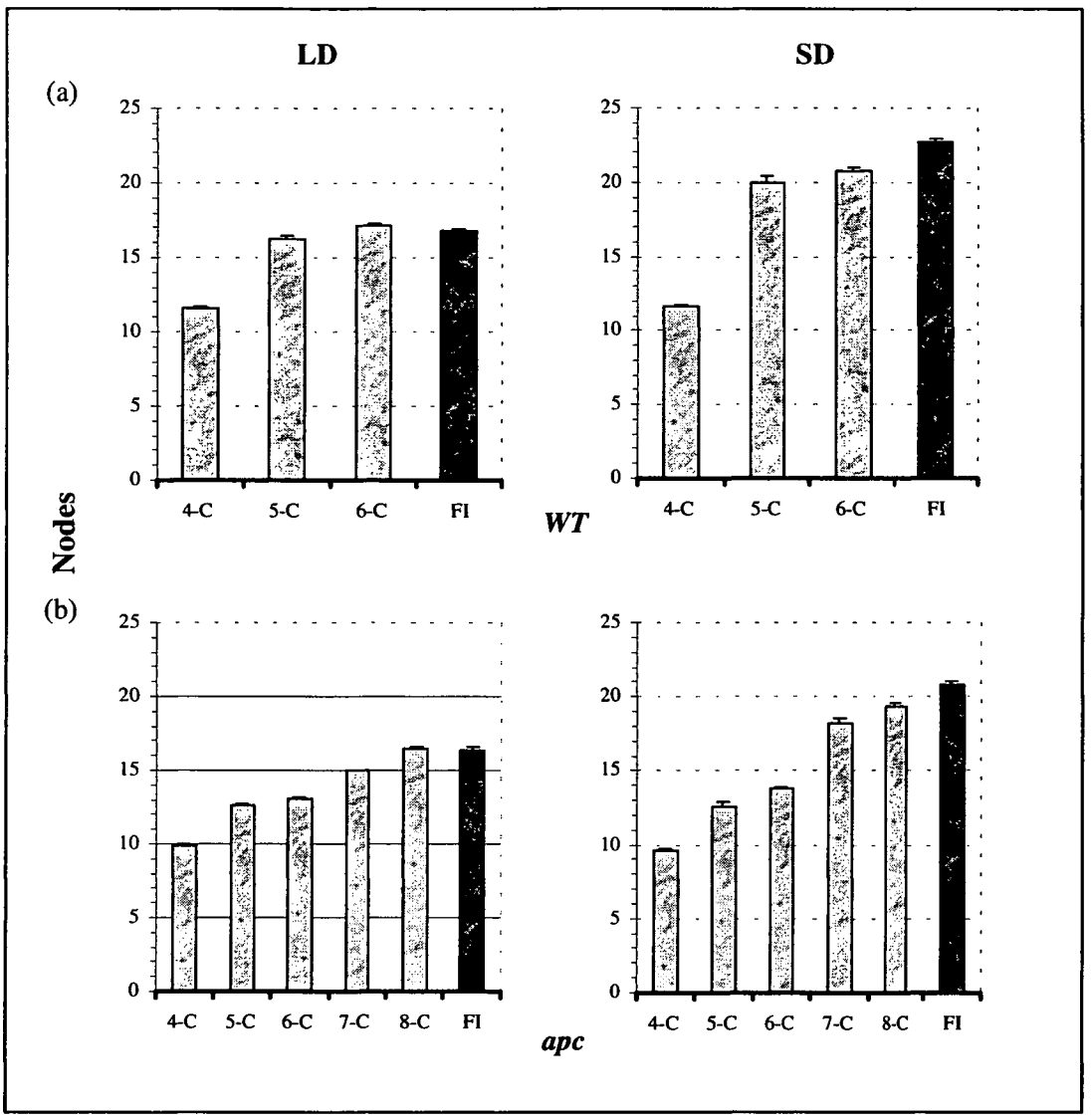

Figure 7.1 The relationship of the node in vegetative (from 4-C to 8-C) and reproductive phase change in *WT* (a) and the *apc* (b) plants grown under LD (18 h) and SD (8 h). Each column represents the mean and standard error of 4-20 plants.

