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**Morphological, physiological and molecular
studies of *Pachycladon exilis*
(Brassicaceae)**

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requirements for the degree of

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Abstract

Pachycladon exilis is a highly endangered endemic species of New Zealand. Due to interest in preventing the extinction of this species and because it is related to the model plant, *Arabidopsis*, floral induction and floral development were investigated. The effects of environmental signals on growth and flowering were investigated by growing plants under factorial combinations of daylength and chilling in controlled environment rooms. The two daylengths were long days (16 h daylength) and short days (8 h daylength), to which plants were exposed following exposure of imbibed seeds for 0, 10, 20 or 30 d at 4°C. The influence of daylength and duration of chilling on the quantitative expression of the key meristem identity genes, *LEAFY* (*LFY*) and *TERMINAL FLOWER 1* (*TFL1*), was also studied using real-time reverse transcriptase-PCR.

Morphological studies on *P. exilis* showed that the plants produced rosette leaves alternately on short internodes and cauline leaves separated by longer internodes. Dissection of whole plants showed that the shoot comprised three levels of branching in a hierarchy, with the leaves of one level subtending the shoots of the next level. Further, it was observed that the plants produced adventitious shoots from roots.

Daylength and chilling treatments influenced development and floral induction. Although flowering started at the same time in plants exposed to long and short days, plants that were exposed to long days and that had passed through cold treatment as imbibed seeds flowered earlier after producing fewer leaves, and flowered more synchronously and to a higher percentage, than plants maintained under short days following chilling or plants with no cold treatment.

At the molecular level, partial sequences of *LFY* and *TFL1* were isolated in *P. exilis* and the expression levels of these genes were investigated using quantitative real-time PCR. It was observed that the up regulation of *PeLFY* coincided with the down regulation of *PeTFL1* at the time of the transition from vegetative to inflorescence development. As expected, *PeTFL1* expression was continued in the vegetative axillary meristems, which suggests that *PeTFL1* is responsible for, or at least involved in, perennality of *P. exilis*.

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Chapter 1. Introduction

1.1 Background

The genus *Pachycladon* belongs to the Brassicaceae and is the New Zealand genus most closely related to *Arabidopsis thaliana* (Mitchell & Heenan, 2000), the model plant used for studying various molecular, physiological and developmental processes in plants.

The genus *Pachycladon* has eight species endemic to the South Island of New Zealand, and an additional species in Tasmania (Heenan et al., 2002). The species *P. exilis* (Heenan) Heenan & A. D. Mitch. is an endangered species and is known from a single locality in North Otago (Luo et al., 2003). It is found in dry, rocky areas of the eastern South Island (Molloy et al., 1999), and is a short-lived perennial.

Previous work was conducted on the genetic variation within the *Pachycladon* complex (Mitchell & Heenan, 2002), and on aspects of embryology of the two threatened species, *P. exilis* and *P. cheesemanii* (Luo et al., 2003). However, neither of these studies was on the environmental signals involved in the flowering processes or flowering phenology.

The developmental turning point in flowering plants is the transition from the vegetative to the reproductive phase. The timing of this transition is crucial for the reproductive success of the flowering plant. The success of this transition may depend on the perception of environmental triggers, which include photoperiod and temperature, and/or developmental cues. The regulation of the floral transition and inflorescence and flower development has been well studied in the model plant *A. thaliana*. The floral structure of *P. exilis* appears similar to *A. thaliana*, so it was hypothesised that the sequence of development of floral structures might be similar in both plants. However, as *P. exilis* is a short-lived perennial and *A. thaliana* is an annual, differences in response to environmental signals and in inflorescence development might be expected.

In this study on *P. exilis*, the influence of photoperiod and duration of seed chilling on the induction of flowering and the quantitative expression of two key meristem identity genes, *LFY* and *TFL1*, were studied.

1.2 Phase change

A plant passes through three phases of development: juvenile, adult vegetative and adult reproductive. Normally, plants flower in the adult reproductive phase. These changes occur in the shoot apical meristem (SAM). In the juvenile phase, SAM is not competent to produce flowers, whereas in the adult phase the meristem is competent to produce reproductive structures and will respond to environmental signals including photoperiod, temperature and nutrients (Fig. 1.1) (McDaniel et al., 1992).

In *Arabidopsis*, differences in juvenile and adult phases can be seen by changes in leaf morphology and the appearance of the trichomes on the abaxial and adaxial surfaces. Juvenile leaves are small and round with trichomes on the adaxial surface, whereas later formed leaves (adult) are large and elongated with serrate margins. Trichomes are distributed on both abaxial and adaxial surfaces (Telfer & Poethig, 1998).

1.3 Flowering pathways

Successful transition to the reproductive phase depends on the perception of favorable external factors and the translation of that information into endogenous developmental signals. Genetic and physiological studies have revealed that there are more than 80 genes that regulate flowering in response to environmental and endogenous signals (Simpson et al., 1999).

The complex network of genetic pathways that controls flowering includes the photoperiodic pathway, the vernalisation pathway and the two pathways that act independently of environmental signals: the autonomous pathway that promotes flowering a few weeks after germination under all conditions, and the gibberellin (GA)

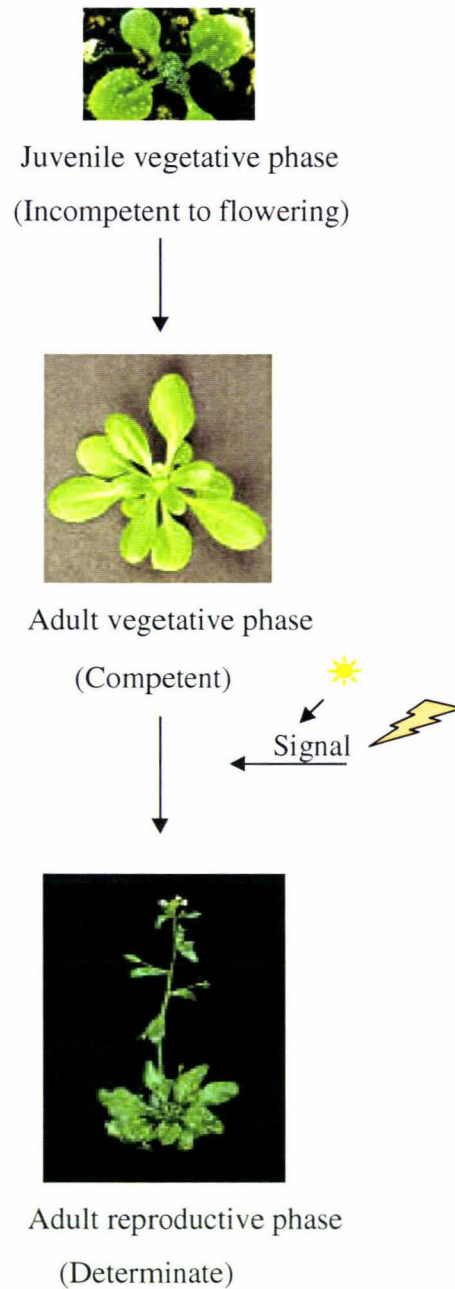


Figure 1.1 A simplified model of the phases of floral development in *Arabidopsis*. Based on McDaniel et al. (1992).

(flowering occurs when the daylength is less than critical value) and Day Neutral Plants (which do not depend on daylength) (Taiz & Zeiger, 1998). *Arabidopsis* is a facultative long day plant i.e. long days promote flowering, but eventually it will flower under short days (Mouradov et al., 2002).

To sense daylength, the two components required are perception and transmission of the signal, and the internal oscillator. Phytochromes A to E and cryptochromes 1 and 2 perceive light in *Arabidopsis* (Johnson et al., 1994). After perception, the signal transfers to the shoot apical meristem. The circadian clock measures the duration of daylength and regulates photoperiodic flowering in addition to regulating internal rhythms (Blazquez et al., 2001).

Genetic and molecular dissections have identified genes involved in the long day pathway. Some of these genes are involved in flowering whereas others are involved in circadian rhythms. In *Arabidopsis*, the mutants *lhy*, *cca1*, *gi*, *elf3*, *toc1*, *zfl*, *fkf1* affect daylength dependent flowering and also disturb other circadian regulated processes (Yanovsky & Kay, 2001; Suarez-Lopez et al., 2001).

Two models have been proposed to explain photoperiodic flowering (Thomas and Vince-Prue, 1997; Samach and Coupland, 2000; Samach and Gover, 2001). The external coincidence model suggests that the interaction of an external signal (light) with an internal light sensitive rhythm and promotes flowering in long day plants (*Arabidopsis*) and delays flowering in short day plants (rice). In the internal coincidence model, under floral inductive conditions the two circadian rhythms are brought into the same phase and coincidence promotes flowering, whereas under non-inductive conditions the rhythms are out of phase.

CONSTANS (CO), *CRYPTOCHROME 2/ FHA (CRY2)*, *GIGANTEA (GI)*, *FLOWERING LOCUS T (FT)* and *FWA* are the genes that act in the long day promoting pathway. *CO* is specific to this pathway and the other genes have general effects and act in other

pathways. *FT* and *FWA* act downstream of *CO* and in other pathways (Onouchi et al., 2000), whereas *GI* and *CRY2* act upstream of *CO* (Suarez-Lopez et al., 2001).

Under long days, the *CO* transcript level shows a diurnal rhythm. A broad biphasic peak is observed between 12 and 24 h after dawn with maximum levels at 16 and 24 h (dawn). Under short days, this peak is narrower and ends 4 h earlier. Plants entrained in long days show a circadian rhythm in *CO* transcript levels when transferred to continuous light, suggesting that this rhythm is controlled by the circadian clock (Suarez-Lopez et al., 2001).

The early target of *CO* is *FT* (Samach et al., 2000). The *FT* transcript level follows a circadian rhythm that peaks after dawn in long days (Suarez-Lopez et al., 2001), whereas this peak is absent in the *co* mutant. Therefore, this model suggests that the circadian clock acts in the light dependent pathway to regulate the expression of downstream genes, such as *CO* and *FT*. *CO*, a transcription factor with two B-box type zinc fingers, acts as a link between the central oscillator and the flowering time *FT* (Coupland, 1997).

1.3.2 Vernalisation pathway

In some plants, exposure to cold temperatures (3-5°C) induces flowering. This is called vernalisation. Susceptibility to this treatment can differ markedly between ecotypes of a species (Mouradov et al., 2002). For instance, many naturally occurring *Arabidopsis* ecotypes will flower late if they are not exposed to a vernalisation treatment. However, they flower early when exposed to low temperatures for about 4-8 weeks (Michaels & Amasino, 2000). The genetic control of vernalisation in *Arabidopsis* was studied by crossing winter annual varieties that require vernalisation and summer annuals that do not. The cross revealed that these varieties differed at two loci: *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). The *FLC* expression was higher in winter annuals than in summer annuals. The higher expression levels of *FLC* suggested a relationship with the vernalisation requirement in winter annuals (Michaels & Amasino, 1999; Sheldon et al., 1999).

The *FLC* encodes a repressor of flowering. The levels of *FLC* show a quantitative inverse relationship with flowering. Vernalisation causes quantitative reduction of the *FLC* levels and enhances flowering. The fact that *FLC* expression is required for *FRI* to delay flowering is supported by the observation that loss-of-function *flc* mutations suppress the effect of *FRI* on flowering time (Mouradov et al., 2002). The *FRIGIDA* and vernalisation pathways act to take control of *FLC*.

The reduction in *FLC* in *Arabidopsis* continues even when the period of vernalising temperatures cease, which indicates that the genes have undergone some kind of epigenetic change that causes it to stay repressed (Marx, 2004).

Screening of mutants that remain late flowering after a long cold treatment led to identification of the genes in the vernalisation pathway. In *Arabidopsis*, the two genes identified in the process of vernalisation are *VRN1* and *VRN2* (Chandler et al., 1996). Studies on *vrn1* and *vrn2* mutants revealed that the repression of *FLC* occurred in the same way during vernalisation in these mutants and in wild type (Gendall et al., 2001; Levy et al., 2002). However, the repression is not stable in *vrn1* and *vrn2* mutants upon return to warm temperatures. Therefore, *VRN1* and *VRN2* maintain the vernalisation state but are not required for the initial establishment. *VRN2* encodes a protein homologous to Polycomb-group proteins (PcG) found in plants and animals (Gendall et al., 2001). The structure of *VRN2* shows that it might be involved in adding methyl groups to the histone proteins associated with the DNA in chromosomes – a change known to lead gene silencing in other species (Marx, 2004).

Recently, Sung & Amasino (2004), identified a gene called *VERNALISATION INSENSITIVE GENE (VIN3)*, a plant-homeodomain-containing protein. They showed that during vernalisation, histone tails of *FLC* chromatin were deacetylated followed by an increased methylation of lysines 9 and 27 on histone H3. *VIN3* plays a role in the initiation of these modifications and it was observed that none of these vernalisation mediated modifications were observed in *FLC* chromatin of vernalised *vin3* mutants (Sung and Amasino, 2004; He and Amasino, 2005).

In wheat, recent studies on the vernalisation pathway showed that the genetically identified targets were not related to *FLC* (Yan et al., 2003). These studies have not yet revealed how the genes in the two species have been found to be different. However, the basic mechanism of sensing the prolonged cold is conserved (Sung & Amasino, 2004).

1.3.3 Autonomous pathway

The autonomous pathway is daylength independent and regulated in an age-dependent manner. The pathway was identified through a group of *Arabidopsis* mutants that flower late under all photoperiods and were highly responsive to vernalisation. These mutants included *fca*, *fy*, *fpa*, *ld*, *fld*, and *fve* (Martinez-Zapater & Somerville, 1990; Koorneef et al., 1991). The similarity between these mutants was that they contained high levels of *FLC* compared to wild-type plants and late flowering mutants of long day and GA pathways (Michaels & Amasino, 1999; Sheldon et al., 1999). Thus, this indicated that the genes in the autonomous pathway acted to maintain low levels of *FLC* transcripts.

Although the genes in the autonomous pathway act by repressing *FLC* expression, genetic evidence suggests that they do not act in the same linear pathway. For instance, *fca fpa* double mutants are much later flowering than would be expected. The simple additive effect of these mutations and combining *fpa* and *fy* mutations appear to be lethal. This shows a broader role for these genes in plant development rather than simply in floral regulation (Koorneef et al., 1998a). Mutations in *LD* do not cause late flowering phenotype in Landsberg erecta ecotype, whereas in other ecotypes such as Columbia, *LD* caused late flowering. This suggests the allele of *FLC* in Columbia, but not in Landsberg responds to loss of function of the *LD* gene (Lee et al., 1994).

FCA encodes a protein containing two copies of an RNA binding domain, the RNP and a WW protein-protein interaction domain (Macknight et al., 1997). In the same way, *FPA* also encodes an RNA-binding protein containing RNA motifs. This suggests *FLC* may be post transcriptionally regulated in the autonomous pathway (Schomburg et al., 2001). *LD* encodes a homeodomain (Lee et al., 1994). *FVE* encodes a WD-40 repeat protein (Blazquez et al., 2001).

1.3.4 Gibberellin Pathway

In the life span of plants, all the hormones have been implicated in flowering at some time. However, gibberellins (GA) are the key hormones that promote flowering in *Arabidopsis*. This pathway was dissected by studying the mutations that disrupt either GA biosynthesis or GA signalling (Wilson et al., 1992).

The *Arabidopsis* mutant *gal-3* contains a deletion in *KAURENE SYNTHASE*, an enzyme that catalyses the first step of GA biosynthesis and thus disrupts GA production in *gal-3* mutants (Sun et al., 1992). Under LD, *gal-3* mutants exhibit a short delay in flowering, whereas under SD (8 h) conditions, *gal-3* mutants do not flower even after vernalisation. They remain vegetative and eventually undergo senescence (Wilson et al., 1992). The *gal-3* mutation converts *Arabidopsis* from a facultative to an obligate LD plant (Michael & Amasino, 1999).

Michaels & Amasino (1999) demonstrated that the *gal-3* mutation does not block the response to vernalisation in intermediate photoperiods or in long day conditions in a late-flowering, vernalisation responsive background. This shows that GA may not have a role in the vernalisation response in *Arabidopsis*. However, it may be required in an alternative pathway that promotes flowering in noninductive photoperiods.

Genes that are involved in GA signalling include *GIBBERELLIC ACID INSENSITIVE (GAI)*, *REPRESSOR OF GAI-3 (RGA)*, and *RGA-LIKE 1 (RGL1)*. Mutations in *RGA* and *GAI* rescued the phenotype of *gal* mutants. Hence, the GA signal involves an up-regulation of the pathway (Dill & Sun, 2001).

Gibberellins have been shown to induce the transcriptional activity of *LFY*, a floral meristem identity gene. Blazquez et al. (1998) found that *gal-3* mutants lack *LFY* promoter expression under short day conditions. The link between these two events was confirmed by the ability of a constitutively expressed *LFY* transgene to restore flowering to *gal-3* mutants in short days. In contrast to plants grown under SD, plants grown under

LD or with sucrose in the dark, GA biosynthesis impairment merely caused a reduction of *LFY* expression.

Recently, He et al. (2004) showed that nitric oxide (NO), a key growth regulator in plants, repressed the transition to the reproductive phase in *Arabidopsis*. The mutant producing less NO (*nos1*) flowered early compared to a mutant overproducing NO (*nox1*). Further, NO suppressed the key genes in the photoperiod pathway, *CO* and *GI* and enhanced *FLC* expression. This shows NO may regulate the photoperiod and autonomous pathways.

1.3.5 Integration pathway

The pathways described above all converge to a few downstream regulatory genes (Komeda, 2004; Fig. 1.3). The *LFY* gene has an important role in this convergence (Borner et al., 2000). *LFY* regulates the transcription of *API*, *AP3* and *AG* and gives floral identity to the SAM tissues. Thus, *LFY* is the switch of floral development but is not of floral evocation, which initially determines flowering (Bernier, 1988; Komeda, 2004). On the other hand, *FT*, which shows homology to *TFL1*, is an important switch of floral evocation (Bradley et al., 1997). The key gene in the long day pathway, *CO* interacts with the *FT* gene (Samach & Gover, 2001), which is in turn regulated by *FLC*. In the integration of pathways, another important gene is *SOC1/AGL20* (Hepworth et al., 2002). *LFY* acts downstream of *SOC1* (Lee et al., 2000).

The expression of *FT* and *SOC1* is positively regulated in the long day and autonomous pathways acting through the repression of *FLC*. Further, exposure to cold increases the expression levels of *SOC1* by repressing *FLC* (Lee et al., 2000). In the GA pathway, *SOC1* expression is up regulated (Borner et al., 2000). Therefore, all the four pathways converge at *SOC1* and *FT* (Hepworth et al., 2002).

TFL1 is an important inflorescence meristem identity gene. Mutations in *TFL1* are semi-dominant and lead to early flowering with determinate inflorescences in *Arabidopsis* (Alvarez et al., 1992). Thus, *TFL1* is a repressor of flowering. *TFL1* encodes a protein

that shows homology to *FT* (Bradley et al., 1997). Although the *tfl1* mutants flowered earlier than wild-type, the transcription of *TFL1* is inhibited by the floral meristem identity genes (Komeda, 2004).

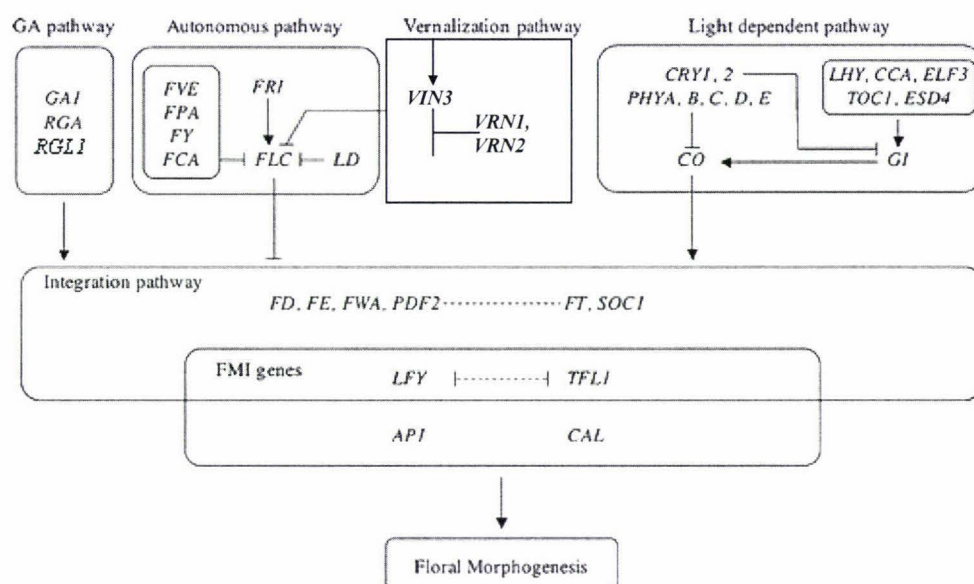


Figure 1.3 The Genetic pathways of flowering in *A. thaliana*. Arrows indicate positive interaction and T lines show negative interaction. Dotted lines show an undescribed interaction. Modified from Amasino (2004) & Komeda (2004).