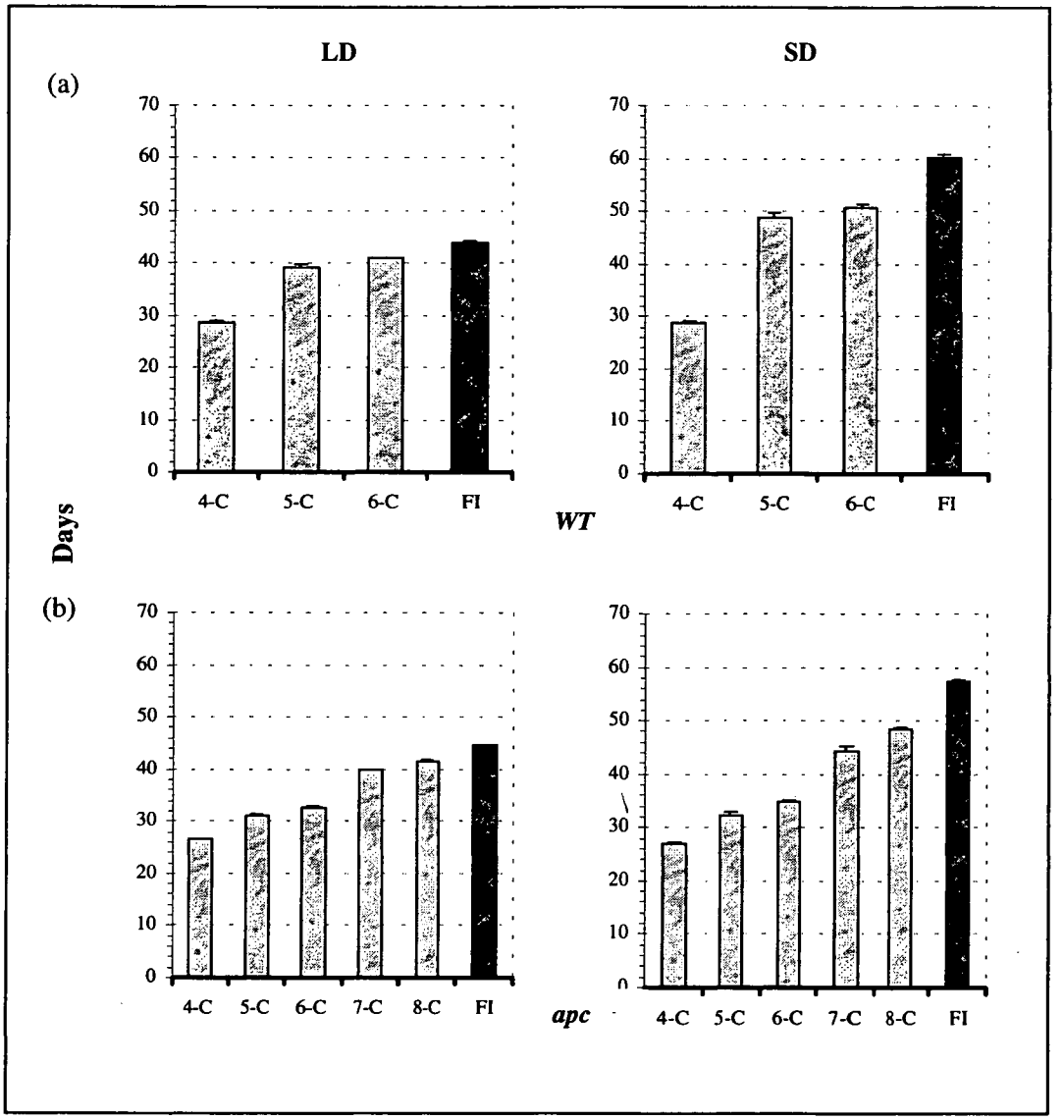

Figure 7.2 The relationship of the time between vegetative (from 4-C to 8-C) and reproductive phase change in *WT* (a) and the *apc* (b) plants grown under LD (18 h) and SD (8 h). Each column represents the mean and standard error of 4-20 plants.