1.4 Meristem identity genes

The critical genes that control meristem identity in *Arabidopsis* have been identified by mutations that disrupt the normal floral development. Mutations in the *TERMINAL FLOWER (TFL)* gene cause early flowering, suggesting that *TFL* inhibits the transition to flowering (Shannon & Meeks-Wagner, 1991), whereas *LFY* and *API* promote flowering. Over expression of these two genes results in earlier flowering (Mandel & Yanofsky, 1995; Weigel & Nilsson, 1995). The meristem identity genes in turn control floral organ identity genes, which specify the type and position of floral organs.

1.4.1 *LEAFY*

In dicots, the transition from vegetative to reproductive phase is due to the plant specific gene *LEAFY* (Schultz & Haughn, 1991; Huala & Sussex, 1992; Weigel et al., 1992). Blazquez et al. (1997) studied in detail the expression of *LFY* during the plant life cycle. They showed that *Arabidopsis* held under long days make the floral transition soon after germination, and this is paralleled by rapid up-regulation of *LFY* expression. In contrast, under short days, *Arabidopsis* plants flower several weeks after germination and the expression of *LFY* increases gradually before flowering. The plant hormone gibberellic acid (GA) increases the expression of *LFY* and hastens flowering under short days.

Blazquez & Weigel (2000) studied *LFY* expression by using plants carrying fusions of the *LFY* promoter to the GUS marker gene and showed that *LFY* expression responded to both long days and to GA. Further, they showed that a deletion in a putative *myb* transcription factor-binding site within the *LFY* promoter prevented activation by GA, but not by long days. Double mutant analysis and the effects of constitutive *LFY* expression in an *fca* mutant background supported the idea of a positive regulation of *LFY* by the autonomous pathway (Page et al., 1999). All these studies showed that *LFY* is the ultimate target of all the flowering pathways.

LFY plays the role of a meristem identity gene, as it is requisite for all the features that differentiate flowers from inflorescence branches. However, after transition from the vegetative to the reproductive phase, *LFY* plays another role in the activation of floral homeotic genes, which are involved in floral organ identity (Weigel & Meyerowitz, 1994). Thus, *LFY* has both the properties of a floral meristem identity gene and a flowering time gene. This shows that *LFY* is a direct link between the global process of floral induction and the regional events associated with the initiation of individual flowers (Blazquez et al., 1997).

At the genetic and molecular levels, the two roles of *LFY* can be separated (Lee et al., 1997; Percy et al., 1998). *LFY* is a transcriptional factor that regulates the developmental events. The three direct target genes of *LFY* are *AGAMOUS* (*AG*),

APETALA3 (*AP3*) and *API* (Wagner et al., 1999; Busch et al., 1999; Lamb et al., 2002; Wagner et al., 2004). The floral homeotic genes, *AG* and *AP3* act directly downstream of *LFY* in floral morphogenesis, whereas *API* is the direct *LFY* gene product target and acts in the meristem identity pathway (Wagner et al., 1999). The *ap1-1* mutation partly suppresses the activity of *LFY* (Weigel & Nilsson, 1995; Liljegren et al., 1999), which indicates *API* acts downstream of *LFY* in floral transition.

LFY controls the transition to the reproductive stage by inducing *API* expression in the SAM that produces flower primordia. Wagner et al. (1999) demonstrated the transcriptional activation of *LFY* by using a steroid hormone-inducible posttranslation *LFY* switch, which caused the early expression of *API*. This *API* induction was independent of protein synthesis and occurred specifically in the development stage tissues in which floral fate are assumed. Later expression of *API* appears to be only indirectly affected by *LFY*. In addition, it has been shown that *LFY* binds to cis-regulatory elements that control *API* expression (Parcy et al., 1998).

LFY null mutations cause severe defects in the transition to floral development. However, the strongest available *API* mutation has a much weaker effect on the transition. This shows that *LFY* has other target genes in the meristem pathway.

By using a combination of transcription profiling and ChIP (chromatin immunoprecipitation), William et al. (2004) showed that *LFY* also directly regulates the transcription of the *API* related gene, *CAULIFLOWER* (*CAL*), and five additional genes, of which three genes encode putative transcription factors and another two encode putative signalling molecules.

1.4.2 *TERMINAL FLOWER1*

TFL1 is an important inflorescence meristem identity gene, responsible for maintaining the inflorescence meristem in the indeterminate state. Mutations in *TFL1* convert the SAM into a floral meristem, and *tfl1* plants flower earlier than wild type plants. This

shows a dual role of *TFL1* in the apical meristem: it maintains inflorescence meristem identity, and controls the timing of the transition to flowering. *TFL1* encodes a protein, which shows a similarity to animal phosphatidyl ethanolamine-binding proteins (Bradley et al., 1997). In mammals, these proteins appear to play multiple roles, including being a precursor for a neuropeptide, and an inhibitor of Raf-1 kinase activity (Tohdoh et al. 1995; Yeung et al. 1999).

Cloning of the flowering-time gene *FT* revealed that it is a homologue of *TFL1* (Kardailsky et al., 1999; Kobayashi et al., 1999). In contrast to *TFL1*, *FT* acts downstream of both light dependent and light independent pathways to promote the floral transition. The opposite phenotypes of loss-of-function and over expression of *FT* and *TFL1* suggest that these two genes act antagonistically in regulation of growth phase transition. The sequence similarity and functional divergence between *TFL1* and *FT* have suggested that other *TFL1*-homologues may have roles different from those of *TFL1* and *FT* (Mimida et al., 2001).

Mimida et al. (2001) isolated a new member of the *TFL1* gene family, which mapped to chromosome 2 of *Arabidopsis*. The gene is named as *Arabidopsis thaliana* *CENTRORADIALIS* (*ATC*) as this gene is more closely related to *CEN* than to other *TFL1* homologues.

Larsson et al. (1998) isolated another mutant, *tfl2*, of *Arabidopsis* that initiates early flowering and terminates the inflorescence with floral structures. Although these phenotypes are similar to *tfl1*, *tfl2* mutants are dwarfed in appearance, have reduced photoperiod sensitivity and have a more variable terminal flower structure. Double mutants of *tfl1* and *tfl2* exhibit different phenotypes compared to that of the *tfl1* single mutant under long day and short day growth conditions. *tfl1 tfl2* double mutants terminate the inflorescence without the development of lateral flowers, whereas in *tfl1* single mutants the inflorescence morphology is not affected by daylength. This shows that *TFL2* acts in a different developmental pathway from *TFL1*. The phenotype of *tfl2*

mutant is complex and this suggests *TFL2* has a regulatory role more global than that of *TFL1*.

1.5 Meristem identity genes in annuals

Orthologues and homologues of *LFY* have been studied in several plant species. In *Antirrhinum*, *FLO* is the *LFY* homologue. The transcript levels of *FLO* are abundant in bracts and in early floral meristems (Coen et al., 1990), whereas in *Arabidopsis*, *LFY* expression is higher in the regions of the inflorescence meristems that form floral meristems, and in newly formed floral meristems. However, *LFY* is expressed at low levels during vegetative development (Weigel et al., 1992).

In *Antirrhinum*, *CENTRORADIALIS (CEN)*, the *TFL1* homologue, maintains inflorescence meristem identity only. Foucher et al. (2003) isolated three *TFL1* homologues in pea, *PsTFL1a*, *PsTFL1b*, and *PsTFL1c*. *PsTFL1a* is similar to *DETERMINATE (DET)* and *PsTFL1c* is similar to the *LATE FLOWERING (LF)* gene. This shows that, in pea, different *TFL1* homologues control different aspects of plant development, whereas in *Arabidopsis*, a single gene, *TFL1* performs both the functions.

1.6 Meristem identity genes in perennials

Walton et al. (2001) isolated a partial orthologue of *LFY (ALF)* from kiwifruit (*Actinidia deliciosa*). *ALF* shows a bimodal pattern of annual expression in developing first order axillary buds and their subsequent shoots.

Sreekantan et al. (2004) isolated a *LFY* equivalent in *Metrosideros excelsa (MEL)*. Temporal expression studies showed a bimodal expression pattern of *MEL*. Expression was seen during early floral initiation in autumn followed by down-regulation during winter and up-regulation in spring as floral organogenesis occurred. They also isolated *METFL1* from *M. excelsa*. They showed that *METFL1* was expressed throughout the inflorescence development period and also in the inflorescence meristem, but no expression was seen in floral meristems, which is similar to *Arabidopsis TFL1*.

Southerton et al. (1998) isolated *ELF1* and *ELF2* in *Eucalyptus*. These genes showed sequence homology to the genes *LFY* in *Arabidopsis* and *FLO* in *Antirrhinum*. They reported that *ELF1* was expressed in the developing floral organs similar to *LFY* in *Arabidopsis* while *ELF2* appeared to be a pseudogene.

Mouradov et al. (1998) isolated *NEEDLY* (*NLY*) from *Pinus radiata*, and showed that it was expressed in both reproductive and vegetative meristems. Jensen et al. (2001), isolated a *TFL1*-like gene in perennial ryegrass (*Lolium perenne*), and named it as *LpTFL1*. They showed that *LpTFL1* was a repressor of flowering and also maintained axillary meristem identity.

Wada et al. (2002) isolated two orthologues of *LFY/FLO*, *AFL1* and *AFL2*, in apple and showed that *AFL1* was expressed only in the floral bud during the transition from vegetative to reproductive growth, whereas *AFL2* was expressed in vegetative shoot apex, floral buds, floral organs and root. Kotoda and Wada (2005) isolated a gene homologous to *TFL1* (*MdTFL1*) in *Malus domestica*. It was shown that *MdTFL1* suppressed the floral meristem identity genes *LFY* and *AP1* and maintained the inflorescence meristems in *Arabidopsis*. Transgenic *Arabidopsis* expressing *MdTFL1* flowered later than wild type plants and the phenotype was similar to that of transgenic *Arabidopsis* over expressing *TFL1*. This shows that *MdTFL1* is analogous to *TFL1* and maintains the vegetative phase in apple.

1.7 Perenniality

Woody perennials are polycarpic: flowering occurs over many years with intervening vegetative growth periods. The key to perennial growth is conservation of vegetative meristems during flowering and a separation of flowering from the senescence programme, whereas in annuals and monocarpic perennials senescence occurs after flowering and the plant dies. The mechanism that prevents some meristems from flowering during induction is not known (Battey & Tooke, 2002). In the blossom cluster of apple tree, the distal portion subtends flowers, whereas one of the proximal nodes

subtends a large vegetative bud, which continues growth in the next year (Abbott, 1984; Battey & Tooke, 2002). In citrange trees that overexpress *LFY* or *API*, the flowering interspersed with vegetative growth is preserved (Pena et al. 2001). This shows that some meristems are incompetent to respond to the effects of these overexpressed genes.

1.8 *Pachycladon* species

Pachycladon species, which are endemic to New Zealand, are limited to the South Island, mainly in mountain habitats. All species occur on rock bluffs, cliffs and outcrops, although they sometimes occur around the margins of these on loose rock or stone debris and in fellfield and herbfield (Heenan & Mitchell, 2003). Mostly they grow on ledges and crevices with roots penetrating cracks in the rocks.

P. cheesemanii (Heenan & A. D. Mitch) has wide latitudinal and altitudinal ranges, occurring in Marlborough, Canterbury, Otago and northern Southland, and is most abundant in the southern South Island. Altitudinal range is about 10 m on the Otago Peninsula to above 1500 m in the Dunstan (Otago) and Eyre (Southland) mountains (Heenan & Mitchell, 2003).

P. enysii (cheeseman) Heenan & A. D. Mitch., is the highest altitude species of *Pachycladon* occurring up to 2492 m in the Liebig Range, Mt Cook (H. D. Wilson, pers. comm., 2002, Heenan & Mitchell, 2003). In contrast, *P. fastigata* (Hook.f.) Heenan & A. D. Mitch grows at a lower altitude and is absent from the high mountains of the Southern Alps. *P. enysii*, *P. fastigata*, and *P. stellata* (Allan) Heenan & A. D. Mitch. are limited to greywacke in the eastern South Island, and are facultatively monocarpic, have serrate leaves and a stout terminal inflorescence (Heenan & Mitchell, 2003).

P. latisiliqua is widespread in north-west Nelson with a narrow latitude range and a mean altitude of 1441 m. *P. novae-zelandiae* occurs in Otago, Southland, and south-west Canterbury on all transitional zones (Heenan & Mitchell, 2003). *P. crenatus* Philipson

appears to group into *P. novae-zelandiae* with respect to leaf characters (Mitchell & Heenan, 2002).

P. wallii is identified from the Eyre Mountains, southern end of the Garvie Mountains, East Dome (all Southland) and the Remarkables (Otago) (Heenan & Mitchell, 2003).

Pachycladon sp. is an unnamed taxon limited to limestone in the Chalk Range on Ben More, Marlborough, with plants from the Chalk Range being considered as strong calcicoles (Druce et al., 1989; Heenan & Mitchell, 2003).

P. exilis distribution is limited to only three sites in north Otago. It occurs on low-altitude (<500 m) calcareous substrates, including limestone and volcanic rock (Heenan & Mitchell, 2003). The two species, *P. cheesmanii* and *P. exilis* are the sister group to all other species of *Pachycladon*. These two are distinguished by a woody stem, heteroblastic leaves, slender and terminal inflorescences, terete siliques and seeds which are uniseriate and lack wings. These two species are morphologically more similar to their closest overseas relatives, *Beringia* and *Crucihimalaya*, than to other *Pachycladon* species (Heenan et al., 2002).

Although geological parent materials may have provided an important stimulus for the adaptive radiation of *Pachycladon*, further research is needed to determine the influence of other environmental factors (Heenan & Mitchell, 2003). *Pachycladon* shows a close relationship with *A. thaliana*, a model plant for genetic, molecular, physiological and evolutionary studies. It may be possible to use these resources to unravel the factors that influence floral induction in *P. exilis*.

1.9 Aims and objectives of this study

Annual and perennial growth is a result of a quantitative relationship between the genes that influence progression through vegetative growth, flowering, senescence and death. The fundamentals of flowering are probably similar in annuals and perennials. It was, therefore, hypothesised that the balance of genes such as *TFL1* maintains vegetative identity in perennials.

The aim of the project was to compare and contrast inflorescence and floral meristem identity gene expression in vegetative and reproductive meristems of a perennial plant readily amenable to molecular analysis.

The focus was on the herbaceous short-lived perennial *Pachycladon exilis*. This plant, which forms a rosette of leaves before bolting occurs, is closely related to *Arabidopsis thaliana* (Heenan et al., 2002). It is believed to be a long-day sensitive plant (Heenan et al., 2002). However, little was known about how the genetic, environmental and physiological factors influence flowering in *P. exilis*.

The objectives, therefore, related to the need to gain a better understanding of flower phenology, floral induction and key floral gene expression in *P. exilis* so that the hypothesis pertaining to the balance of vegetative and reproductive identity would be tested. Specifically:

- To examine morphological features of *P. exilis* and to view floral development.
- To establish the effect of long day and short day conditions on growth and flowering.
- To determine the effect of chilling on growth and flowering.
- To isolate from putative vegetative and floral meristems, the floral and inflorescence meristem identity genes equivalents of *LFY* and *TFL1* from *P. exilis* during floral induction.
- To investigate the effects of daylength and chilling on *PeLFY* and *PeTFL1* expression using real-time reverse transcriptase-PCR.

Chapter 2. Morphology

2.1 Introduction

The morphology of a plant has usually included both the comparative study of mature forms and the study of primordium/organ morphogenesis (Goebel, 1900; Arber, 1950; Hempel, 1996). In flowering plants, distinction of morphological changes occurs with the onset of reproductive structures such as inflorescence and floral development. *Arabidopsis* produces a rosette of leaves at ground level during vegetative development and produces an elevated flowering shoot system during reproductive phase. The distinction between the vegetative region of the plant and inflorescence is difficult to interpret. Structures such as trichomes and leaf shape were used to distinguish the different phases in shoot development (Allsopp, 1967; Martinez-Zapater et al., 1995).

Pachycladon exilis is a native species of New Zealand. Previous studies have shown that it is a close relative of *Arabidopsis thaliana* (Heenan et al., 2002). The species has short aerial branches, slender and terminal inflorescences, terete siliques, and is generally found in dry, rocky areas of the eastern South Island (Molloy et al., 1999). Ovule development and embryogenesis studies by Luo et al. (2003) have shown that the structures of megasporogenesis and embryogenesis of *P. exilis* and *P. cheesemanii* were similar to those of *A. thaliana*. However, they differ in the timing of events, with an extended delay apparent in *Pachycladon* between anther dehiscence and first cell divisions of the embryo and endosperm.

In this thesis other morphological characters such as trichomes and leaf characters were examined in *P. exilis*. Flowers and floral buds were dissected to determine the number of organs and the sequence of organ development.

2.2 Materials and methods

2.2.1 Plant material

Seed (105 mg) obtained from Dr P. B. Heenan (Landcare Research, Lincoln) was sown (36 mg) on 4 April 2003 in a tray containing sterile seedling mix. The tray was kept at 4°C in the dark for 5 d, wrapped with cling film and then with aluminium foil to conserve moisture and to exclude light. On 9 April 2003, the tray was transferred to a glasshouse and the coverings were removed, and replaced with shade cloth. After observing germination on 15 April, the shade cloth was removed to provide the seedlings sufficient aeration and sunlight. Then on 11 May, seedlings (c. 69) were individually potted into separate 100 mm x 100 mm pots containing potting mix and slow release NPK fertilizer, and the pots were transferred to the Plant Growth Unit (PGU), Massey University. Plants were maintained in the PGU under an ambient daylength with supplementary heating to give a day/night temperature of 20/15°C. The seed harvested from these plants was subsequently used for the next experiment (Chapter 3).

Following flowering, four whole plants were dissected, one on 9 Dec 2003 by Dr John Clemens and three on 7 Jan 2004 to study the hierarchy of branching. Further, floral buds at different stages from the base of the inflorescence rachis to the tip were collected into the liquid nitrogen and stored at -80°C for later use. These buds were later sectioned to track floral development.

2.2.2 Fixation, dehydration and wax infiltration

The buds were immersed in the fixative, FAA, under vacuum for 2 h and then left overnight. The tissue was rinsed with 70% ethanol and then dehydrated in an ethanol series starting with 70% ethanol, with changes for every 2 h through 80%, 95% and 100% ethanol, and left overnight at room temperature. The next morning the tissue was immersed in fresh 100% absolute ethanol for 2 h followed by Histo-clearII (National Diagnostics, USA): ethanol (EtOH) series (75%EtOH:25%Histo-clearII for 2 h followed

by changes of 50% EtOH:50% Histo-clearII, 25% EtOH:75% Histo-clearII and 100% Histo-clearII at 2 h interval each) Finally, two changes of Histo-clearII at 2 h interval was done followed by wax infiltration. In wax infiltration, to ¼ volume of Histo-clearII, paraplast wax was added and incubated at 42°C overnight. Next morning the tissue with wax and Histo-clearII was transferred to an oven at 60°C to melt the wax. The molten mix was replaced with fresh molten wax and left at 60°C. In the evening again the wax was changed. Similarly, changes (twice daily) were made for about 3 d. The tissue was embedded in paraplast wax using a Leica EG 1160 embedder, and the blocks were stored at 4°C for later sectioning. Later, the tissue was sectioned using a microtome (Leica RM 2145) to 10 µm sections. The sections were transferred to a 42°C water bath, placed onto Polysine microscopic slides (BDH Laboratory Supplies) and dried. Later, tissues were stained with Fast Green with the series of hydration, staining, and dehydration. In this, slides were placed in Histo-clearII for 10 min, then to Histo-clearII/EtOH (50:50) for 5 min followed by absolute EtOH for 5 min, again absolute EtOH for 5 min, 95%EtOH -5 min, 85%EtOH-5 min, 70%EtOH-5 min, Fast Green-<15 s and then rinsed with clove oil followed by clove oil-OH:Histo-clearII -5-10 s, Histo-clearII:EtoH-5 min, Histo-clearII- 5 min, Histo-clearII-5 min and finally mounted coverslips onto slides using DPX and observed them under light microscope. Then sections were viewed and photographed using an Olympus BX51 microscope, an Optonics digital camera and MagnaFire software.

2.3 Results

2.3.1. Plant morphology

P. exilis produces cotyledonary leaves, rosette leaves, and cauline leaves and the primary axis terminates with an inflorescence. Dissection of flowering plants revealed that the shoot was made up of (apparently) three levels of branching in a hierarchy, with the leaves of one level subtending the shoots of the next level. Primary and secondary axes were floral and senescing; tertiary axes were vegetative and green.