CHAPTER 8

Consideration of the *apc* mutant as a new ABA-deficient mutant

8.1 Introduction

Phytohormone mutants are a powerful tool for investigating physiological and developmental processes in plant growth (Reid, 1993; Reid and Ross, 1993). Synthesis mutants affecting the production of endogenous hormones in plants have been particularly useful in identifying physiological roles for gibberellins, auxin, ethylene, cytokinin and abscisic acid (Reid, 1993) and for genetic dissection of the synthesis pathways. In ABA–deficient mutants, for instance, there are blocks in the steps leading to the synthesis of active ABA, e.g. oxidation of ABA aldehyde to ABA (Taylor *et al.*, 1988; Walker-Simmons *et al.*, 1989) and in carotenoid biosynthesis (Neil *et al.*, 1986) that lead to reduced endogenous ABA levels in the mutant compared with the *WT*. This reduction then causes characteristic physiological abnormalities in the mutant, such as a tendency to wilt through excessive transpiration as a function of increased stomatal conductance (Neill and Horgan 1985; Quarrie, 1987). ABA-deficient mutants have been isolated and characterized in various plants, including: *flacca* (*flc*), *sitiens* (*sit*) and *notabilis* (*not*) from tomato (Tal 1966; Tal and Nevo, 1973); *droopy* (*dr*) from potato (Simmonds 1965); *aba* from *Arabidopsis* (Koornneef *et al.*, 1982); and *viviparous* (*v*) from *Zea mays* (Moore and Smith,1985; Neil *et al.*, 1986).

A similar mutant isolated in pea, named *wilty* (*wil*) (Marx, 1976), also has characteristic physiological differences from the WT: lower percentage water content; lower water potential; and a lower diffusive resistance in the leaves (Donkin *et al.*, 1983), associated with reduced levels of ABA (Wang *et al.*, 1984).

ABA plays an important role in the control of a wide range of essential physiological processes, including: seed development; dormancy; and adaptation to environmental stress (Zeevaart and Creelman, 1988). A possible role of ABA in the vegetative phase

change has been suggested in number of species; however, the results are contradictory. Studies in a woody species (ivy) and a heterophyllic aquatic species suggested that changes in ABA levels seem to be crucial in inducing the juvenile-adult transition (Frydman and Wareing 1973a; Anderson, 1978; Goliber and Feldman, 1989), whereas study of a *viviparous8* mutant of maize proposed that vegetative phase change is not affected by change in ABA level (Evans and Poethig, 1997).

In **Chapter 5**, the shoot apex of *apc* mutant was shown to possess significantly lower levels of ABA than the *WT* plant, using GC-MS-SIM measurement. This study examines whether the *apc* mutant displays the characteristic physiological profile of an ABA-deficient genotype, and if the altered ABA levels in the shoot apical meristem of a known ABA mutant (*wil*) also confer accelerated vegetative phase change.

8.2 Materials and Methods

8.2.1 Plant materials

Plant materials used in this study were the pure line L107 (*Apc*) and Af3 (*apc;* F_7), and Line 5843 (*Wil*) and the F4 progeny of a Line 5843 x L233 cross (*wil*). The seeds of the *Wil* and the *wil* plants used in the present study were kindly provided by Shona Batge (University of Tasmania, Hobart). Genetic background of LI07 and Af3 lines are given in **Chapter 2.**

8.2.2 Growing conditions

Seeds were nicked and sown in a 14 cm slim pot containing the standard pot mix. The plants were grown under LD (18 hr) under glasshouse conditions. The natural daylight was extended using a mixture of 8 fluorescent tubes (L40W/20S cool white, Osram Germany) and 4 incandescent globes (100 W Pearl, Mazda Australia). The intensity of the supplementary lighting was 25 $\mu mol.m^{-2}.s^{-1}$ at the pot top. Plants were watered once or more a week, depending on the weather, to maintain the plants in a mildly droughted condition. Nutrient solution (Aquasol) was provided once a week.

8.2.3 Characters scored

Physiological characteristics (transpiration rate and stomatal conductance) were compared between the *apc* and *WT* plants and between the known ABA-deficient *wil* and the *Wil* plants to verify that the *apc* mutant is ABA-deficient. These characters show consistent differences in most ABA-deficient mutants when compared to the *WT* plants. The transpiration rate and stomatal conductance were measured using a diffusion photometer (The Analytical Development Co. Ltd., Hoddesdon England). Flow of air (CO_2) into the system was at a range of 399 – 400 ml/m^{-1}. The average of temperatures during measurement was 28.36 ± 0.07. Measurements were made on one of the basal leaflets of a mature leaf, the third leaf removed from the most recently fully-expanded leaf in each plant. Because of the complicated measurement of an intact leaflet using a chamber of the photometer, the data representing the physiological traits between the *Apc* and the *apc*, and the *Wil* and the *wil* were obtained from direct measurements.

The rate of vegetative phase change in the segregating F_4 progeny of the known ABA-deficient mutant was measured as the first node with four- (4-C) and six-leaflets (6-C), counting the cotyledons as node zero. The node of flower initiation was also measured.

8.3.4 Data analysis

Each leaf (or each plant) for the measurement of the physiological characters and the phase change characters was subjected to replication. Tests for significant differences in the means of the physiological traits and the phase change traits of the *Apc* and the *apc*, and the *Wil* and the *wil* were performed with Student's *t*-test in Excel (Microsoft Office 97).

8.3 Results

8.3.1 Physiological characterization

Transpiration rate and stomatal conductance were compared between the leaves (leaflets) of the *apc* mutant and WT *Apc* plants and between those of the known ABA-deficient *wil* and WT *Wil* plants (**Table 8.1**). The physiological traits in the *apc* and the *Apc* and the *wil* and the *Wil* plants showed similar results. The mean transpiration rate was higher in the *apc* (1.70 ml/cm^2/m^{-1}) leaflets than in the *Apc* (1.21 ml/cm^2/m^{-1})($P <$ 0.001) and higher in the *wil* than in the *Wil* plants (2.52 and 1.86 ml/cm^2/m^{-1}, respectively). The mean stomatal conductance was also significantly higher in the *apc* (0.05) than in *Apc* (0.03), and higher in the *wil* (0.09) than in *Wil* (0.05) ($P < 0.001$, **Table 8.1**).

8.3.2 Phase change traits

To establish whether a reduction in ABA level is associated with an earlier vegetative phase change, the mean first initiation of four leaflet (4-C) and six leaflet (6-C) leaves were compared between the *apc* and the *Apc* and between the *wil* and *Wil* (**Table 8.1**). There was a significant difference in rate of initiation of the vegetative phase change between *apc* and the *Apc*, but no significant ontogenetic differences were found between the *wil* and the *Wil* plants. The first leaf with 4 leaflets (4-C) was about two nodes earlier in the *apc* than the *Apc*, whereas that transition occurred at the same node in both the *wil* and the *Wil* plants. The six leaflet (6-C) condition was initiated nearly 5 nodes earlier in the *apc* than in *Apc* (12.6 vs. 17.4 nodes, *P<0.00001*). Only 25 % of the *Wil* progeny produced leaves with six leaflets, but there were none in the *wil* plants. The node of flowering initiation occurred one-half a node earlier in *apc* than in *Apc* ($P = 0.001$) but there was no significant difference in the first flower initiation between *wil* and the *Wil* plants (**Table 8.1**).

8.4 Discussion

8.4.1 Physiological characterization

When grown under controlled-drought conditions in the glasshouse, the *apc* mutant showed a greater transpiration rate than the WT *Apc*, a similar proportional increase to that of the known ABA-deficient mutant, *wil* over the WT *Wil,* under the same conditions. This result is in accord with studies on other mutants; for example in *not, sit* and *flacca* of tomato (Tal and Imber, 1970; Nevo and Tal, 1973), *aba* of *Arabidopsis* (Koornneef *et al.*, 1982), and *dr* of potato (Quarrie, 1982; 1987).