After 38 weeks of germination the following observations were recorded:

The primary shoot axis:

- had ≥ 12 rosette leaves, and 3-5 cauline leaves
- these leaves now all senesced and dry or missing
- difficult to see presence/absence of marginal serrations
- primary bolt branched, bearing ~50 siliques
- cauline leaves subtend further inflorescence branches
- all leaves in primary rosette subtend secondary axes:

Secondary axes:

- secondary axes had 3-11 rosette leaves, and 2-5 cauline leaves
- these leaves now all senesced and dry, again difficult to gauge leaf serration
- all secondary axes floral (except 3 that were vegetative and/or had died before floral development apparent), each bearing 8 to >22 siliques
- 3-11 leaves in secondary rosettes subtend tertiary axes:

Tertiary axes:

- 3-11 tertiary axes have rosettes in various stages of development and expansion
- tertiary rosettes all (apparently) vegetative, leaves all green
- most proximal 3-6 tertiary shoots only 1-2mm in length, with 2-4 leaves <1-2mm long
- more distal tertiary shoots show gradual change in leaf morphology, shape and size
- proximal 1-3 leaves sessile, 5 then 7 leaf serrations
- more distal leaves increasingly petiolate, not serrated

Plants of *P. exilis* produced adventitious shoots from roots extending from the base of the container (Fig. 2.1). To confirm whether the growth was from roots or not, the branch on which adventitious shoots occurred was sectioned. The section of the tissue from which the shoot arose confirmed it was a root, in that the vascular tissue was



Figure 2.1 Root system of a *P. exilis* plant showing adventitious shoot growth

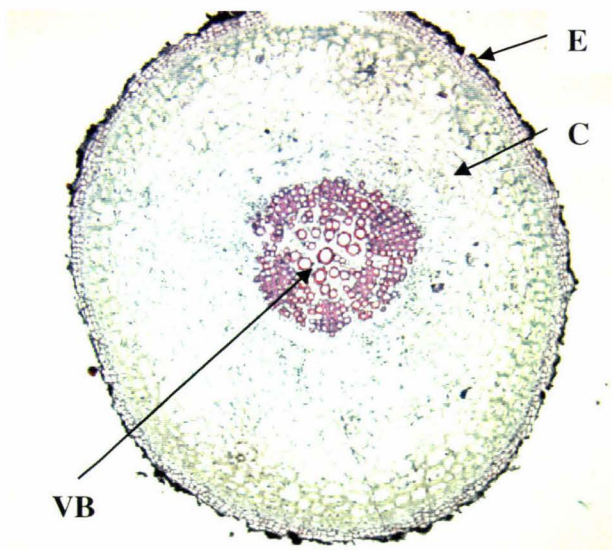


Figure 2.2 Section of a *P. exilis* root from which an adventitious shoot appeared.
E : epidermis, C: cortex, VB: vascular bundle

confined to the centre (Fig. 2.2), whereas in dicot stems the vascular bundles occur around the periphery.

2.3.2. Leaf morphology

P. exilis plants produced rosette and cauline leaves. Rosette leaves were produced alternately on short internodes and cauline leaves were separated by longer internodes. Rosette leaves also had long petioles, whereas cauline leaves had short petioles (Fig. 2.3). Cotyledonary leaves were small and round with no trichomes. The first leaf had trichomes only on the adaxial surface, whereas the later leaves produced trichomes also on their abaxial surface.

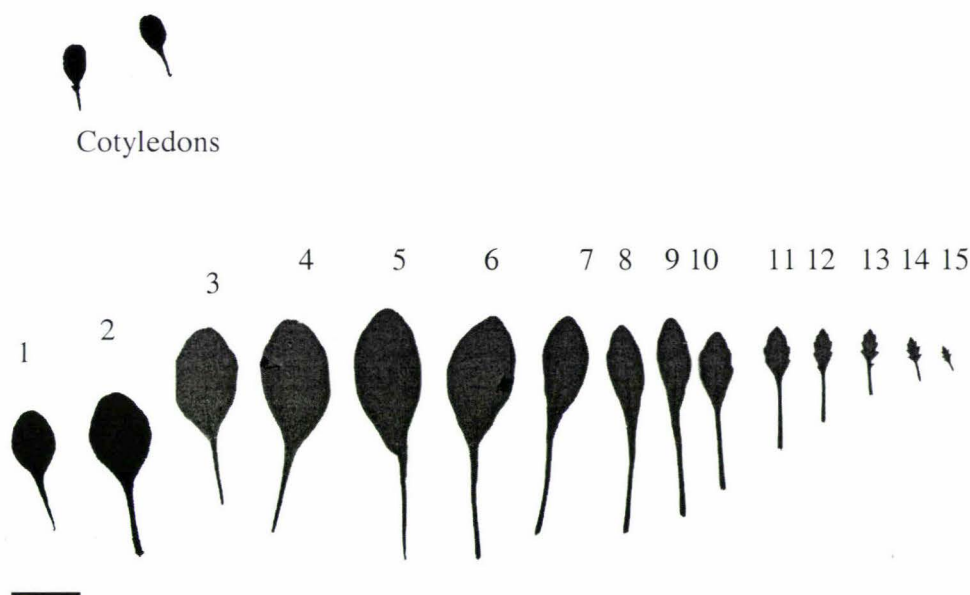


Figure 2.3 Cotyledons and rosette leaves on primary axis of one bolted *P. exilis* plant
Scale bar, 2.3 cm

2.3.3. Floral development

The inflorescence in the *P. exilis* plants is a simple indeterminate raceme. Flowers are arranged alternately on the rachis. The dissection of flowers of *P. exilis* showed four

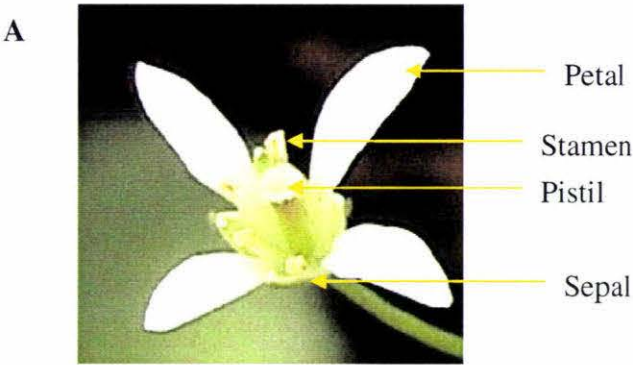


Figure 2.4A Flower of *P. exilis* showing all floral parts

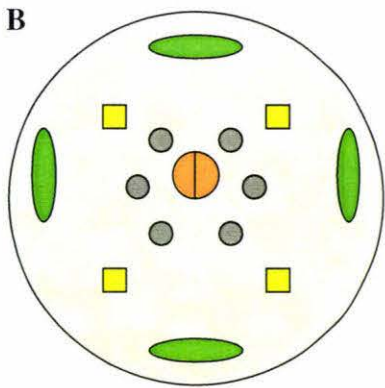
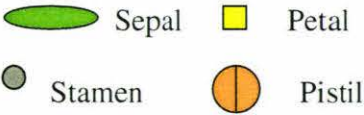


Figure 2.4B Floral diagram showing all floral parts arranged in definite whorls



sepals in the outer whorl, four petals in the second whorl, six stamens in the third whorl, and two carpels fused to form the pistil (Fig. 2.4A and 2.4B).

Sectioning of floral buds at different stages enabled the development of floral organs to be tracked. The buds at the top of the inflorescence were smaller and the floral organs were as yet undifferentiated (Fig. 2.5A). In the next stage of development, the sepals, petals and stamens were differentiated, and the stamens were arranged in two whorls, four stamens in one whorl and another two in the second whorl (Fig. 2.5B), whereas the gynoecium was not yet differentiated (Fig. 2.5C). The next stage buds had fully developed female reproductive organs. The ovary of *P. exilis* was long and elongated with a short style and a papillary stigma (Fig. 2.5D). The fully developed and opened flowers at the base of the inflorescence showed a condensed and bulged ovary (Fig. 2.5E).

2.4 Discussion

P. exilis, like *Arabidopsis*, has a rosette of leaves at ground level and a prominent flowering shoot. It produces three levels of branching in a hierarchy. Primary and secondary branches were floral, whereas tertiary branches were still vegetative at 38 weeks after germination. Furthermore, *P. exilis* produced adventitious shoots from roots. All these might be responsible for the perennial nature of this plant.

Higher plants pass through several phases of shoot growth during which they may produce morphologically distinct vegetative structures (Telfer and Poethig, 1997). In many plants, leaf shape is the obvious phase change marker (Allsopp, 1967). In some species, juvenile and adult leaves can be distinguished by the density and/or distribution of trichomes (Schaffalitzky de Muckadell, 1954; Wareing and Frydman, 1976; Brand & Lineberger, 1992; Lawson & Poethig, 1995). In *Arabidopsis*, both leaf shape and trichomes play an important role in the distinction of phases, in which the first two juvenile leaves are round with only adaxial trichomes, whereas adult leaves are oblong with both adaxial and abaxial trichomes. Similarly, in *P. exilis*, cotyledons are small and

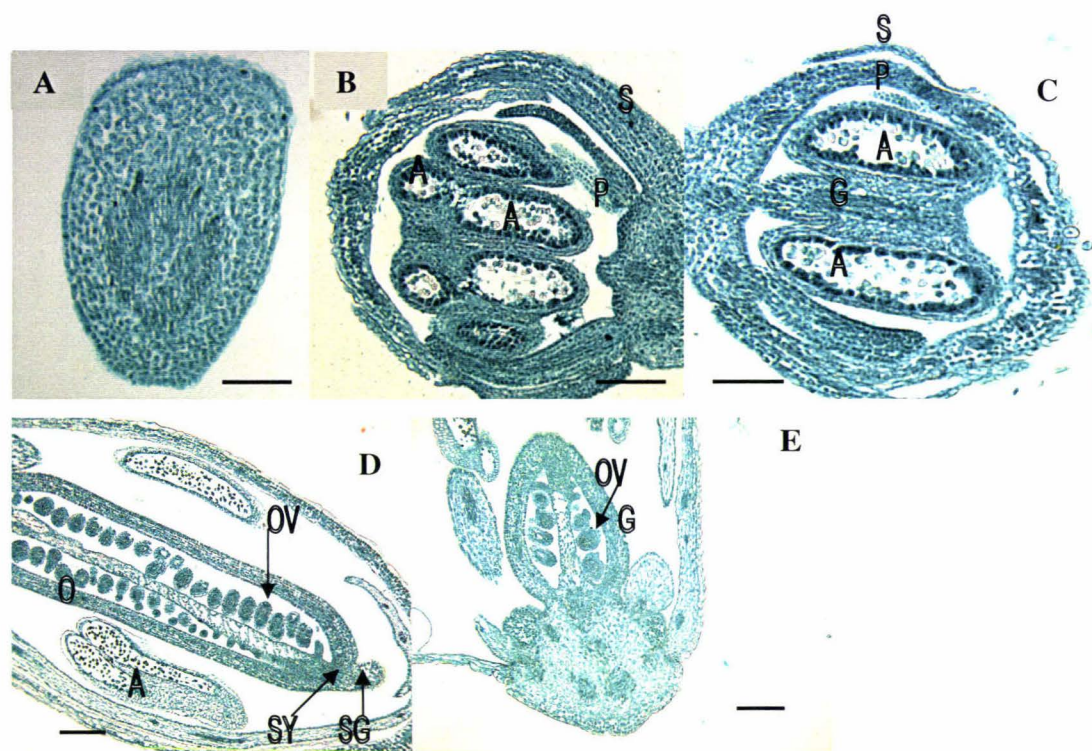


Figure 2.5 Foral development. **A.** No floral parts differentiated in the very young bud at the top of the inflorescence. **B.** All floral parts including sepals, petals and stamens were differentiated and stamens were arranged in two whorls, two stamens in the outer whorl and the other four in the inner whorl. **C.** Undifferentiated gynoecium. **D.** Characteristic gynoecium with well-differentiated ovules and papillary stigma. **E.** Fully opened flower with well developed ovules and condensed gynoecium. Scale bars, 200 μ m.

S-sepals, P-petals, A-anthers, G-gynoecium, O-ovary, OV-ovule, SY-style, SG-stigma

round without any trichomes. Leaves produced early in rosette development do not have trichomes on the abaxial surface, whereas leaves produced later possess trichomes on both their adaxial and abaxial surfaces. Therefore, as with *Arabidopsis* trichomes may serve as phase change markers in *P. exilis*.

The dissection of floral buds at various stages of development tracked the sequence of floral organ development and also the type of female parts, which play a major role in the evolution of angiosperms. Although the dissected buds did not show the sequence of development of sepals and petals, they showed the development of the remaining two whorls of floral parts, i.e. stamens and gynoecium. Characteristic of member of the Brassicaceae, the stamens are arranged in two whorls, two stamens in the outer whorl and the remaining four in the inner whorl. As in *Arabidopsis*, the gynoecium of *P. exilis* consists of an elongated ovary with a short style and a papillary stigma.

The arrangement of leaves on the primary axis, appearance of trichomes on leaves and the initiation and arrangement floral organs in *P. exilis* appears to be similar to that of the model plant, *Arabidopsis*. In the following Chapter the effects of environmental signals on the growth and flowering behaviour of *P. exilis* are reported.

Chapter 3. Influence of photoperiod and duration of seed chilling on growth and flowering in *Pachycladon exilis*

3.1 Introduction

The timing of the transition from the vegetative to reproductive phase is an important feature in agriculture, horticulture and plant breeding, because flowering is the first step of sexual reproduction (Bernier et al., 1993). Many studies have been carried out to determine how this transition is regulated. Most plants use environmental signals to regulate the transition to flowering, two of the most important being photoperiod and temperature (Bernier et al., 1993).

The environmental factors interact with each other, so that the threshold value of effectiveness of one factor can be changed by the others. The model plant, *A. thaliana*, is a facultative long day plant: long days induce flowering, although it eventually flowers under short days. Exposure to low temperature prepares the plant for floral evocation. Nordborg & Bergelson (1999) showed that a month-long cold treatment of *A. thaliana* ecotypes at the seed stage decreased the time to flower, whereas a short cold treatment (3 d) had little or the opposing effect. Moreover, they showed that cold treatment at the rosette stage was less effective than treatment of seed.

The morphology of *Pachycladon exilis* resembles *A. thaliana* and, based on earlier studies made by Heenan et al. (2002), the two species are closely related. Based on informal observation, it was believed that *P. exilis* was a long day plant (Heenan et al., 2002). Therefore, it was the objective to test the hypothesis that long days would induce flowering more readily than short days in *P. exilis*. In addition, it was highly likely, based on published literature for *A. thaliana*, that chilling of the seed would have interactive effects with daylength on flowering. Therefore, it was hypothesised that an increase in the duration of chilling of the imbibed seed and exposure to long days would cause earlier flowering. These two factors were studied in factorial combination in an

experiment carried out at the Controlled Environment Laboratory (CEL), HortResearch, Palmerston North.

3.2 Materials and methods

3.2.1 Chilling experimental conditions

The seed obtained from the preliminary experiment (Chapter 2) was used for this study. On 8 December 2003, about 60 liters of soil was mixed with a controlled release fertilizer. The medium was held in 100 mm x 100 mm square pots, and watered thoroughly. Seed (250 mg) of *P. exilis* was sowed on the top of the medium in each pot, and covered with fine gravel (2-5 mm diameter). From preliminary observations (Chapter 2), enough seed was sown in each pot that would result in c.500 germinants in total. The pots were placed in trays and wrapped with cling film and aluminium foil as in the first experiment to prevent moisture loss and to maintain the seeds in the dark during the application of the chilling treatment. Seeds were sown in four different phases to achieve four different chilling regimes. The first seed lot was subjected to the most days of chilling: the pots were kept at 4°C for 30 d. Ten days later a second seed lot was sown and placed in the same 4°C room for 20 d of chilling. Ten days later, a third seed lot was sown for 10 d of chilling. Finally, ten days later a fourth lot of seeds were sown just one day before the start of daylength treatment in CEL, the seeds were kept at room temperature, so they were subjected to 0 d of chilling.

3.2.2 Daylength experimental conditions

Two controlled environment rooms, located at the Controlled Environment Laboratory (CEL), The Horticultural and Food Research Institute of New Zealand Ltd (HortResearch), Palmerston North, were used to study the effects of four long and short daylength regimes in factorial combination with the different chilling treatments on growth and reproduction of *P. exilis*. The pots with seed that had been chilled at 4°C for 0, 10, 20 or 30 d were transferred to the CEL on 8 January 2004. Daylength treatments

were applied for 9 weeks until 15 March 2004 in the LD treatment, and for 15 weeks until 24 April 2004 in other treatments.

In both rooms, day/night temperatures were maintained at 24/18°C. Air moisture content, controlled as vapour pressure deficit, was 0.4/0.3 kPa (day/night). Relative humidity (RH) was 87/86% (day/night). CO₂ was c. 350 ppm, and photosynthetically active radiation (PAR) was 480 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 8 h each day. Main lighting was provided by four metal halide (1.0 kW) and four tungsten halogen (1.0 kW) lights per room between 0900 and 1700 NZST each day.

Short day (SD) conditions were those occurring between 0900 and 1700 each day without daylength extension. Long day (LD) conditions were applied in the second room between 0600 and 2300 NZST by daylength extension outside the main light period provided by six auxiliary tungsten lights (150 W) with PFD 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

In addition, one set of SD plants were transferred to LD one week after germination and remained under LD conditions for one week, before being returned to SD conditions. And another set of SD plants were transferred to LD, four weeks after germination and remained under LD conditions for one week before being returned to SD conditions. These transfer treatments are referred to as SD/LD/SD1 and SD/LD/SD4, respectively, below.

Every 14 d, the room conditions in each of the rooms were reversed and the plants transferred during the light period, i.e., the SD room became the LD, and *vice versa*, to minimize any effects created by the rooms rather than the treatments applied in each room.

The experiment was arranged as an A x B randomized complete block design with four levels of chilling pre treatment x four levels of daylength and 20 blocks (individual plant parts).

In the days immediately after transfer to the CEL, the pots were arranged on capillary matting held in metal trays on six trolleys in each room and covered with cling film to prevent moisture loss. This was progressively removed as germination occurred. The pots were programmed to be watered with a 5 min pulse daily at 0900 (NZST), and the surface of the growing medium was regularly watered by hand with a fine water mist/sprinkler until 4-5 leaves were seen. Seedlings emerging in each pot were counted on 12 January, 19 January, 26 January, and 28 February 2004 (4, 11, 18, 51 d after transfer to the CEL).

3.2.3 Seedling emergence, leaf count and time to bolting

In each pot of each of the 20 blocks, one representative plant was labelled with a coloured head pin for tracking of leaf and floral development. Number of days to first flower from the time of transfer of the pots into the controlled environments was recorded in each plant. These days represented time from the start of warm conditions suitable for seed germination. Number of leaves was recorded, periodically, a leaf being considered as emergent when 2-3 mm in length.

3.2.4 Leaf measurements and trichome count

Approximately ten representative whole plants in each treatment were collected from 20 blocks randomly during the main lighting period, for study of leaf morphology and the occurrence of trichomes. Fresh leaves were observed under a dissecting microscope. The number of trichomes was recorded for the petiole, lamina margin, and abaxial and adaxial surfaces. After trichome counting, each leaf was stuck on a transparent sheet and photocopied for later measurement of leaf dimension parameters. Leaf length and width, and petiole length were measured using electronic vernier callipers (Sylvac). Measurements were taken from fully expanded leaves, typically from leaf 1 (most proximal in the rosette) to leaf 10 (most distal), and up to leaf 14 in some plants. Harvests were made between 22 January 2004 and 1 April 2004. As there were

insufficient plants in 30 d treatment, only plants from 0, 10 and 20 d chilling were collected for the study.

3.2.5 Silique, inflorescence branch count

Silique and inflorescence branch count was taken on 20 March 2004 in LD, SD and in treated SD plants.

3.2.6 Shoot apical meristem histology

After the removal of leaves for trichome counting and leaf measurements, each of the remaining shoots, each with its shoot apical meristem (SAM), was fixed in formalin-acetic acid (FAA) fixative for 24 h. Then the tissue was dehydrated, wax infiltrated, embedded and sectioned as described earlier for the microscopy of floral buds (Chapter #2). After sectioning the tissue was stained with Fastgreen/Safranin following the hydration/staining/dehydration gradients shown (Fig 3.1).

3.2.7 Statistical analyses

The experiment design was 4 x 4 factorial design with 10 replicates in each of the 20 blocks per daylength and chilling combination. All variables were tested by analysis of variance (ANOVA) using Minitab software (Ver. 14.1). The General Linear Model of ANOVA was used to test the effects.