In the present study, the increase in the transpiration rate of the *apc* mutant is likely to be caused by the increased stomatal conductance observed in each mutant. Studies in three non-allelic, recessive, wilty mutants of tomato (*not, flc*, and *sit*) found that the mutants possessed higher rates of transpiration than normal plants because their stomata open wider and resist closure in the dark (Tal and Nevo, 1973). In the *dr* mutant of tomato, increase in stomatal conductance also led to excessive transpiration (Quarrie, 1987).

The increased transpiration rate induced by an increase in stomatal conductance is linked to the ability of plants to produce a signal required in stomatal control. The signal causing the stomata to close has been identified as ABA (Jones and Mansfield, 1970; Zeevaart and Creelman, 1988; Giraudat *et al.,* 1994). Evidence describing the link was obtained by several studies (e.g. Davies and Zhang, 1991), including the tight correlation between the degree of stomatal closure in the leaves and the ABA concentrations in xylem (Zhang and Davies,1990; Tardieu *et al.,* 1991). Studies in potato have also shown that excessive transpiration induced by increased stomatal conductance was found in a genotype with a low endogenous ABA content and insufficient capacity to synthesize this hormone (Quarrie, 1982, 1987) and that conductance can be reduced by applying ABA (Quarrie, 1982). The ABA levels in *not, flc* and *sit* mutants of tomato were much lower than in the normal plants (Neil and Horgan, 1985). These mutants cover a range of the phenotypic expression of water stress from relatively mild in *not*, in which endogenous ABA concentration were

between one-third and one-half those in the *WT*, to relatively severe in *sit*, where ABA levels were less than 15 % of the *WT* plants (Jones *et al.*, 1987). Application of ABA restored the wild phenotype (Bradford, 1983; Taylor, 1984; 1987).

The present study, therefore, provides additional evidence to support the suggestion (**Chapter 5**) that the *apc* mutant is new ABA-deficient mutant in pea plants. The *wil* and *apc* mutants are clearly not allelic forms of the same gene, for *wil* is positioned in linkage group III (Marx 1976) whereas *apc* is located in linkage group II of pea (**Chapter 3**).

8.4.2 Phase change

The present study confirms the significant difference in the initiation of the vegetative phase change in *Apc* and *apc* plants. The *apc* mutant is clearly heterochronic, with an earlier vegetative phase change to both the four and six leaflet form than in the *WT*. By comparison, the ABA-deficient *wil* mutant showed no significant difference in the rate of the vegetative phase change when compared to the WT *Wil* plant. The results indicate that the ABA level of the shoot apex is not the principal influence on vegetative phase change in pea.

This finding is supported by a previous study on maize (Evans and Poethig, 1997) that suggests that the postponement of vegetative phase change in the *vp8* is not caused by a reduction of ABA level (Evans and Poethig, 1997). However some studies have reported that ABA can induce changes in leaf morphology in various plants e.g. in a fern (Liu 1984), a monocot (Anderson 1982), and some dicots (Mohan Ram and Rao, 1982; Deschamp and Cooke, 1984; Young and Horton, 1985; Kane and Albert, 1987). Application of ABA to submerged shoot (or juvenile shoot) of *Callitriche heterophylla* induces the formation of the aerial-type leaf (adult leaf) (Deschamp and Cooke, 1984). Therefore, it can be suggested that change in ABA level during the growth process produces a range of different responses to vegetative phase change in different species but is not the controlling factor in the vegetative phase change in pea.

Table 8.1 Comparison of the means of physiological and phase change traits in the *apc* and the *Apc*, and in the *wil* and the *Wil* grown under drought conditions and LD (18 hr)

Parameters	*apc*		*Apc*		*p*
	X ± SE	n	*X ± SE*	n	
Transpiration rate (ml/cm^2/m^{-1})	1.70 ± 0.13	14	1.21 ±0.09	14	0.001
Stomatal Conductance	0.05 ±0.00	14	0.03 ± 0.00	14	0.0005
Node of 4-C Initiation	9.86 ± 0.10	14	12.07 ±0.13	14	<0.00001
Node of 5-C Initiation	11.20 ± 0.20	5	16.80 ± 0.20	5	<0.00001
Node of 6-C Initiation	12.57 ± 0.17	14	17.38 ±0.14	14	<0.00001
Flowering Initiation	16.50 ±0.14	14	17.14 ± 0.10	14	0.001
	wil		*Wil*		
Transpiration rate (ml/cm^2/m^{-1})	2.58 ± 0.16	25	1.86 ±0.12	15	0.003
Stomatal Conductance	0.09 ± 0.01	25	0.05 ±0.00	15	0.006
Node of 4-C Initiation	11.48 ± 0.09	25	11.60 ± 0.91	15	ns
Node of 6-C Initiation	-		15.71 ± 0.64	6	ns
Flowering Initiation	15.20 ± 0.13	25	14.80 ± 0.75	15	ns

Chapter 9

Conclusions

9.1 Vegetative phase change

The nature of vegetative phase change has been reported widely for different species by a number of investigators (e.g. Allsopp, 1967; Poethig, 1990; Evans and Poethig, 1997; Itoh *et al.*, 1998), however the regulation of this ontogenetic change is less adequately described (Poethig, 1990). One possible reason is that the model species used do not display a clear demarcation between different phases of growth. In the present study, the *apc* mutant of the pea garden (*Pisum sativum*) was used as a model of the vegetative phase change because this mutant showed a clear difference in the rate of vegetative phase change, when compared to the wild type plants free from the confounding effects of reproductive phase change or marked differences in size or other characteristics (**Chapter 1 and 2**). The vegetative phase change from the four- to the six-leaflet condition occurs at an earlier node in the mutant than the *WT*. The linkage analysis has revealed that the *apc* locus is positioned between the *aat-p* and *a* genes of Linkage group II (**Chapter 3**).

As shown in **Chapter 7** and **8**, the vegetative phase change in the *apc* and the *WT* took place at a predictable position along the shoot, even though both plants were grown under identical conditions. The vegetative phase change to the 6-leaflet condition occurred initially at node < 15 in the *apc* and at node ≥ 15 in the WT. The predictable position of the vegetative phase change has also been shown in maize for four observable traits (leaf width, the presence of epicuticular wax, epidermal hairs and the staining reaction with toluidine blue), with vegetative phase change occurring between nodes 6 and 8 in *tp2* plants, but much higher in *Tp2* plants (Dudley and Poethig, 1993). Another study in the *gl15* maize mutant, found that juvenile characteristics were replaced with adult characteristics in the leaf epidermis at node 2 or 3 (Avato, 1987; Coe *et al.*, 1988). The predictable nature of these positional changes suggests that the nature of the vegetative phase change is genetically controlled (Galinat, 1966; Poethig

1988a; Bertrand-Garcia and Freeling, 1991; Dudley and Poethig, 1993) as are shifts in timing of expression of phase-specific characteristics (Bachman, 1983; Hilu, 1983; Gottlieb, 1984).

Previous studies have indicated a correlation between vegetative phase change and a change in size of the shoot apical meristem (e.g. Allsopp, 1954; Crotty, 1955; McLellan, 1990; Clearwater and Gould, 1994), with an increase in size of the SAM associated with an increase in leaf complexity. This finding is consistent with the results from the present study in which the *apc* mutant showed acceleration in vegetative phase change and a significantly larger shoot apical meristem when compared to the *WT* (**Table 4.2**). An increase in size of the shoot apical meristem is a product of an increase in number of cells within shoot apical meristem through cell division (Hackett, 1980; Medford *et al.*, 1992; Laufs *et al.*, 1998), presumably as a function of cell division activity in the meristem (Itoh *et al.*, 1998). Therefore, the accelerated vegetative phase change of pea produced by the *apc* mutation may also be controlled genetically by a change in the rate of cell division within the shoot apical meristem.