3.2.8 Partial repeated experiment

On 5 February 2004, because of a fault in the management of the CEL, the auxiliary lights remained on for about 2 h in SD room after the nominal end of the light period. The experiment for this treatment was repeated. In this, the pots with seeds were chilled for 7 d and then transferred to SD room in CEL. For these plants, number of leaves to

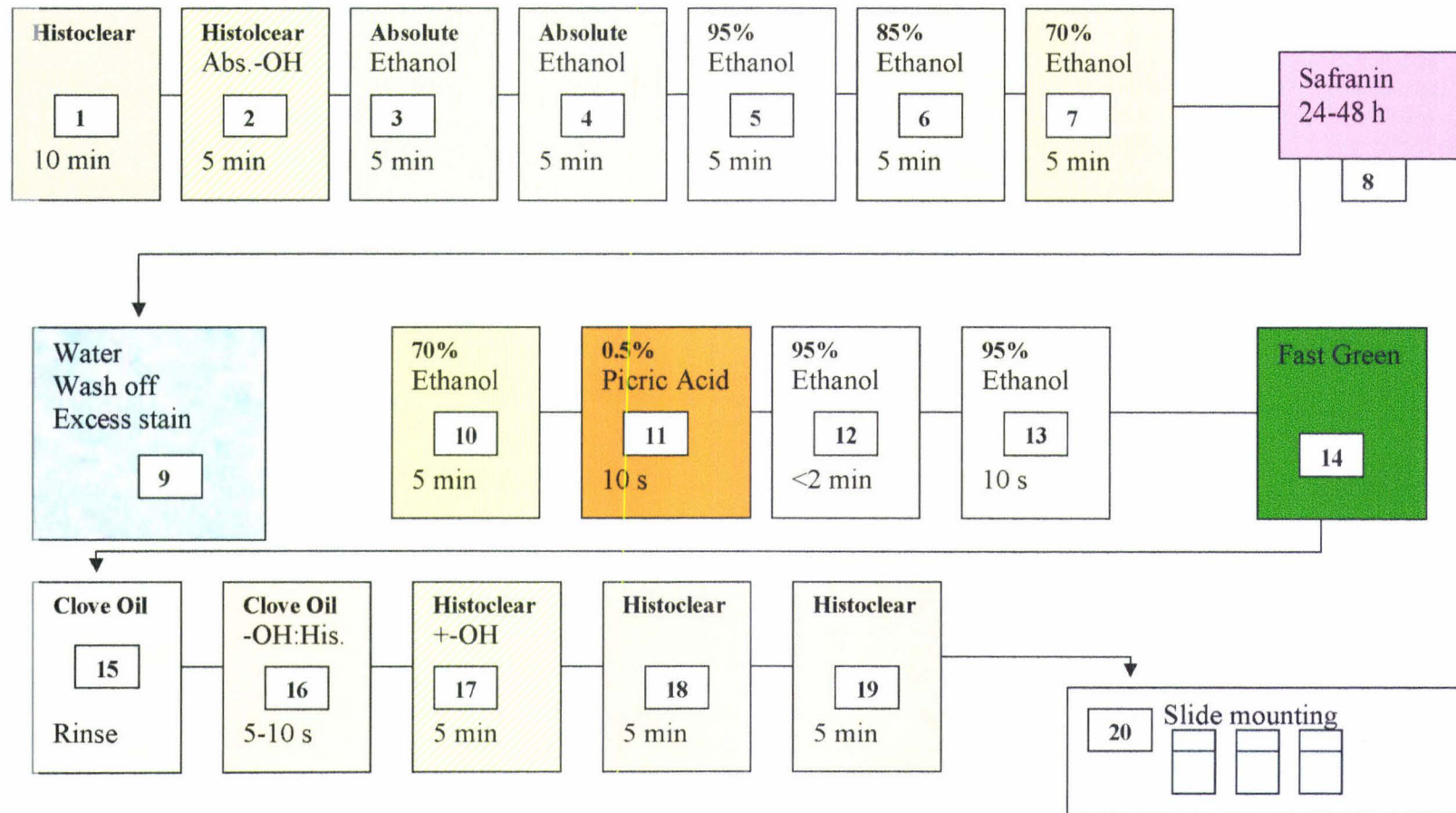


Figure 3.1 Staining schedule with safranin and fast green
(Adapted from Nickless, L.)

first bolt and time to first bolt was observed. Their behaviour was not distinguishable from the SD plants in the main experimental plants. No further data were collected from these plants.

3.3 Results

3.3.1 Seedling emergence

Four days after transfer from 4°C to the growth conditions in the CEL, almost complete seedling emergence had occurred for seed treated for 10, 20, and 30 d, with final emergence decreasing with increasing length of chilling treatment. Emergence of seedlings in the treatment that received no chilling was initially low. However, this steadily increased over the next 47 d to ultimately exceed that of all the other chilling treatments. Emergence was not affected by daylength (Fig 3.2 A, B).

3.3.2 Leaf emergence

There was a significant effect of daylength and chilling on the number of leaves to first bolt ($p < 0.001$). The leaf count was greater in plants grown under short days and in transferred treatments, compared to plants under long days. Further, with more days of chilling, the number of leaves to first bolt was less in long and short days (Fig 3.3). However, there was no significant interaction effect of daylength and chilling on the leaf number to first bolt ($p = 0.155$, Appendix II).

3.3.3 Flowering

Although the earliest flowering appeared in treated plants (SD/LD/SD1) exposed to 20 d of chilling (Fig 3.4 C), the flowering percentage was higher and occurred more synchronously in plants exposed to long days and 10 or 20 d of chilling. The chilling treatments applied, 0 d, 10 d, 20 d, and 30 d, prior to LD exposure had a significant effect on the percentage of plants flowering. Plants that had undergone 10 d and 20 d of

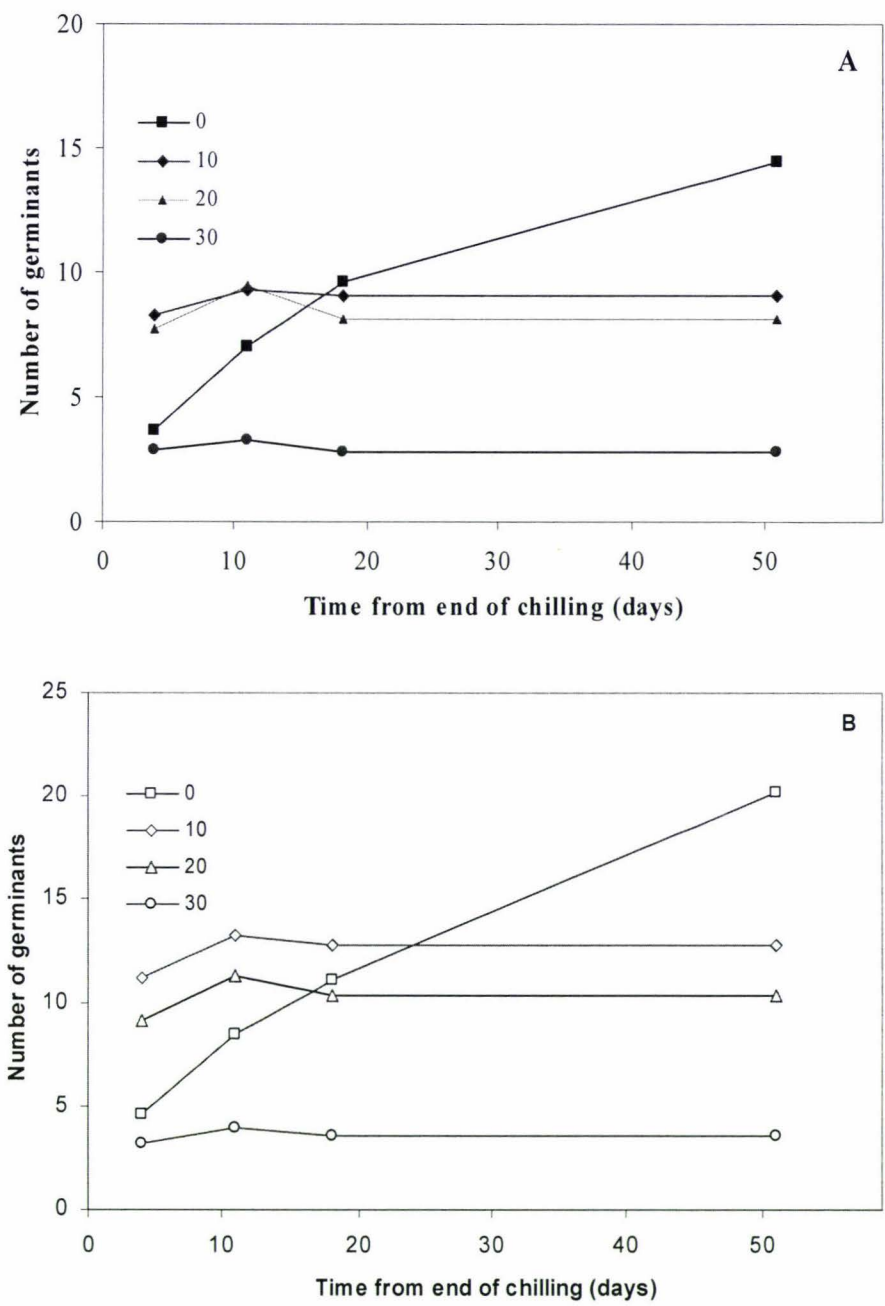


Figure 3.2 Mean number of seedlings emerging per pot after chilling treatments applied to imbibed seed for 0, 10, 20 or 30 days following transfer to controlled environments under A, LD conditions; B, SD conditions.

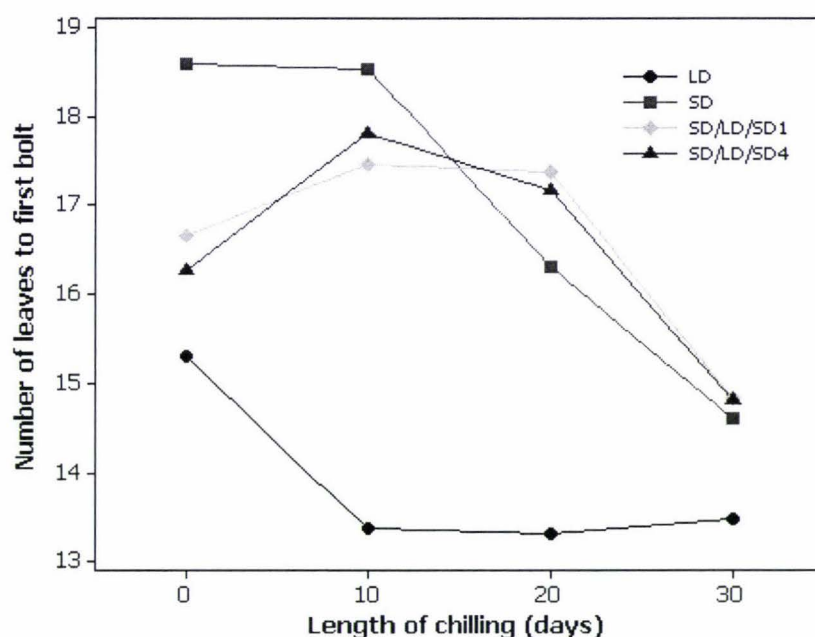


Figure 3.3 Number of leaves to first bolt in 0, 10, 20 and 30 d of chilling under the LD, SD and two SD/LD/SD treatments.

chilling prior to LD reached nearly 100 % bolting (Fig 3.4 B, C), whereas the plants with 30 d of chilling prior to LD reached nearly 80% of bolting (Fig 3.4D), and the plants under LD and 0 d chilling reached 70% of bolting (Fig 3.4A). On the other hand, there was no significant difference in percentage of bolting among short daylength and treated plants. Even though there was no effect of chilling prior to short day exposure, bolting started in a small number of plants at the same time as in long days. However, the flowering was non-synchronous and spread over time compared to the synchronous bolting observed in the plants exposed to LD after 10 or 20 d of chilling.

3.3.4 Leaf measurements

The environmental treatments applied to *P. exilis* plants had different effects on petiole length, leaf length, and leaf width of leaves 1-10. Daylength and chilling had significant effects on petiole length ($p < 0.001$). Further, the interaction between daylength and chilling also had a significant effect on petiole length ($p < 0.001$) (Fig 3.5 A).

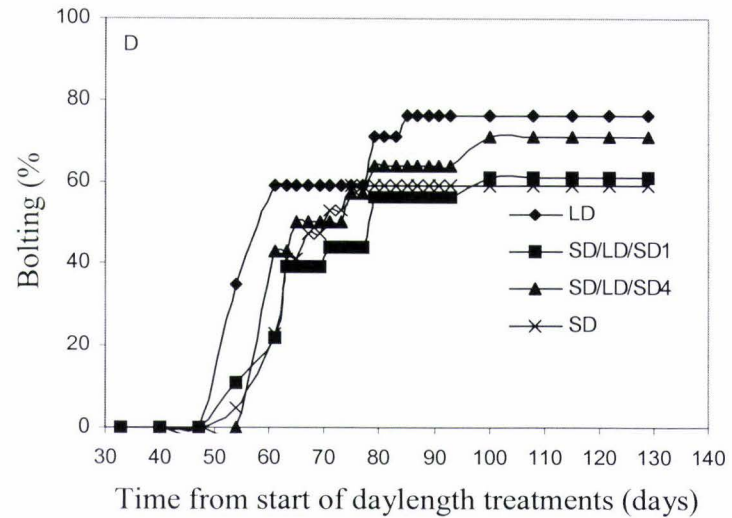
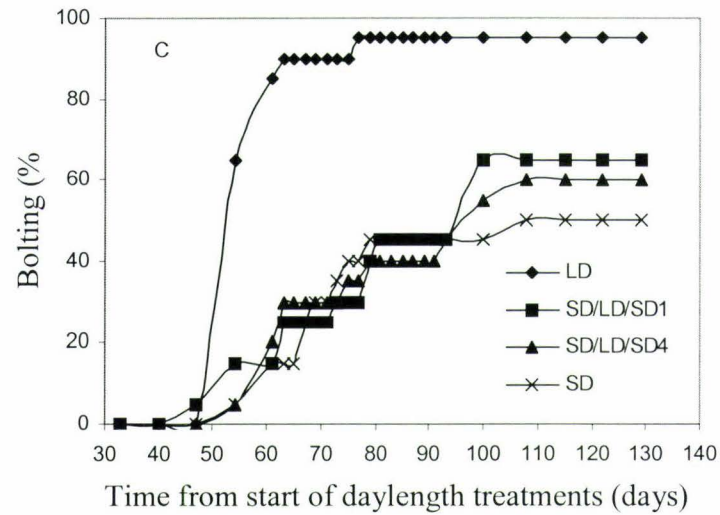
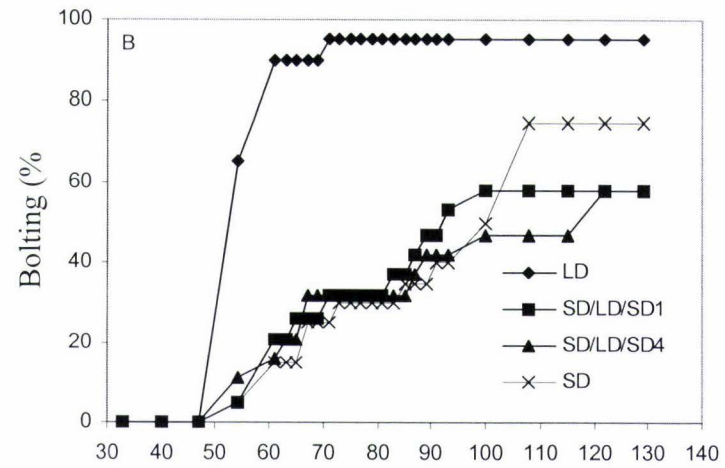
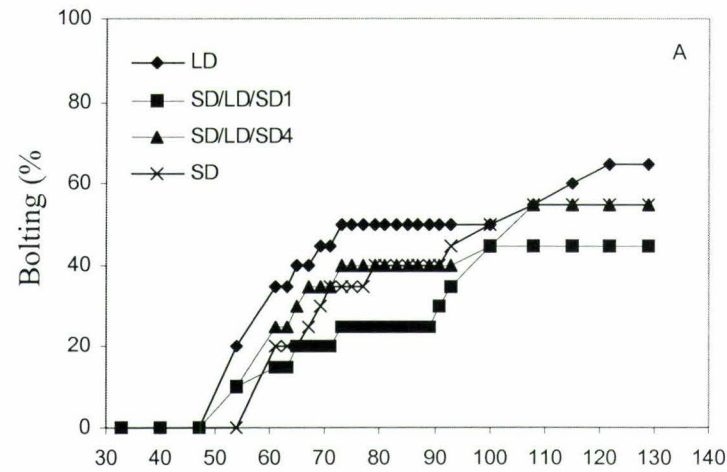


Figure 3.4 Bolting percentages in LD, SD and in two SD/LD/SD treatments after A, 0, B, 10, C, 20 and D, 30 d of chilling.

Daylength and chilling also had a significant effect on blade length ($p < 0.001$). However, the daylength*chilling interaction was not significant ($p = 0.073$) (Fig 3.5B). ANOVA performed on blade width showed different effects of daylength and chilling. Daylength had a significant effect on blade width ($p < 0.001$), whereas chilling and the interaction between daylength and chilling had no significant effect ($p > 0.005$) (Fig 3.5C). The ratio between blade length and width was higher in the plants with 10 d of chilling compared to plants with 0 and 20 d of chilling under SD, whereas in other plants there was not much difference (Fig 3.5 D).

By taking leaf number into account, there was no significant difference in any of the treatments (Fig 3.6, 3.7, 3.8; Appendix II).

3.3.5 Trichomes

Daylength, chilling and the interaction between daylength and chilling had a significant effect on the number of trichomes on the abaxial surface ($p = 0.004$, $p < 0.001$, $p = 0.002$, respectively). The duration of chilling showed a clear effect on the number of trichomes on the abaxial surface. Trichome count was higher on the abaxial surface of plants with no chilling. However, with an increase in duration of chilling the trichome count on the abaxial surface was lower (Fig. 3.9; Appendix II) The environmental treatments also had a significant effect on the number of trichomes on margins and petiole ($p < 0.001$). However, the interaction had no significant effect on trichome number of margins and petiole ($p = 0.66$, $p = 0.151$ respectively) (Fig. 3.10; Fig. 3.12; Appendix II). On the other hand, daylength, chilling and their interaction had no significant effect on trichomes on the adaxial surface ($p = 0.125$, $p = 0.886$ and $p = 0.726$, respectively) (Fig. 3.11; Appendix II).

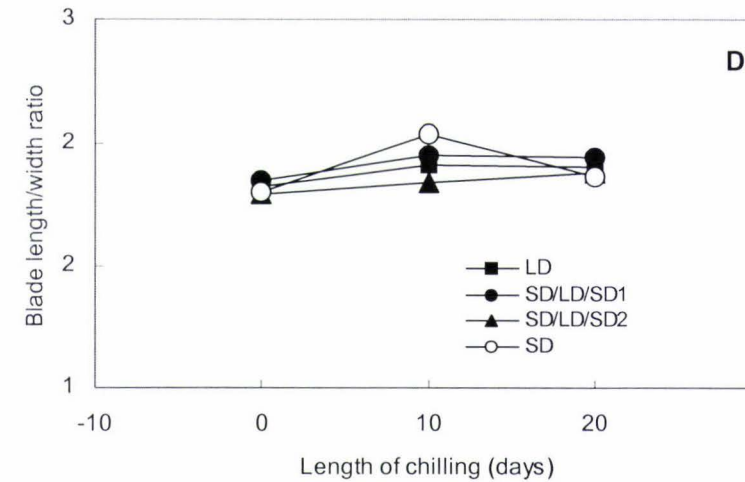
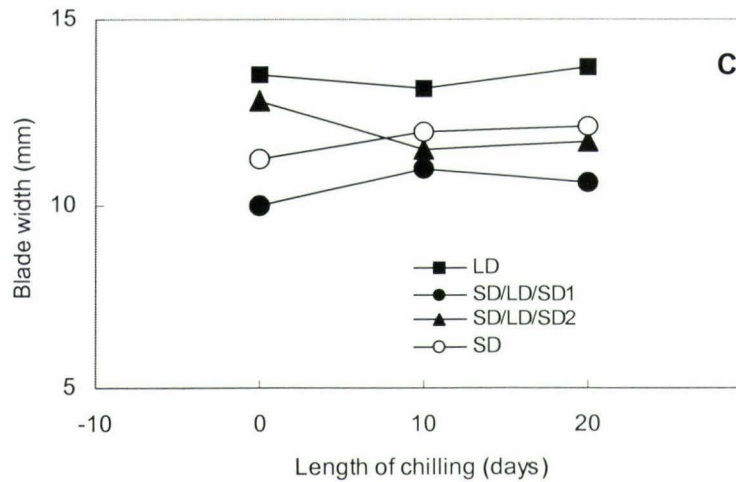
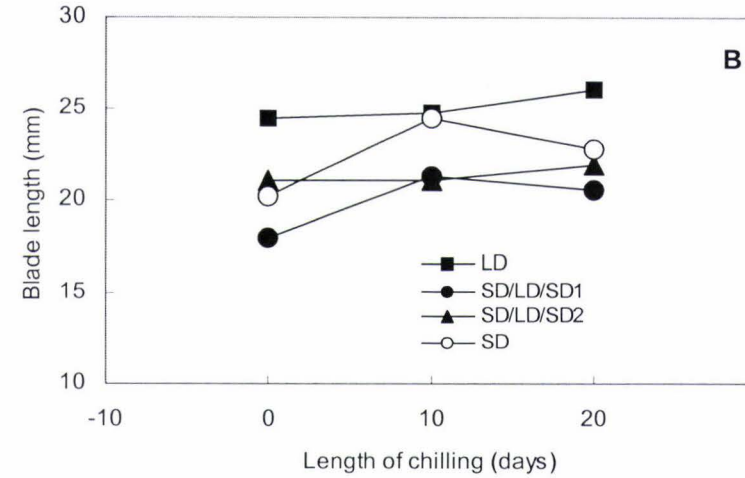
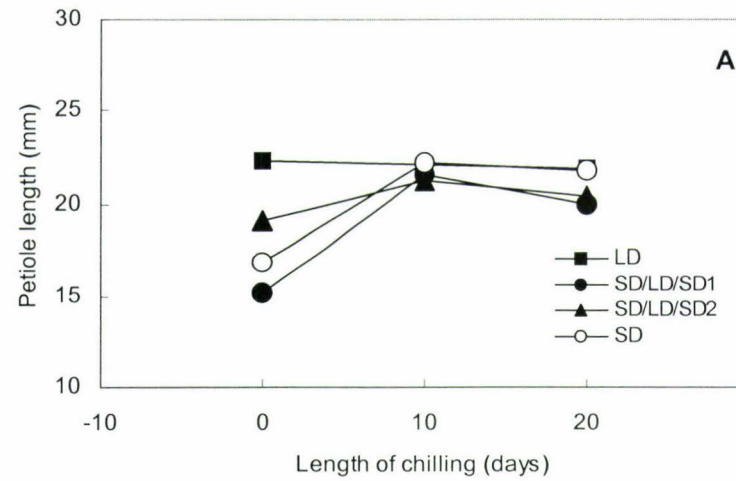


Figure 3.5 Effect of daylength and length of chilling across leaves 1-10 on A, petiole length; B, blade length; C, blade width; D, blade length/width ratio.

3.3.6 Silique and inflorescence branches count

Daylength had a significant effect on the number of siliques per plant ($p < 0.001$), in which the number of siliques per plant was higher in the LD treatment than in the other treatments, which did not differ significantly (Fig 3.13A).

The interaction of daylength and chilling treatments approached significance ($p = 0.07$). While the silique number per plant rose and then fell with increasing length of chilling in the LD and SD treatments, there was no consistent response to chilling evident in the two SD/LD/SD treatments (Fig 3.13A).

As for siliques per plant, there was an increase in number of inflorescence branches (primary bolt) per plant and then a decline with increasing length of chilling in the LD and SD treatments, and an inconsistent response to chilling in the SD/LD/SD treatments (Fig. 3.13B). Neither of the main effects, or the interaction of daylength and duration of chilling, was significant ($p > 0.05$) for branch number.

3.3.7 Histology

To observe any developmental changes occurring in the meristem in *P. exilis*, under different daylength conditions, ten plants from each CEL room with 20 d of chilling were collected at fortnight intervals and sectioned. Representative pictures of sections are included in this report.

After 14-45 days after transfer to CEL, in both SD and LD treatments plants were at different vegetative states. At 14 d after the start of daylength treatments, in both LD and SD plants slight protrusion of the apical meristem was seen and it appears to be at the same stage under both conditions (Fig 3.14A, E). At 30 d, developmental differences were seen. Under long days, protrusions of axillary meristems and accelerated cell

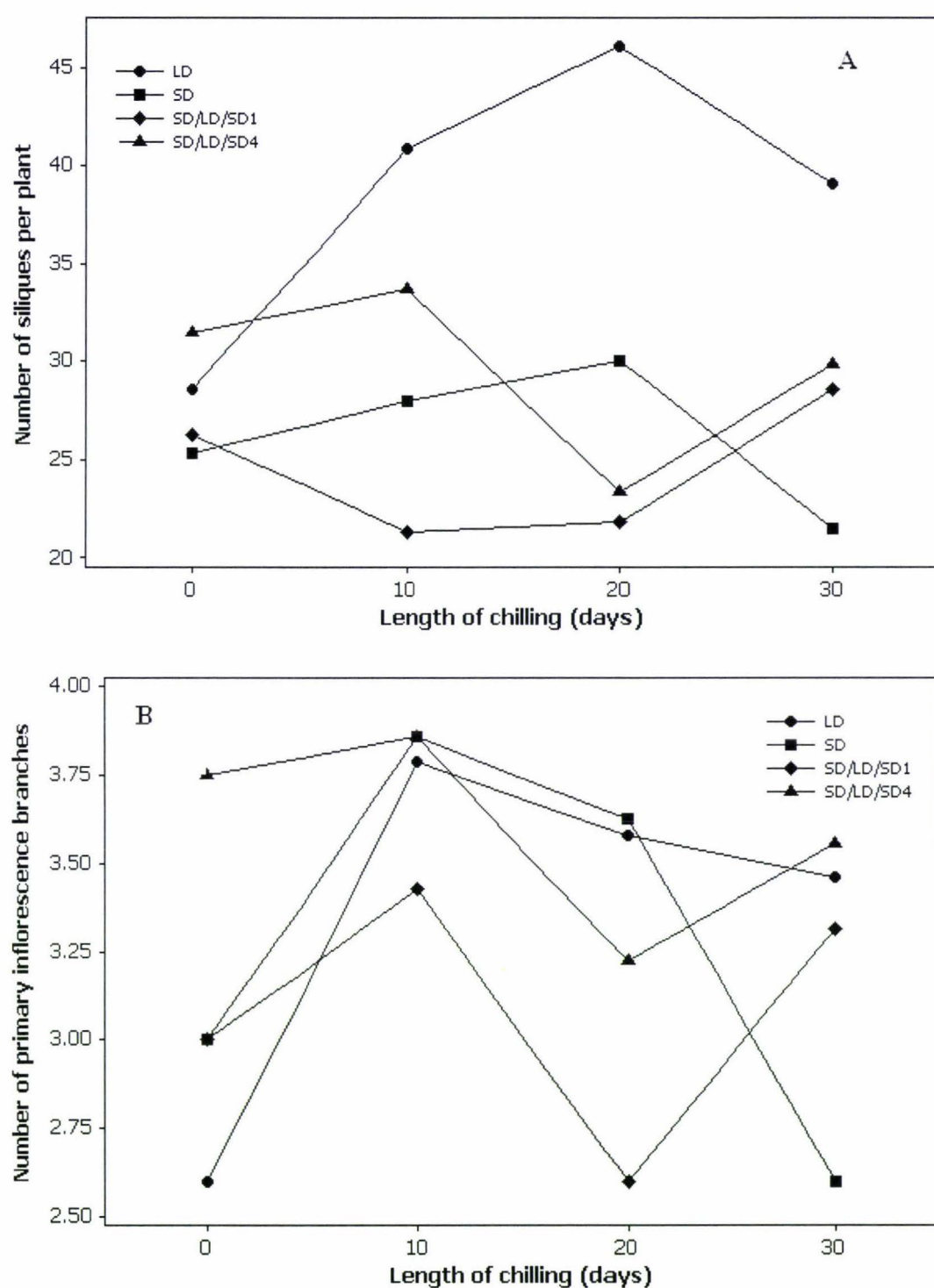


Figure 3.13 Effect of daylength and length of chilling on A, number of siliques per plant; B, number of primary inflorescence branches per plant.

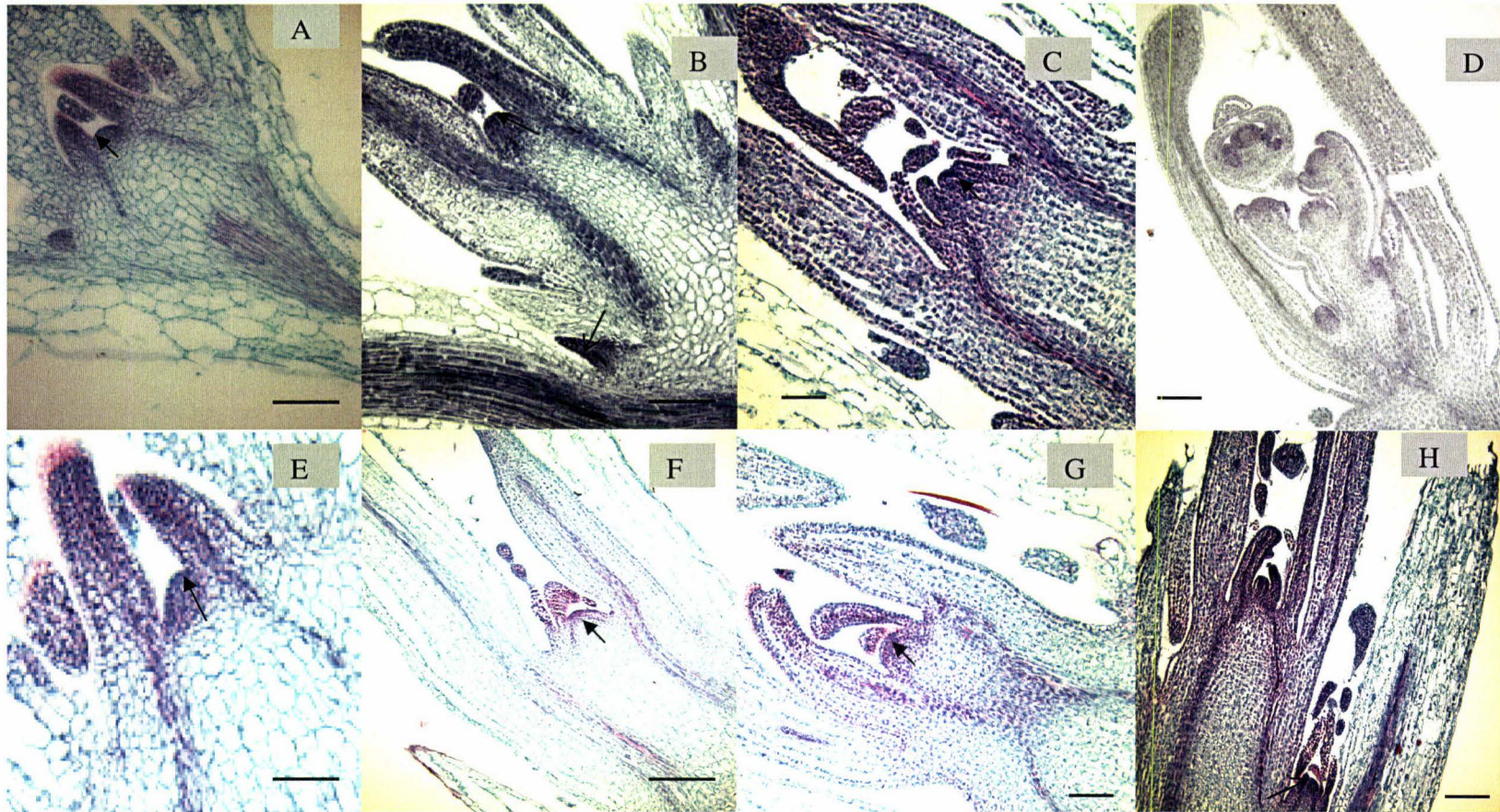


Figure 3.14 Sections of meristems of *P. exilis* plants exposed to 20 d of chilling under long and short days from 15 d to 60 d after the start of daylength treatments. A, E, 15 d under long days and short days, respectively; B, F, 30 d under long days and short days, respectively; more axillary growth was seen under long days. C, G, 45 d under long days and short days, respectively; D, H, 60 d under long and short days, respectively; floral initiation was seen in most of the sections under long days. (These pictures are the representatives of ten sections in each treatment) → SAM; → axillary meristem. Scale bars, 100 μ m.

divisions were seen, whereas under short days such axillary growth was not seen (Fig 3.14B, F). At 45 short days, protrusions of axillary meristems were seen and under long days, rapid cell divisions were seen (Fig 3.14C, G). At 60 d, emergence of inflorescence could be seen in LD plants while most of the SD plants were still in vegetative stage producing axillary meristems (Fig 3.14D, H).

3.4 Discussion

In many plant species, including *A. thaliana*, flowering is promoted by a period of exposure to low temperature through a process known as vernalisation. The process of vernalisation is a quantitative response to prolonged periods of cold (Sheldon et al., 2000), in which the extent of the promotion of flowering by cold treatment is proportional to the duration of the cold treatment.

In *P. exilis*, chilling of imbibed seeds increased the speed of germination. However, there was no absolute requirement of chilling for germination. Nordborg & Bergelson (1999), on a study in *A. thaliana* ecotypes, showed that a short cold treatment had a positive effect on the probability of germination compared to no cold treatment, while a long cold treatment could have either a negative or a positive effect compared to the short cold treatment. Similarly, in the present study on *P. exilis*, germination was greater in seeds exposed to 10 or 20 d of chilling compared to no chilling. However, exposure to 30 d of chilling had a negative effect on germination. On the other hand, daylength had no effect on germination of seeds in *P. exilis*.

In the present study, the flowering response in *P. exilis* was positively correlated to daylength and chilling. The plants that were exposed to LD with 10 or 20 d of chilling reached maximum bolting and bolted more synchronously compared to other treatments, in which the bolting was non-synchronous, although they started to bolt at the same time as long day plants with 10 or 20 d of chilling. This shows the combined effects of daylength and chilling on bolting, which supports that flowering behaviour depends strongly on light conditions as well as cold treatments, and that these effects are known to interact (Martinez-Zapater et al., 1994). On the other hand, similar to germination,

long exposure to chilling, 30 d, reduced the percentage of bolting. This supports the idea that there is a point of saturation for cold treatment (Martinez-Zapater et al., 1994; Lee and Amasino, 1995). In *P. exilis*, 10-20 days of chilling is sufficient to accelerate bolting. However, with further increase in chilling length there was a decrease in bolting percentage as well as in germination. The extended chilling might have resulted in the seeds being non-viable or there was imposition of secondary dormancy in seeds. However, the decrease in bolting percentage needs further investigation.

Sectioning of the apical meristems showed that floral induction was earliest in plants exposed to long days. Under LD, floral induction was evident in between 45-60 days after transfer to CEL, whereas in SD the floral induction was slower. This strongly supported the physiological study on flowering percentage.

In contrast to germination and bolting percentage, the effects of chilling and daylength were clear with respect to number of leaves to primary bolt. In this, plants under long days had fewer leaves compared to plants under short days. Further, with increase in length of chilling the leaf number to primary bolt decreased.

Thus, in *P. exilis*, the duration of cold treatment is not quantitatively regulated in the promotion of flowering. However, certain levels of chilling enhanced the flowering. Further, long days promoted flowering. This shows that, for flowering, *P. exilis* is facultative to both long days and cold treatment.

Chapter 4. Isolation of meristem identity genes from *Pachycladon exilis*

4.1 Introduction

To study some basic aspects of plant biology, such as flowering, the model species *Arabidopsis* has been used, and the genes involved have been identified through molecular and genetic approaches. This basic information has since been used in comparative studies among species. In studies with *Arabidopsis*, the process of flowering involves some 80 genes, which includes the flowering time genes and meristem identity genes. The meristem identity genes are subdivided into inflorescence (shoot) meristem identity genes, which include *TFL1*, and floral meristem identity genes, which include *LFY*. Homologues and orthologues of *LFY* and *TFL1* have been studied in other species. In some cases, different regulatory processes or new functions have been found.

In *Arabidopsis*, *LFY* is a transcription factor that plays an important role in determining floral meristem identity. In *Antirrhinum*, *FLO*, a homologue of *LFY* has almost the same role. Loss of function mutants of these genes results in the transformation of flowers into indeterminate secondary shoots (Coen et al., 1990; Weigel et al., 1992). On the other hand, it has been shown that *LFY* and its homologues are sufficient to advance floral initiation and development in *Arabidopsis*, poplar (Weigel & Nilsson, 1995), and citrus (Pena et al., 2001). In addition, modification of inflorescence architecture, from indeterminate to determinate, was observed in *Arabidopsis* transformed with a chimeric *LFY* gene expressed constitutively under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Esumi et al., 2005).

TFL1 is a putative regulator gene involved in the control of flowering time and floral development, which encodes a protein that plays a role in signalling, possibly as an inhibitor of mitogen-activated protein kinase pathways (Corbit et al., 2003). A loss-of-function mutant of this gene converts the inflorescence into a terminal flower and the mutants bolt and flower earlier than wild type (Shannon & Meeks-Wagner, 1991; Bradley et al., 1997). In *Antirrhinum*, *CEN*, the homologue of *TFL1*, plays the same role

as in *Arabidopsis*. *cen* mutants result in the conversion of the inflorescence from indeterminate to determinate (Bradley et al., 1996). A study in pea showed three homologues of *TFL1*, among which one is involved in inflorescence development and another in the floral transition (Foucher et al., 2003).

To investigate the biological functions of *LFY* and *TFL1* in *P. exilis*, which is a short-lived perennial, equivalents were isolated and sequences were compared with other species.

4.2 Materials and methods

4.2.1 RNA extraction

Young floral buds from *P. exilis* were collected and immersed immediately in liquid nitrogen and then stored at -80°C for later use. RNA was extracted from these floral buds using TRI reagent method modified from the supplier's (ProGENZ Limited, Auckland, New Zealand) instructions for a mini-preparation.

Approximately 100 mg of floral buds were taken and ground to a fine powder using liquid N_2 in a baked mortar and pestle. The powder was transferred to an Eppendorf containing 1 ml of TRI reagent and the solution was homogenized by vortexing for 10-20 s. Later, the homogenized mix was centrifuged for 2 min at 10,000 rpm at 4°C . The supernatant was transferred to a new tube and stored at room temperature for 5 min. To this, 200 μl of chloroform (isoamyl alcohol-free) was added and the mix was shaken vigorously for 15 s and stored at room temperature for 10 min. After spinning for 15 min at 10,000 rpm at 4°C , the upper aqueous phase was transferred to a new tube. To this, 300 μl of isopropanol and 300 μl of salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) were added and spun at 10,000 rpm for 8 min at 4°C . The upper supernatant was decanted and the RNA pellet was washed by vortexing with 1.2 ml of 75% (v/v) ethanol and centrifuged at 700 rpm for 5 min at 4°C and ethanol was removed. This washing was repeated three times and the RNA pellet was air-dried for 3-5 min. Finally the RNA pellet was dissolved in 30 μl RNase-free water and 1.2 μl of 25

X RNasecure (Ambion) was added. The mix was heated to 65°C for 20 min and stored at -20°C.

4.2.2 Estimation of nucleic acids

The concentration of nucleic acids was measured using NanoDrop ND 1000 without any dilution of the samples. Blanking was done using TE buffer or water for DNA or RNA respectively. Then 1 µl of sample was loaded onto the measurement pedestal and the concentration was measured. Successive samples were measured by simple wiping with a soft wipe in between samples.

4.2.3 Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcriptase reactions were performed using Expand Reverse Transcriptase (Roche) and oligo (dT)₁₅ to prime the reactions. 1 µg of total RNA was added to 1 µl of oligo (dT)₁₅ and DEPC treated water to make a total volume of 10 µl. This mix was heated to 65°C for 10 min to denature RNA and primers and cooled immediately on ice. To this, 10 µl of RT cocktail was added, which contains 4 µl of 5X RT buffer, 2 µl of 100 mM DTT, 0.5 µl of RNase Inhibitor (40U/µl) (Roche), 1 µl of 20 mM dNTPs, 1 µl of Expand RT (50 U/µl) and 1.5 µl of DEPC treated water to make a final volume of 10 µl. This final reaction mix was incubated at 48°C for 30 min and stored at -20°C.

4.2.4 Polymerase chain reaction (PCR)

An aliquot (2 µl) of cDNA was amplified using 0.2 µl of 5 U. µl⁻¹ Taq polymerase in 2 µl of 10 X Taq buffer and in the presence of 1.25 mM of MgCl₂, 0.2 µM dNTPs, primers (0.5 µM each), dH₂O (to 20 µl).

Reactions were carried out using initial denaturation step of 4 min at 94°C followed by 30 s at 94°C, 30 s at 48°C, 60 s at 72 °C for 30 cycles. The final extension was carried out for 7 min at 72°C. The same cycling conditions were used for all the primer sets.

4.2.5 Degenerate primer sequences

Two sets of primers were used for the isolation of *LFY* from *P. exilis*. The LFY1 and LFY2 primer set was taken from Oh & Potter (2003) and LFYL and LFYR set was taken from Sreekantan et al. (2004). The sequences of the primers are shown below:

LFY1: CAC CCA CGA CCI TTY ATI GTI ACI GAR CCI GGI GA
LFY2: CCT GCC IAC RTA RTG ICK CAT YTT IGG YTT

LFYL: GCG AAT TCA CIA AYC ARG TIT TYM GIY AYG C
LFYR: CGG ATC CGT GIC KIA RIY KIG TIG GIA CRT A

The published primers of *TFL1* (Mimida et al., 2001) were used for the isolation of *TFL1* from *P. exilis*. These primers span two introns of *TFL1*. The primer sequences were:

TFL1: ATGAYAGACCCWGATGTKCC
TFL2: AAANACRAACCGRTGKATMCC

The *ACTIN* primers used by Sreekantan et al. (2004) were used for the isolation of the equivalents in *P. exilis*. The sequences of the primers were:

ActinF: GCGAATTCTTCACCACYACHGCGYGARCG
ActinR: GCGGATCCCCRATCCARACACTGTAYTTCC

4.2.6 Gel electrophoresis and extraction

PCR products were run on a 2% agarose gel and visualized after ethidium bromide staining. Bands of expected size were cut under a UV transilluminator and the product was purified using a Qiagen Gel Extraction kit according to the manufacture's instructions. The purified product was then quantified on a 1.5% agarose gel using a low DNA mass ladder (Roche).

4.2.7 Sequencing

Sequencing was carried out by the Allan Wilson Sequencing Service, Palmerston North, New Zealand, using the Big Dye Terminator Reaction V 3.0 and capillary separation.

4.2.8 Tree building

The alignment of sequences and building of phylogenetic trees were done using vectorNT software 9.0.0 (InforMax). Vector NTI Advance builds a phylogenetic tree using the neighbour joining method (NJ) of Saitou & Nei, which works on a matrix of distances between all pairs of sequences to be examined. The value in the parenthesis following the molecule name is the calculated distance value.