Such a change in activity of the shoot apical meristem may be mediated by an alteration of hormonal status. Indeed, many studies have suggested that vegetative phase is associated with changes in endogenous levels of hormones (Allsopp, 1967; Hackett, 1985; Poethig, 1990; Evans *et al.*, 1994). Chien and Sussex (1996) have shown that gibberellins, in particular, are involved in vegetative phase change transition in *Arabidopsis*, especially in the regulation of trichome formation on the adaxial and abaxial leaf surfaces. The present study suggests that gibberellins (GA_1 and GA_{20}) and auxin (IAA) are not involved in the vegetative phase change in pea because quantification of GA_1, GA_{20} and IAA levels in the shoot apex revealed no consistent differences between the *WT* and the *apc* at the time of initiation of the crucial node (**Chapter 5**). This is an agreement with the study on the *sin1* (*short integument1*) mutant of *Arabidopsis* that suggested that a delay of vegetative phase change in the mutant was not caused by change in gibberellin metabolism (Ray *et al.*, 1996).

Although there appears to be a significant decrease in the ABA level in the *apc* mutant (**Chapter 5**) at the initiation of the phase change to the six-leaflet condition, compared to the WT pea of the same age and size, this is probably not a cause of acceleration of vegetative phase in the mutant (**Chapter 8**). A previous study in *vp8* mutant of *Zea* (Iris and Jegla, 1997) found that the decrease in ABA level in that mutant was not the crucial factor in vegetative phase change.

Rather than responding to a control, such as a plant growth hormone, from an external source, the grafting studies of **Chapter 6** indicate that the control of shoot apical meristem activity (or size), and the acceleration of phase change, is internally regulated. This study found that the site of action of *apc* mutation is neither in the root nor in expanded leaves, in contrast to studies that have suggested both organs as sources of transmissible signals for vegetative phase change (Rogler and Hackett, 1975a,b; Evan and Poethig, 1995). Therefore, if vegetative phase change in the pea is mediated by hormones, they are produced within the shoot apical meristem or leaf primordia, or in both tissues.

Although many studies have shown that environmental cues such as photoperiod also affect vegetative phase change (Rogler and Hacket, 1975a), it does not mean that photoperiod determines the process directly. The present study has demonstrated that the significant effect of photoperiod on vegetative phase change occurred in both *apc* and *WT* plants (**Chapter 7**). Hence, it is suggested that the vegetative phase change in pea seems to be controlled genetically via an increase in responsiveness, or unresponsiveness of SAM to photoperiod (Martines-Zapater *et al.*, 1994; Coupland, 1995).

9.2 The vegetative phase change *vs* the reproductive phase change

Experiments under different photoperiods clearly indicated that vegetative and reproductive phase change are separable phenomena, since the number of nodes from the vegetative phase change initiation to the reproductive transition of both the *apc* and *WT* was markedly different under LD and SD conditions (**Chapter 7**). This study is consistent with some studies on mutants of *Zea* that concluded that the timing of

reproductive phase change is not dependent on the transition to the adult vegetative phase (Bassiri *et al.*, 1992; Evans and Poethig, 1997). In *vp2*, for instance, the mutant produce significantly more leaves than *WT* plants but initiated a tassel (reproductive phase marker) in the same time as the *WT*. A study in *amp1* mutant of *Zea* also showed that the mutation prolongs the juvenile phase without affecting the timing of reproductive phase change (Telfer *et al.*, 1997). Other studies in both herbaceous and woody plants have suggested that the vegetative phase change is a prerequisite for reproductive phase change (Allsopp, 1967; Bruck and Kaplan, 1980; Hacket, 1985; Zimmerman *et al.*, 1985). Heterochronic mutants such as *apc*, that can shift the developmental timing of these ontogenetic events more or less independently, provide the tools to examine the interdependence of these processes.

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