4.3 Results

4.3.1 Isolation of *PeLFY*

The sequence of about 150bp (Fig 4.1A) was isolated from the cDNA of *P. exilis* using degenerate primers LFY1 and LFY2 and also another sequence of about 300bp (Fig 4.1B) was isolated using LFYL and LFYR degenerate primers. The sequences isolated overlapped with each other. So both the sequences were combined and specific primers were built.

The sequence of the identified gene fragment covered about 32% of the expected size of the gene. The nucleotide sequence of this 375 bp cDNA fragment was 95% identical to the *A. lyrata* LFY gene (Gen Bank accession number AF466802.1) and 93% identical to *A. thaliana* floral meristem identity control protein LEAFY (LFY) mRNA (Gen Bank accession number NM 125579.1, Fig 4.2).

The deduced amino acid sequence of the protein encoded by the isolated cDNA fragment showed similarity with the *FLORICAULA/LEAFY* sequence. By comparing the expected *P. exilis* LFY protein sequence with data from conserved domain databases

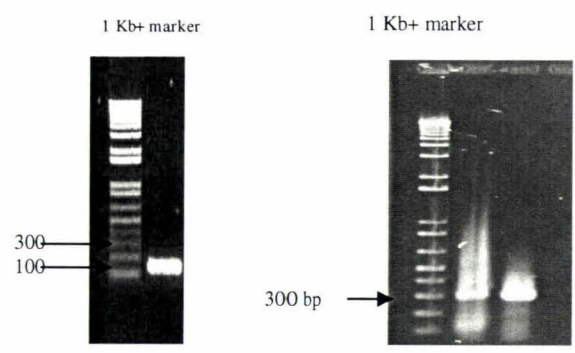


Figure 4.1 Isolation of *PeLFY* by RT-PCR. A, Bands approximately 150 bp in size that sequenced to give a fragment of *PeLFY*; B, Bands approximately 300 bp in size that sequenced to give a fragment of *PeLFY*.

<i>P. exilis</i>	1	CCGTTTATGGTGACGGAGCCGGGGAAGTGGCACGTGGCAAAAAGAACGGTATAGATTAT	60
<i>A. thaliana</i>	706	CCGTTTATCGTAACGGAGCCTGGGGAAGTGGCACGTGGCAAAAAGAACGGCTTAGATTAT	765
<i>A. lyrata</i>	2291	CCGTTTATCGTAACGGAGCCTGGGGAAGTGGCACGTGGCAAAAAGAACGGTTTAGATTAT	2350
***** ** ***** *****			
<i>P. exilis</i>	61	CTGTTCATTTGTACGAACAATGCCGTGAGTTCCTTCTTCAGGTTCAGACTATAGCTAAA	120
<i>A. thaliana</i>	766	CTGTTCACCTTGTACGAACAATGCCGTGAGTTCCTTCTTCAGGTCCAGACAATTGCTAAA	825
<i>A. lyrata</i>	2351	CTGTTCACCTTGTACGAACAATGCCGTGAGTTCCTTCTTCAGGTCCAGACAATTGCTAAA	2410
***** ***** ***** ** *****			
<i>P. exilis</i>	121	GACCGTGGCGAAAAATGCCCCACCAAGGTGACGAACCAAGTGTTCAGGTACGCGAAGAAA	180
<i>A. thaliana</i>	826	GACCGTGGCGAAAAATGCCCCACCAAGGTGACGAACCAAGTATTCAGGTACGCGAAGAAA	885
<i>A. lyrata</i>	2411	GACCGTGGCGAAAAATGTCCCACCAAGGTGACGAACCAAGTGTTCAGGTACGCGAAGAAA	3646
***** ***** ** *****			
<i>P. exilis</i>	181	TCTGGAGCGAGTTACATAAACAAGCCCAAATGCGCCACTACGTCCACTGTTATGCTCTC	240
<i>A. thaliana</i>	886	TCAGGAGCGAGTTACATAAACAAGCCTAAAATGCGACACTACGTTCCTGTTACGCTCTC	945
<i>A. lyrata</i>	3647	TCGGGAGCGAGTTACATAAACAAGCCCAAATGCGACACTACGTCCACTGTTACGCTCTC	3706
** ***** ***** ***** *****			
<i>P. exilis</i>	241	CACTGCCTAGACGAAGACGCTTCAAACGCTCTCCGAAAAGCGTTTAAAGAACGCGGTGAG	300
<i>A. thaliana</i>	946	CACTGCCTAGACGAAGAAGCTTCAAATGCTCTCAGAAGAGCGTTTAAAGAACGCGGTGAG	1005
<i>A. lyrata</i>	3707	CACTGCCTAGACGAAGACGCTTCAAACGCTCTCCGAAAGAGCGTTTAAAGAACGCGGTGAG	3766
***** ***** ***** ** *****			
<i>P. exilis</i>	301	AACGTTGGCTCGTGGCGTCAGGCTTGTTACAGGCCACTTGTTAACATCGCTTGTCGTCAT	360
<i>A. thaliana</i>	1006	AACGTTGGCTCATGGCGTCAGGCTTGTTACAAGCCACTTGTTGAACATCGCTTGTCGTCAT	1065
<i>A. lyrata</i>	3767	AACGTTGGCTCGTGGCGTCAGGCTTGTTACAAGCCACTTGTTGAACATGCTTGTCGTCAT	3826
***** ***** ***** *****			
<i>P. exilis</i>	361	GGCTGGGATATAGAC	375
<i>A. thaliana</i>	1066	GGCTGGGATATAGAC	1080
<i>A. lyrata</i>	3827	GGCTGGGATATAGAC	3841

Figure 4.2 Alignment of predicted nucleotide sequence of *PeLFY* of *P. exilis* with *LFY* sequence of *A. thaliana* and *A. lyrata*. The sequences were obtained from GenBank (accession numbers are given in parentheses): *A. thaliana* (NM 125579. 1), *A. lyrata* (AF466802.1). Asterisks indicate identical nucleotides.

A

```
PeLFY : 1 PFMVTEPGEVARGKKNGIDYLFHLYEQCREFLQVQTIAKDRGEKCPTKVTNQVFRYAKK 60
FLO_LFY: 238 PFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLQVQSIAKERGEKCPTKVTNQVFRYAKK 297
** *****
PeLFY : 61 SGASYINKPKMRHYVHCYALHCLDEASNALRKAFKERGENVGSWRQACYRPLVNIACRH 120
FLO_LFY: 298 AGASYINKPKMRHYVHCYALHCLDEASNALRRAFKERGENVGAWRQACYKPLVAIAARQ 357
*****
PeLFY : 121 GWDID 125
FLO_LFY: 358 GWDID 362
*****
```

B

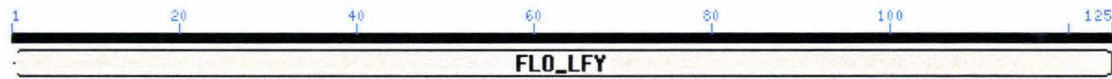


Figure 4.3 Alignment of the predicted amino acid *P. exilis* LFY sequence with a conserved domain of FLORICAULA/LEAFY protein. A, the deduced *P. exilis* LFY sequence shows high similarity to the conserved domain of FLO/LFY protein (CDD accession number 6544). Asterisks indicate identical amino acid residues. B, the part of FLO_LFY conserved domain with which the deduced PeLFY shows similarity (the bottom rectangle indicated as FLO_LFY domain and the digits represent numbers of amino acid residues) (based on RPS-BLAST alignment).

(CDD) revealed that the predicted *P. exilis* LFY protein showed identity to the C-terminal part of the 393 residues long conserved domain of the FLO/LFY protein (CDD accession number 6544; Fig 4.3A, B).

4.3.2 Isolation of *PeTFL1*

The sequence of about 160 bp (Fig 4.4) was isolated from the cDNA of *P. exilis* using degenerate primers TFL1 and TFL2. The isolated nucleotide sequence of 163 bp cDNA fragment showed 97% identity with *A. thaliana* terminal flower 1 (*TFL1*) mRNA (GenBank accession number U77674.1) and 96% identity with the exon sequences of *A. lyrata* terminal flower 1 gene (GenBank accession number AF466817.1, Fig 4.5).

A CDD search revealed that the deduced amino acid sequence of the protein encoded by the isolated cDNA fragment matched with Raf Kinase Inhibitor Protein (RKIP), bacterial/archaeal Phosphatidylethanolamine-Binding Protein (PEBP_RKIP), Phosphatidylethanolamine-binding protein (PBP) (Fig 4.6A, B). The accession numbers of the sequences are given in parentheses: RKIP (27619), PEBP_RKIP (27617), and PBP (25700).

4.3.3 Isolation of actin

The house keeping gene actin was isolated from *P. exilis* using degenerate primers. Approximately 420 bp band was seen on the gel (Fig 4.7). The isolated nucleotide sequence showed 96% identity with *A. thaliana* putative actin2 protein mRNA (GenBank accession number AYO96381.1, Fig 4.8). The deduced amino acid sequence of the protein encoded by the isolated fragment showed 100% identity with actin2 protein of *A. thaliana* (GenBank accession number AAM65287.1, Fig 4.9).

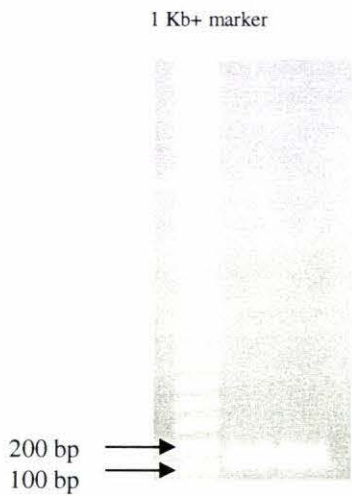


Figure 4.4 Isolation of *PeTFL1* by RT-PCR from *P. exilis*. Agarose gel showing a band at approximately 160 bp in size.

<i>P. exilis</i>	1	ATAGACCCAGATGTTCCAGGTCCTAGTGATCCCTTTCTAAAAGAACACCTGCACTGGATC	60
<i>A. thaliana</i>	232	ATAGACCCAGATGTTCCAGGTCCTAGTGACCCCTTTCTAAAAGAACACCTGCACTGGATC	291
<i>A. lyrata</i>	1239	ATTGATCCAGATGTTCCAGGTCCTAGTGACCCCTTTCTAAAAGAACACCTGCACTGGATC	1483
** ** *****			
<i>P. exilis</i>	61	GTTACAAACATTCCCGGTACAACAGATGCTACATTGGGAAAAGAGGTGGTGAGCTATGAA	120
<i>A. thaliana</i>	292	GTTACAAACATTCCCGGCACAACAGATGCTACGTTGGCAAAGAGGTGGTGAGCTATGAA	351
<i>A. lyrata</i>	1484	GTTACAAACATTCCCGGCACAACAGATGCTACGTTGGCAAAGAGGTGGTGAGCTATGAA	1627

<i>P. exilis</i>	121	TTGCCAAGGCCAAGCATAGGGAT	143
<i>A. thaliana</i>	352	TTGCCAAGGCCAAGCATAGGGAT	374
<i>A. lyrata</i>	1628	TTGCCAAGGCCAAGCATAGGGAT	1650

Figure 4.5 Alignment of predicted nucleotide sequence of *PeTFL1* of *P. exilis* with *TFL1* sequences of *A. thaliana* and *A. lyrata*. The sequences were obtained from GenBank (accession numbers are given in parentheses): *A. thaliana* (U77674.1), *A. lyrata* (AF466817.1). Asterisks indicate identical nucleotides.

A

PeLFY	:	1	IDPDVPGPSDPFLKEHLHWIVTNIPGTTDATFGKEVVS	YELPRPSI--GIHRFVF	53
RKIP	:	62	TDPDAPSRSNPKYREWHLWLVVNI	PGTNDISSGNTLSDYVGPGPKGTGLHRYVF	116
PeLFY	:	1	IDPDVPGPSDPFLKEHLHWIVTNIPGTT-----	DATFGKEVVS-----YELPRPSI-	46
PEBP_RKIP:		46	TDPDAPSRK-PKGREWLHWLVVNI	PGTDLELPAGGNISGGVQGRNDFGNIGYGGFCPPKG	104
PeLFY	:	47	-GIHRFVFK		54
PEBP_RKIP:		105	TGPHRYVFL		113
PeLFY	:	1	IDPDVPGPSDPFLKEHLHWIVTNIPGTTDATFGKEVV---	SYELPRPS--IGIHRFVF	53
PBP	:	51	VDPDAPSRSNPDLREWHLWIVVNI	PGTNDITQGTNTSGEIGYEGCPPKGTGIHRYVF	108

B

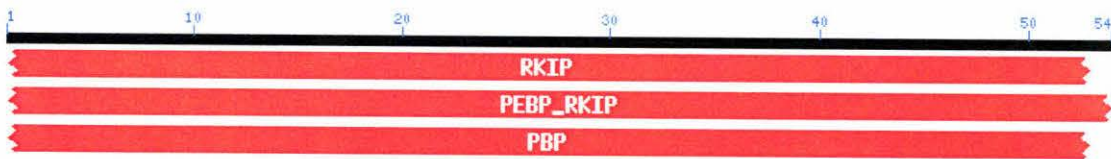


Figure 4.6 Alignment of the predicted amino acid *P. exilis* TFL1 sequence with a conserved domain of RKIP, PEBP_RKIP, PBP proteins. A, the deduced *P. exilis* TFL1 sequence shows high similarity to the conserved domains of RKIP, PEBP_RKIP, PBP proteins (CDD accession number 27619, 27617, 25700, respectively). Dashes are introduced to optimise alignment. B, the part of RKIP, PEBP_RKIP, and PBP conserved domains with which the deduced PeTFL1 showing similarity (the bottom rectangles indicated as RKIP, PEBP_RKIP and PBP domains and the digits represent numbers of amino acid residues) (based on RPS-BLAST alignment).

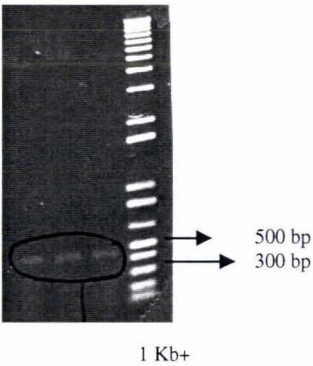


Figure 4.7 Isolation of *PeActin* by RT-PCR from *P. exilis*. Agarose gel showing a band at approximately 450 bp in size.

```

P. exilis 1 GAAATTGTGAGAGACATCAAGGAGAAGCTTTCCTTTGTTGCTGTTGACTACGAGCAGGAG 60
A. thaliana 625 GAAATTGTAAGAGACATCAAGGAGAAGCTCTCCTTTGTTGCTGTTGACTACGAGCAGGAG 684
*****

P. exilis 61 ATGGAGACCTCAAAAACCAGCTCTTCCATCGAGAAGAACTATGAATTACCCGATGGACAA 120
A. thaliana 685 ATGGAAACCTCAAAGACCAGCTCTTCCATCGAGAAGAACTATGAATTACCCGATGGGCAA 744
*****

P. exilis 121 GTGATCACAATCGGTGCTGAGAGATTCAGATGCCAGAAGTCTTGTTCCAGCCCTCGTTT 180
A. thaliana 745 GTCATCACGATTGGTGCTGAGAGATTCAGATGCCAGAAGTCTTGTTCCAGCCCTCGTTT 804
** *****

P. exilis 181 GTGGGAATGGAAGCTGCTGGAATCCACGAGACAACCTACAACCTCAATCATGAAGTGTGAT 240
A. thaliana 805 GTGGGAATGGAAGCTGCTGGAATCCACGAGACAACCTATAACTCAATCATGAAGTGTGAT 864
*****

P. exilis 241 GTGGATATCAGGAAGGATCTGTACGGTAACATTGTGCTCAGTGGTGAACCACTATGTTC 300
A. thaliana 865 GTGGATATCAGGAAGGATCTGTACGGTAACATTGTGCTCAGTGGTGAACCACTATGTTC 924
*****

P. exilis 301 TCTGGTATTGCGGACCGTATGAGCAAAGAGATCACAGCTCTTGACCAAGCAGCATGAAG 360
A. thaliana 925 TCAGGTATCGCTGACCGTATGAGCAAAGAAATCACAGCACTTGACCAAGCAGCATGAAG 984
** *****

P. exilis 361 ATTAAGGTCGT 371
A. thaliana 985 ATTAAGGTCGT 995
*****

```

Figure 4.8 Alignment of predicted nucleotide sequence of *PeActin* of *P. exilis* with actin2 sequence of *A. thaliana*. The sequence was obtained from GenBank (accession number AAM65287.1). Asterisks indicate identical nucleotides.

```
PeActin      1  EIVRDIKEKLSFVAVDYEQEMETSKTSSSIEKNYELPDGQVITIGAERFRCPEVLFQPSF 60
actin2      209 EIVRDIKEKLSFVAVDYEQEMETSKTSSSIEKNYELPDGQVITIGAERFRCPEVLFQPSF 268
          *****

PeActin      61  VGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTMFSGIADRSKEITALAPSSMK 120
actin2      269 VGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTMFSGIADRSKEITALAPSSMK 328
          *****

PeActin      121 IKVV 124
actin2      329 IKVV 332
          ****
```

Figure 4.9 Alignment of the predicted amino acid *P. exilis* actin sequence with actin2 protein sequence of *A. thaliana* (GenBank accession number AAM65287.1).

4.3.4 Phylogenetic trees

Phylogenetic trees were built based on deduced amino acid sequences of LFY, TFL1 and actin (Appendix III). The phylogenetic trees that were built based on LFY and TFL1 amino acid sequences showed a close relation with *A. lyrata* and *A. thaliana* than to other species (Fig 4.10; 4.11). The tree that was built based on actin sequences showed a close relationship with actin2 of *A. thaliana* (Fig 4.12).

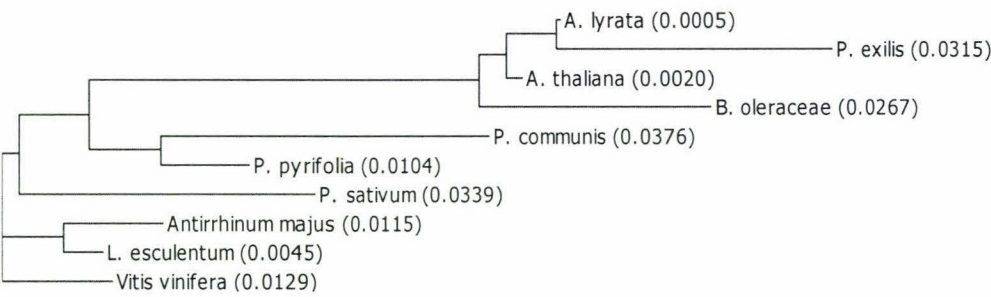


Figure 4.10 Phylogenetic tree based on different LFY-like amino acid sequences. The length of horizontal lines is proportional to the similarity between predicted protein sequences. The value in the parenthesis is the calculated distance value.

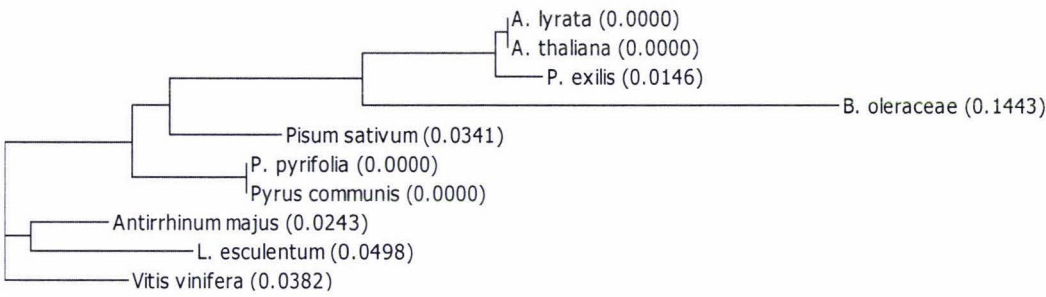


Figure 4.11 Phylogenetic tree based on different TFL1-like amino acid sequences. The length of horizontal lines is proportional to the similarity between predicted protein sequences. The value in the parenthesis is the calculated distance value.

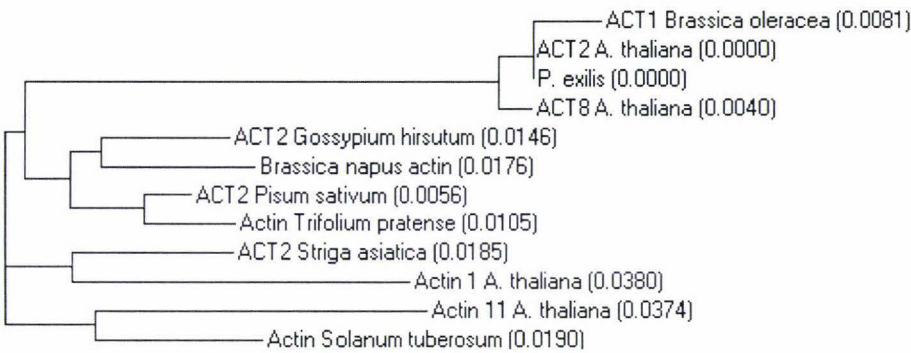


Figure 4.12 Phylogenetic tree based on different actin-like amino acid sequences. The length of horizontal lines is proportional to the similarity between predicted protein sequences. Each value in parenthesis is the calculated distance value.

4.4 Discussion

Flower development can be divided into four major steps: the switch from vegetative to reproductive growth, the development of the inflorescence meristem, development and identity determination of floral organs, and growth and maturation of floral organs (Mouradov et al., 1997). Flowering mutants in *Arabidopsis thaliana* and *Antirrhinum* have led to the identification of genes that regulate the formation of flowers. These include *LEAFY (LFY)* (Weigel et al., 1992), *FLORICAULA (FLO)* (Coen et al., 1990), *APETALA1 (API)* (Mandel et al., 1992), and *TERMINAL FLOWER (TFL1)* (Shannon

and Meeks-Wagner, 1991). The principal assumption of the present work was that similar genes would be present in *P. exilis* to regulate floral development and to maintain vegetative development. On the other hand, it was possible that *LFY* and *TFL1*, might be somewhat different in perennial plants such as *P. exilis*. *TFL*-, and *LFY*-like cDNAs were isolated and sequenced from *P. exilis*.

The analysis of the isolated *LFY*-like cDNA sequence from *P. exilis* showed that the *PeLFY* had slightly greater sequence similarity to *A. lyrata* than to *A. thaliana*. The deduced amino acid alignment with *LFY*-like proteins in other species showed that the deduced protein sequence from *P. exilis* shared slightly greater similarity with *A. lyrata* than *A. thaliana* (Appendix III).

The FLORICAULA/ LEAFY protein family consists of various plant development proteins which are homologues of FLORICAULA (FLO) and LEAFY (LFY) proteins which are floral meristem identity proteins. Mutations in the sequences of these proteins affect flower and leaf development. A search in conserved domain database (rpsblast) with deduced amino acid sequence had shown similarity with FLO_LFY protein family. Therefore, the isolated sequence was *LFY*-like and orthologous to *LFY* in *Arabidopsis*.

A phylogenetic tree was constructed by aligning the partial amino acid sequence from *P. exilis* with *LFY*-like sequences in other species. It showed that *P. exilis*, *A. lyrata* and *A. thaliana* grouped together. This shows that, with reference to the *LFY* sequence, *P. exilis* shows a closer relationship with *Arabidopsis* than with other species.

The *TFL1* gene is one of the genes essential for controlling the identity of the inflorescence meristem in the shoot apex of *Arabidopsis* (Shannon & Meeks-Wagner 1991, 1993; Alvarez et al., 1992). It has been shown that the *TFL1* gene and the genes such as *LFY* and *API*, which control floral meristem identity, function antagonistically in the maintenance of the inflorescence and floral meristems or in the transition of floral development (Shannon & Meeks-Wagner 1991, 1993; Alvarez et al., 1992; Weigel

et al., 1992; Mandel et al., 1992; Schultz & Haughn, 1993; Gustafson-Brown et al., 1994; Weigel & Nilsson, 1995).

Previous studies have shown that *TFL1* belongs to a small gene family, CETS that contains six *TFL1*-like genes (Kardailsky et al., 1999; Kobayashi et al., 1999). A phylogenetic tree was built based on the deduced amino acid sequence of *P. exilis* and other TFL1-like proteins. PeTFL1 formed a clade with TFL1 of *A. thaliana* and *A. lyrata*, which again confirmed the evolutionary analysis that *P. exilis* is a close relative of *Arabidopsis* (Heenan et al., 2002).

In the present thesis, a partial *TFL1*-like sequence was isolated and the sequence comparisons showed that the identified gene was an orthologue of *TFL1* in *Arabidopsis*. A comparison of nucleotide sequences of *P. exilis* with *A. thaliana* and *A. lyrata* showed that the isolated sequence shares 97% identity with *A. thaliana* and 96% identity with *A. lyrata*, which is a perennial plant.

It has been shown that TFL1 and CEN show a high similarity to a family of mammalian phosphatidylethanolamine-binding proteins (PBP) (Grandy et al., 1990; Bucquoy et al., 1994; Bradley et al., 1996; Ohshima et al., 1997). A search in conserved domain database (rpsblast) with the deduced amino acid sequence matched with RKIP (CDD accession no 27619), PEBP_RKIP (CDD accession number 27617) and PBP (CDD accession number 25700). This confirmed that the isolated sequence was a *TFL1*-like that shares homology with mammalian PBP.

In summary, the results suggest that the predicted amino acid sequences of PeLFY and PeTFL1 of *P. exilis* show a high similarity with LFY and TFL1 of *Arabidopsis*. Previous studies using nuclear ribosomal DNA ITS sequence data showed that the *Pachycladon* complex occurs within an Arabidopsoid clade that includes *Arabidopsis* and the recently recognised segregate genera *Beringia*, *Crucihimalaya*, *Olimarabidopsis*, and *Pseudoarabidopsis*, and the *Arabis* segregates *Boechera* and *Turritis* (Heenan et al., 2002). This shows that the present study is in harmony with the past study.

Chapter 5. Effects of daylength and chilling on *LEAFY* and *TERMINAL FLOWER1* expression in *Pachycladon exilis*

5.1 Introduction

The physiological studies in the Chapter 3 showed the effects of daylength and chilling on trichomes, leaf parameters, flowering time and percentage and silique count. To further investigate the effect of light and chilling on floral induction in *P. exilis*, gene expression studies were carried out. In *Arabidopsis* and in other species, *LFY* acts as a floral meristem identity gene that promotes flowering and *TFL1* acts as an inflorescence meristem identity gene which suppresses flowering and which is antagonistic to *LFY*. In the present study, the expression levels of *LFY* and *TFL1* were analysed in shoot apical meristems of *P. exilis*. Based on the physiological studies described in Chapter 3 and earlier studies in different species, it was hypothesised that *LFY* would be more highly expressed in long days and plants exposed to chilling compared to plants exposed to short days and no chilling. It was hypothesised that *TFL1* expression might be responsible for the perennial habit of the plant.

Different methods have been used to study the expression of genes at different stages and in different tissues/organs. These methods include northern blot analysis, RNase protection assay (RPA), DNase I footprinting assay, the electrophoretic mobility shift assay (EMSA), western blot analysis, serial analysis of gene expression (SAGE), RNA differential display analysis and DNA microarray analysis. However, the recent technique of real-time reverse transcriptase PCR is the most sensitive and is a preferable alternative to other forms to quantify gene expression. In this method, the fluorescence emitted during the reaction can be monitored, which is an indicator of amplicon production during each PCR cycle.

Real-time PCR on the LightCycler System (Roche), using the fluorescent dye SYBR Green I for detection, provides rapid cycling for fast analysis (Missel et al., QIAGEN GmbH, Hilden, Germany). To allow very short heating and cooling cycles, PCR is

carried out in small volumes in capillaries. Because of these short heating and cooling cycles, optimisation of parameters, such as Mg^{2+} concentration, is necessary. Further, parameters such as length of PCR product, primer concentration and annealing time can affect the reaction.

In the present study, the expression of *PeLFY* and *PeTFL1* were studied under different light conditions and chilling levels using Roche LightCycler and SYBR Green I dye that binds double stranded DNA.

5.2 Materials and methods

5.2.1 Plant material

Whole plants were collected at fortnightly intervals from six of the treatments (two daylength and three chilling) described in Chapter 3. Plants were immersed in liquid nitrogen and stored at $-80^{\circ}C$. Plants from the 30 d chilling treatment were not collected, as there were insufficient plants. Later, at the time of RNA extraction, shoot apical meristems (SAM) along with some leaf bases were dissected in liquid nitrogen and approximately 100 mg of SAM were used for RNA extraction. As the LightCycler can accommodate only 32 samples at a time, to study the relative expression of *PeLFY* and *PeTFL1* following various chilling regimes (0, 10, 20 days), meristems extracted from samples collected at monthly intervals following transfer to CEL were used instead of samples at fortnight intervals. To study the effect of daylength on the expression of *PeLFY* and *PeTFL1*, meristems extracted from samples collected at fortnight intervals following 20 d of chilling were used (Table 5.1).

5.2.2 RNA extraction

RNA was extracted from SAM using TRI reagent as described in Section 4.1.2.

Table 5.1 Timing and type of tissue collected for the quantitative expression studies in *P. exilis*.

Sample	Tissue	Collection date
LD20	vegetative SAM	23/1/05
SD20	vegetative SAM	23/1/05
LD20	vegetative SAM	5/2/05
SD20	vegetative SAM	5/2/05
LD20	vegetative SAM	19/2/0
SD20	vegetative SAM	19/2/05
LD20	just bolted SAM	6/3/05
SD20	vegetative SAM	6/3/05
LD20	Fully bolted SAM+ Axillary meristems	18/3/05
SD20	just bolted SAM	18/3/05
LD20	Fully bolted SAM+ Axillary meristems	1/4/05
SD20	unbolted vegetative SAM	1/4/05

5.2.3 Deoxynuclease (DNase) treatment and Reverse Transcriptase (RT) reaction

Extracted RNA was treated with DNase I to remove any DNA contamination using the following protocol as recommended by Sigma: To 1 µg of RNA sample, 1 µl of 10 x reaction buffer and 1 µl of amplification grade DNase I (1 U/µl) were added. The reaction was incubated for 15 min at room temperature. Stop solution (1µl) was added to the incubated mix to bind calcium and magnesium ions and to inactivate the DNase I and the mix heated to 70°C for 10 min to denature both the DNase I and the RNA and then chilled on ice. To the cooled reaction, the following RT mix was added:

RT mix

Oligo (dT)15	1.0 µl
5X RT buffer	4.0 µl
100 mM DTT	2.0 µl
RNAse Inhibitor (40U/µl) (Roche)	0.5 µl
20 mM dNTPs	1.0 µl
Expand RT (50 U/µl)	1.0 µl

This final reaction mix (20.5 µl) was incubated at 48°C for 30 min and stored at -20°C

5.2.4 Primer design

Real-time PCR primers for *LFY* and *TFLI* were designed based on the sequences obtained from the PCR products using degenerate primers. Whenever possible, primers were designed at the exon/intron boundary to eliminate any DNA contamination (Appendix IV). The primers were designed so that the resulting sequence should not be greater than 150 bp for efficient quantification. Primer Premier 5.0 software (Premier Biosoft, 1999) was used to design the primers. The sequence of primers were as follows

PeLFYF: ACC AAG GTG ACG AAC CAA GT
PeLFYR: GCT TTT CGG AGA GCG TTT GA

PeTFL1: TGA TAG ACC CAG ATG TTC CA

PETFL2: CCA CCT CTT TTC CAA ATG TA

5.2.5 Housekeeping gene

To standardise the amount of sample added to a reaction, and to control the errors introduced during cDNA synthesis and PCR reaction, amplification of an endogenous control should be performed. In the present study, for the quantitation of gene expression, β -actin was used as the endogenous control. Specific primers were designed from the sequence obtained using degenerate primers in Chapter 4. The sequences of primers were as follows

PeActin1: ACG AGC AGG AGA TGG AGA CC

PeActin2: GGT TCC ACC ACT GAG CAC AA

5.2.6 PCR reaction and gel electrophoresis

Primers were tested using PCR and running the PCR products on 2% Agarose gel (Fig 5.1A, B). Bands were excised and purified using a Qiagen Gel extraction kit as per the manufacture's instructions and the products were sequenced (Appendix V).

5.2.7 Real-time PCR

Real-time PCR reactions were carried out using a LightCycler (Roche) and software version 3.5. The reactions were performed as single reactions. The same samples were used to study the expressing levels of *LFY* and *TFL1*. For the real-time PCR, a total volume of 15 μ l PCR mix was used.

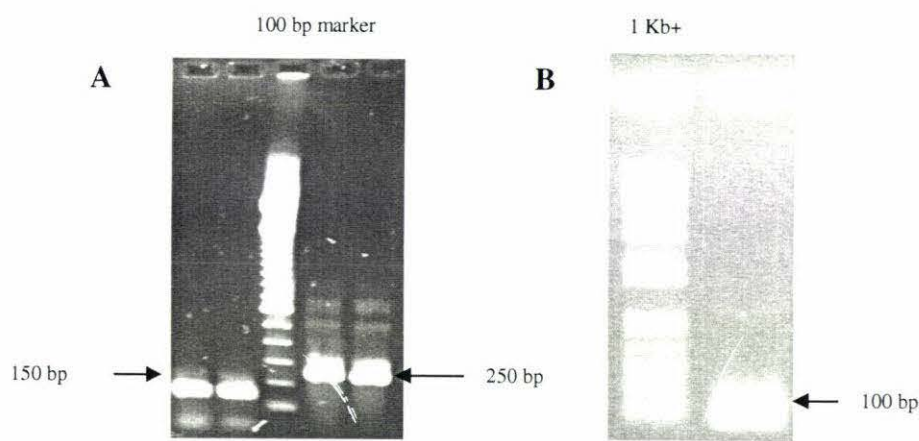


Figure 5.1 Testing of specific primers on a gel. A, Bands of approximately 150 bp in size that sequenced to give *PeLFY*, and 250 bp in size that sequenced to give *PeActin*; B, Band of approximately 100 bp in size that sequenced to give *PeTFL1*.

The 15 µl PCR mix consisted of:

Components	Volume	Final concentration
PCR grade water	9.25 µl	
10X SYBR Green Master	1.5 µl	1x
25mM MgCl ₂	0.75 µl	2.25 mM
Primer A	1 µl	1.0 µM
Primer B	1 µl	1.0 µM
Template cDNA	1.5 µl	1 ng

5.2.7.1 Protocol

To activate Faststart Taq DNA Polymerase the initial denaturing step of heating at 95°C for 10 min was included in the experimental protocol. This step was followed by an amplification cycle of 95°C for 10 s, 52°C for 5 s, 72°C for 15 s, and 78°C for 5 s, repeated 45 times. The aim of the 78°C step is to increase the fluorescence acquisition temperature to minimise the primer dimer effects. These temperature changes were made at a rate of 20°C s⁻¹. To view the final products generated, the following melting curve program was used: heated to 95°C at the rate of 20°C for 0 s, decreased to 65°C at the rate of 20°C s⁻¹ for 15 s and finally increased to 95°C at the rate of 0.1°C s⁻¹. The final real-time PCR products were checked on 2% agarose gel to confirm the size (<150 bp) of amplified products. Once the products were confirmed, melting curves generated during the run on the LightCycler were used to confirm the products instead of running the products on a gel every time.

5.2.7.2 Mg optimisation

The optimal MgCl₂ concentration was determined for each primer set (PeLFY1 and 2, PeTFL1 and 2, PeActin1 and 2). Three concentrations of MgCl₂ were used, which were 4 mM, 3 mM and 2.25 mM. The optimal MgCl₂ concentration will have the lowest crossing point (Cp), the highest fluorescence intensity and steepest curve slope (Roche Molecular Biochemical Note no. LC9/2000). In Figure 5.2, for *PeLFY* and *PeTFL1*, the

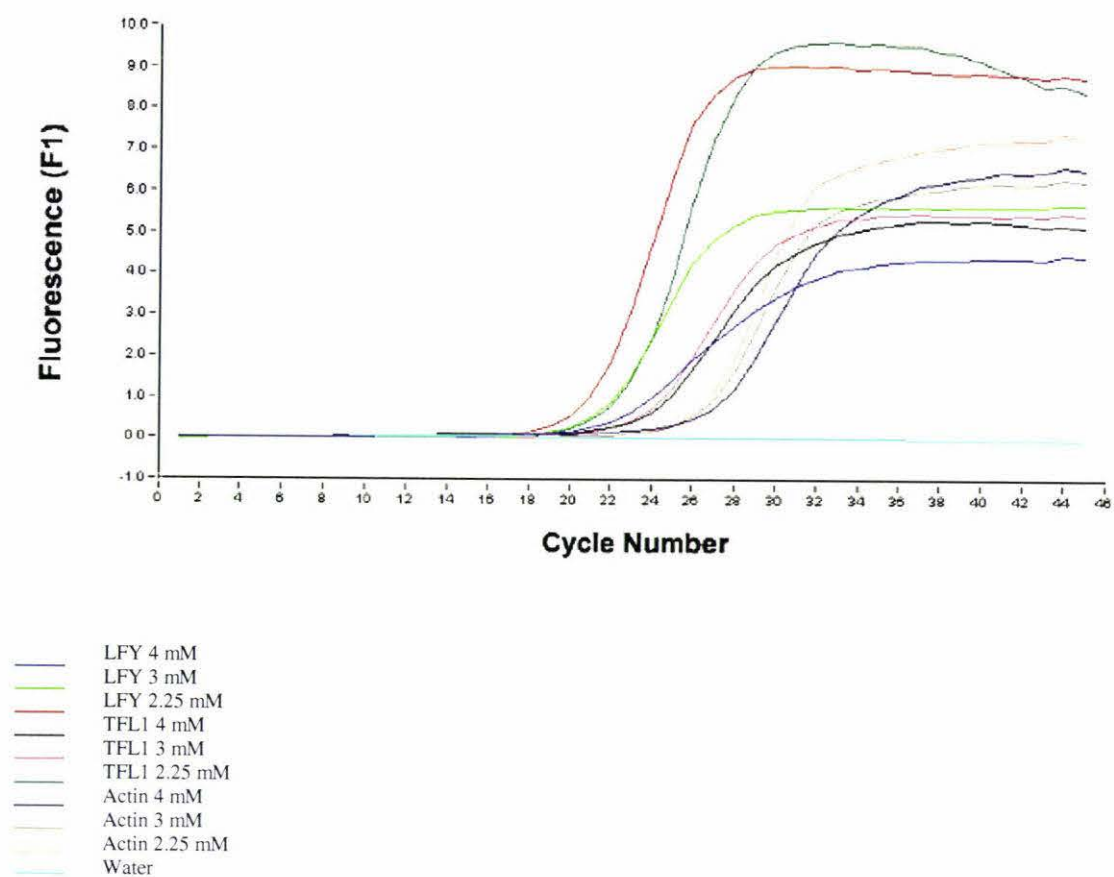


Figure 5.2 Mg^{+2} optimisation for each primer set. Three concentration of Mg^{+2} used include 4 mM, 3 mM and 2.5 mM and the one with low Cp (crossing point) value is optimal.

MgCl₂ concentration of 2.25 mM satisfied the optimal conditions, whereas for the PeActin primer set, all three concentrations were similar. Therefore, 2.25 mM MgCl₂ was used in all the subsequent reactions.

5.2.7.3 Quantitation method

The relative expression of the target gene was corrected by reference to the housekeeping gene. For this, the following equation was used to obtain the relative expression value:

$$\text{Relative expression value} = 2^{-(\text{sample Cp} - \text{house keeping Cp})}$$

Then the biggest value of the different samples included in the same experimental treatment was made to 1 and other values were then calculated relative to 1.

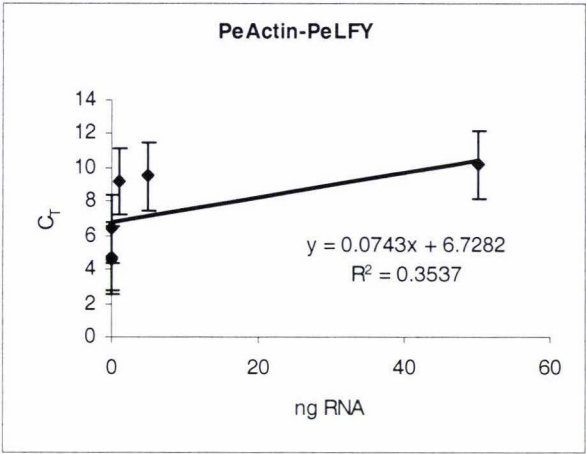
The slopes for *PeLFY* (0.07) and *PeTFL1* (0.003) were calculated, which were <0.1 (Fig 5.3A, B).

5.3 Results

5.3.1 *PeLFY* and *PeTFL1* expression levels in the developing meristems of *P. exilis* in response to daylength and chilling treatments

To quantitate the levels of *PeLFY* and *PeTFL1* RNAs that accumulate under floral inductive conditions, real-time PCR was performed. Real-time PCR measures the threshold cycle values (Ct) which is the PCR cycle at which a detectable increase in product amplification is obtained. Therefore, Ct is inversely related to the initial amount of template present in sample. In this study, the Ct values from unknown samples were normalized against PeActin to account for variation among RT-PCR reactions. Based on these Ct values, quantities of *PeLFY* and *PeTFL1* transcripts were extrapolated and shown in Fig. 5.4 and 5.5.

A



B

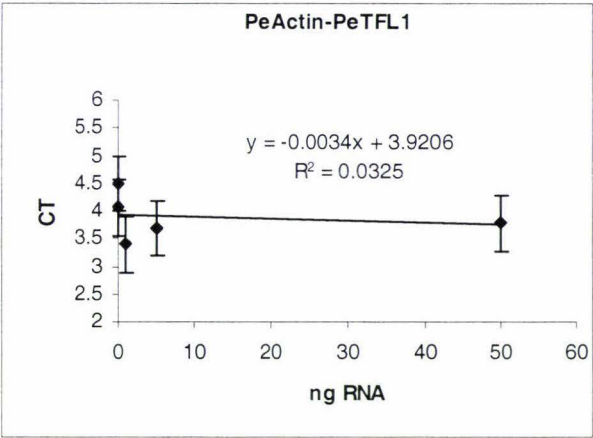


Figure 5.3 Calculation of slopes for *PeLFY* and *PeTFL1*. A, The difference between the slopes of *PeLFY* and *PeActin* was 0.07; B, The difference between the slopes of *PeTFL1* and *PeActin* was 0.003; hence the *PeLFY* and *PeTFL1* passed the efficiency test.

A total of four runs were carried out in the LightCycler. In the first two runs, *PeLFY* and *PeTFL1* were compared against the house keeping gene in samples collected at 14, 42, 69 d from plants exposed to 0, 10 and 20 d of chilling and subsequently grown under long and short days. In the last two runs, *PeLFY* expression under long days and short days, and *PeTFL1* expression under long and short days respectively, were done normalising against *PeActin* gene.

5.3.1.1 Expression levels of *PeLFY* and *PeTFL1* with various chilling levels

In long days, at 14 d after the start of daylength treatment (DAT), the relative expression of *PeLFY* was similar in plants with 0, 10 and 20 d of chilling. At 42 d after the start of daylength treatments, *PeLFY* expression was higher in plants with 20 d of chilling compared to plants with no or 10 d of chilling (Fig 5.4A). However, there was a steady increase in *PeLFY* RNA levels in plants with no chilling. In contrast, at 14 d, the relative expression of *PeTFL1* was higher in plants with no chilling compared with 10 and 20 d of chilling (Fig 5.4B). In further contrast to *PeLFY*, at 42 d, the *PeTFL1* expression had decreased but increased slightly by 69 d.

On the other hand, in short days, at 14 DAT, the expression of *PeLFY* was higher in plants with 10 d of chilling compared to plants with no and 20 d of chilling. However, the expression was higher in all the three at 69 DAT, when the tissue was collected from just bolting plants (Fig. 5.4C). The relative expression of *PeTFL1* was different in three chilling levels. In plants with 10 and 20 d of chilling the expression of *PeTFL1* contrasted with that was seen for *PeLFY*. However, in plants with no chilling there was a steady increase in *PeTFL1* RNA levels (Fig. 5.4D).

5.3.1.2 Expression levels of *PeLFY* and *PeTFL1* under long and short days after 20 d of chilling

Under long days, *PeLFY* expression increased over the time floral organogenesis occurred at 57 DAT and thereafter the expression dropped in the tissues that were fully

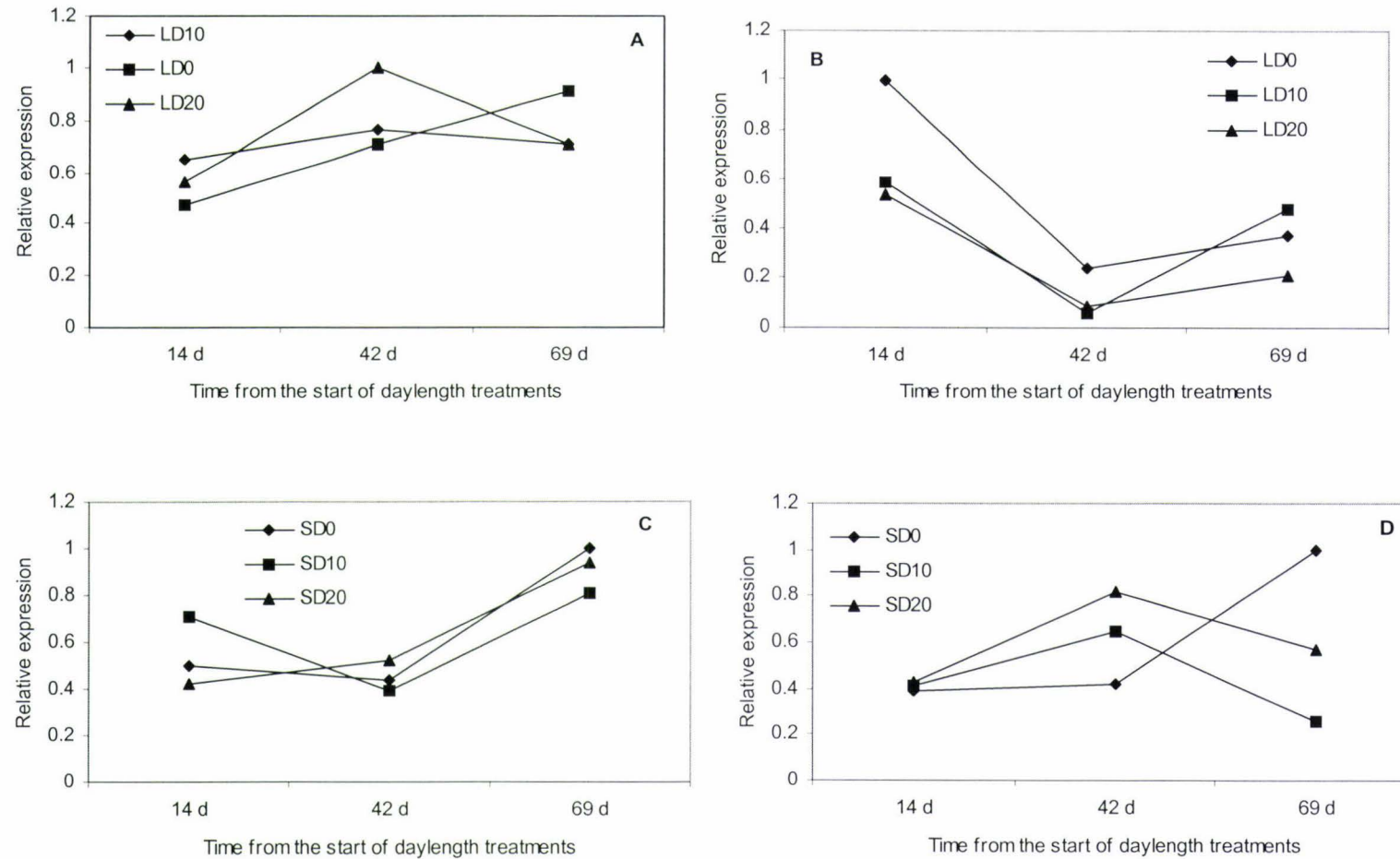


Figure 5.4 Relative expression of *PeLFY* and *PeTFL1* after 0, 10 or 20 d of chilling. A, *PeLFY* expression under LD; B, *PeTFL1* expression under LD; C, *PeLFY* expression under SD; D, *PeTFL1* expression under SD.

bolted with some axillary meristems bearing small leaves. In short day plants, the *PeLFY* expression showed a similar pattern but with the expression peaking at 69 DAT. These are the tissues that were just bolting in short days. However, in tissues that were still in vegetative stage, *PeLFY* expression was at low levels (84 DAT) (Fig. 5.5A). The expression of *PeTFLI* contrasted with the expression of *PeLFY*. Under long days, the expression of *PeTFLI* was low at 57 d (Fig. 5.5B), whereas *PeLFY* transcripts had peaked (Fig. 5.5A). On the other hand, the relative expression of *PeTFLI* was high under long days after silique formation had occurred by 84 d. A similar trend was seen in plants that were grown under short days, where *PeTFLI* expression declined in bolting tissues at 69 d and in the tissues that were still in vegetative state (84 d), the expression continued.

5.4 Discussion

In *Arabidopsis*, flowering is controlled by a daylength-dependent pathway in which long days induce flowering and a daylength-independent pathway in which flowering is ensured in the absence of inductive conditions (Simpson et al., 1999; Koorneef et al., 1998b; Pineiro & Coupland, 1998). At the genetic level these pathways are separable as mutations that delay flowering in long days but not in short days (Koorneef et al., 1991). Further, mutations that block GA synthesis stop flowering in short days whereas in long days it has only a minor effect (Wilson et al., 1992). It is important to understand how similar environmental or endogenous cues can obtain opposite flowering responses in different plants. In the present study, the relative expression of two meristem identity genes, *LFY* and *TFLI* in *P. exilis*, were investigated under different daylengths and chilling regimes in order to deduce a possible function in floral transition.

Based upon morphological (Chapter 2) and molecular (Chapter 4) data, and previous studies (Heenan et al., 2002), it was considered that the expression patterns of flowering genes would be similar to *Arabidopsis*. In *P. exilis*, up regulation of *PeLFY* occurred earlier under long days, compared to short days, where there was a steady increase in the expression of *LFY* and the flowering was also slightly delayed compared to long days.

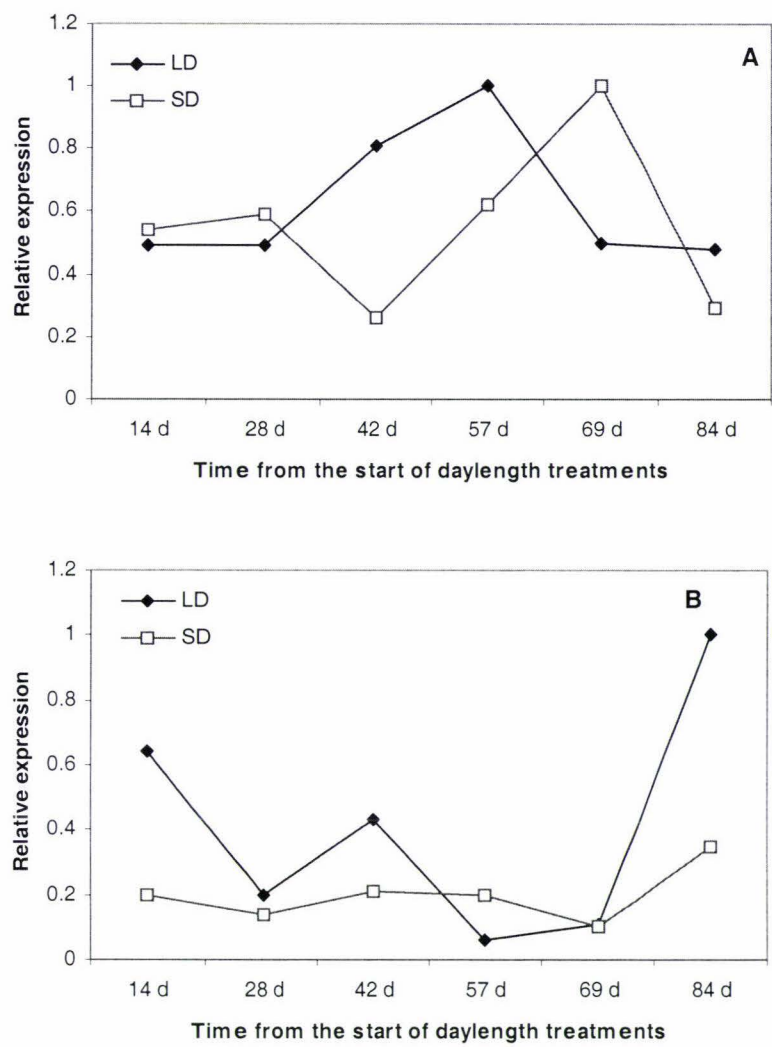


Figure 5.5 Relative expression levels of *PeLFY* and *PeTFL1* under two daylengths following 20 d of chilling. A, *PeLFY* expression; B, *PeTFL1* expression.

Similarly, in *A. thaliana*, *LFY* expression differs under different environmental conditions. Expression is rapidly up regulated in long days, when *A. thaliana* plants flower soon after germination, whereas under short days, it increases gradually and flowering is delayed relative to plants under LD (Blazquez et al., 1997).

In *Arabidopsis*, it has been shown that indeterminate architecture of the inflorescence is due to the interaction between the genes that give floral identity, including *LFY* and *TFL1*, such that *TFL1* inhibits *LFY* expression in shoots and *LFY* inhibits *TFL1* expression in flowers (Shannon & Meeks-Wagner, 1991; Alvarez et al., 1992; Weigel et al., 1992; Bradley et al., 1997). Similarly to *LFY*, *TFL1* is expressed both in vegetative and reproductive phases, and *tfl1* mutants flower early (Shannon & Meeks-Wagner, 1991; Bradley et al., 1997). In the same manner, in *P. exilis*, *PeTFL1* expression was seen both in vegetative and reproductive phases. However, the expression was at a relatively low level during the reproductive phase. On the other hand, the expression of *PeTFL1* again increased after full opening of the flowers (84 d) and this might be due the vegetative growth of axillary meristems. This thus tends to confirm the hypothesis that the perennial nature of *P. exilis* is due, or at least in part to the expression of *TFL1*. In addition, the quantitative real-time PCR studies presented here are consistent with a model where *PeLFY* and *PeTFL1* function in a manner similar to the *Arabidopsis LFY* and *TFL1*.

In addition to daylength, temperature is another environmental signal used by many plants to elicit flowering. It should be highlighted that flowering behaviour depends strongly on daylength conditions and cold treatment, and that these effects are known to act together (Martinez-Zapater et al., 1994). Nordborg & Bergelson (1999) observed that, in *A. thaliana* ecotypes, a cold treatment of one month at the seed or rosette stage decreased flowering times. In this study, the relative expression of *PeLFY* and *PeTFL1* in *P. exilis* was investigated following different lengths of seed chilling followed by different daylengths. In some studies, it has been shown that a month-long cold treatment at the seed stage decreased the flowering time compared to three days cold treatment which had little effect or the opposing effect on flowering time (Nordborg &

Bergelson, 1999). Similarly, in *P. exilis*, under long days, plants from seeds exposed to 20 d of chilling had a higher level of expression of *PeLFY* compared to 10 d and no chilling, whereas the expression of *PeTFL1* showed an opposite effect in which plants from seeds with no chilling had a higher level of expression compared to chilled plants. However, under short days not much difference was seen among the chilled and non-chilled plants in terms of *PeLFY* expression. Perhaps this was due to the interaction of daylength and temperature. Therefore, plants with long daylength and more days of chilling had high level of expression of *PeLFY*, which is responsible for early flowering in many plants.

There are no real-time studies on the quantitative expression of flowering time genes either in *A. thaliana* or in *A. lyrata*. The results on *P. exilis* suggest that it might be a good perennial model due to the interesting expression of *PeTFL1*.

Chapter 6. Final discussion and conclusions

The Cruciferae/ Brassicaceae or Mustard family is a large family with a diverse variety of crop plants. It consists of over 3,000 species in about 350 genera. *Arabidopsis thaliana*, which belongs to this family, has been well studied by molecular biologists because of its small genome structure and short life cycle. This species has become a model plant in functional plant biology as well as in plant ecology and evolution (Pigliucci, 1998; Mitchell-Olds, 2001). There is morphological and ecological diversity among the relatives of *Arabidopsis* (Mitchell-Olds, 2001).

Recent studies by Heenan et al. (2002) on nITS sequence variation in the Brassicaceae imply a close phylogenetic relationship between species of the New Zealand *Pachycladon* complex and *Arabidopsis thaliana*. The *Pachycladon* complex is considered to have radiated in New Zealand probably within the last million years. Some *Pachycladon* species are perennial and polycarpic (flowering occurs many times) whereas others are annual and monocarpic (flowering occurs only once and the plant dies).

In the present thesis, the perennial *P. exilis*, which is an endangered species (de Lange et al. 1999) and is known from a single locality in North Otago of New Zealand (Luo et al. 2003), was studied. The aim of the work was to investigate the effects of environmental signals on growth and flowering in *P. exilis*. A central question to this thesis was whether the genes that control the process of flowering were conserved in a perennial plant such as *P. exilis*. Further, the genetic basis for perenniality was investigated.

The morphology and organisation of the leaves on the shoots of *P. exilis* was similar to *A. thaliana*, in which rosette leaves were separated by short internodes and cauline leaves were separated by long internodes. However, in *P. exilis*, three levels of branching in a hierarchy were seen. Primary and secondary branches were floral, whereas tertiary branches were vegetative. In addition, adventitious shoots arising from

roots were observed. All these characteristic features might be responsible for maintaining the perennial nature of *P. exilis*.

Flowering in *P. exilis* occurred seven to eight weeks after the start of daylength treatments. Although flowering started at the same time in plants exposed to long and short days, plants that were exposed to long days and that had passed through cold treatment as imbibed seeds flowered after producing fewer leaves, and flowered more synchronously and to a higher percentage, than plants maintained under short days following chilling or plants grown from seed that had no cold treatment, in which non-simultaneous bolting was seen. This suggests that *P. exilis* is a facultative long day plant. To further investigate the effect of daylength on floral initiation, microscopic sections were made at regular intervals starting from vegetative to until tiny floral buds were seen. The sections clearly showed that in plants that were grown under long days had early floral induction. Therefore, these sections once again showed that long days induce flowering in *P. exilis*.

The genes that were equivalents of the inflorescence meristem identity gene *TFL1*, and the floral meristem identity gene *LFY*, were chosen for the present study as these genes occupy a central position in the hierarchy of genes involved in the floral transition. It was hypothesised that these gene equivalents would be present in *P. exilis* and perform the same function as in *A. thaliana*. In the present study, partial orthologues of *LFY* (*PeLFY*) and *TFL1* (*PeTFL1*) were isolated from *P. exilis*. Homology analysis confirmed that they were fragments of the equivalents of *LFY* and *TFL1*. Putative conserved domains for *PeLFY* and *PeTFL1* were detected. A FLO_LFY domain was detected for *PeLFY*, and for *PeTFL1*, all the three domains RKIP, PEBP_RKIP, PBP were detected. Phylogenetic trees that were built based on putative amino acid sequences of *PeLFY* and *PeTFL1* showed a closer relationship of *P. exilis* with *Arabidopsis* than other species, and within *Arabidopsis*, closer to *A. lyrata* – the perennial than to *A. thaliana* – the annual, based on *LFY*-tree preferably.

Quantitative expression patterns of these two genes showed a pattern similar to *A. thaliana*. In *Arabidopsis*, *LFY* expression has been seen throughout plant development

and even during early stages of vegetative development. However, its expression has been found to up-regulate sharply around the time of flowering (Blazquez et al., 1997). Quantitative regulation of flowering genes could play a very important role in flowering and *LFY* appeared to be tightly regulated as small changes in its activity affected flowering time or shoot morphology (Pineiro & Coupland, 1998). Similarly, in *P. exilis*, quantitative regulation of *PeLFY* seems important in view of the low levels of expression in vegetative tissue and higher levels during floral development. There is significant difference considering the interaction between *PeLFY* and daylength in *P. exilis*. In plants under long days, maximum expression of *PeLFY* was seen around 57 d after the start of daylength treatments, whereas in plants under short days, maximum expression of *PeLFY* was observed around 69 d co-incidents with bolting. However, the duration of chilling didn't significantly affect the expression of the two genes. Anatomical studies and physiological studies revealed that floral induction was occurring at the time when *PeLFY* expression was increasing to maximum levels (55 d in LD, 67 d in SD).

Similar to *Arabidopsis*, *PeTFL1* expression was seen at higher levels in vegetative tissues and low levels around floral induction. This once again showed the opposite effect of these two genes in *P. exilis*. Noteworthy is the observation that after floral opening and silique formation, the *PeTFL1* expression was seen at higher levels in the tissue containing vegetative axillary meristems. This supports the hypothesis that *PeTFL1* may contribute to the perennial nature of the plant.

In this thesis, the morphological structure of *P. exilis* and floral development is presented. Evidence is provided showing similar expression patterns of a floral and an inflorescence meristem identity gene in this perennial in comparison to the annual model plant, *Arabidopsis*, using real-time PCR. *P. exilis* may be a useful model perennial plant.

For future work, it would be interesting to isolate *LFY* and *TFL1* sequences containing an intron sequence to assess the relationship between *P. exilis*, *A. thaliana* and *A. lyrata*. Further, it is important to evaluate the reasons behind adventitious shoots on roots.

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Appendix I

Formulations of common buffers

EDTA (0.5M pH8.0)

Na ₂ EDTA	186.1 g
ddH ₂ O to	500 ml
Total volume	1000 ml

Note : Dissolve in approximately 400 ml ddH₂O, adjust pH to 8.0 with 10 N NaOH, and adjust to 1 litre final volume with sterile water. Autoclave to sterilise.

Tris-acetate-EDTA buffer (50x TAE)

Tris	242 g
0.5 M EDTA	100 ml
Glacial acetic acid	57.1 ml
MilliQ water	to 1.0 L

TAE buffer (1x)

50 x TAE buffer	20 ml
MilliQ water	to 1.0 L

TE buffer:	10mM Tris base pH 8.0
	1 mM EDTA (Na salt)
	MilliQ to 1.0 L

Ethidium bromide (5 mg/ml)

Ethidium bromide	500 mg
ddH ₂ O to	100 ml
Total volume	100 ml

In staining use a final concentration of 0.5 $\mu\text{g ml}^{-1}$

Agarose gel loading dye (10X)

Ficoll	1.5 g
Bromophenol blue	0.02 g
Xylene cyanole FF	0.02 g
ddH ₂ O to	10 ml

Salt precipitation solution (SPS)

Sodium citrate	0.8 M
NaCl	1.2 M

Appendix II

Statistical analyses

General Linear Model: Petiole length, Blade length, Blade width versus Daylength (DL), Chilling(V)

Factor	Type	Levels	Values
daylength	fixed	4	LD, SD, SD/LD/SD1, SD/LD/SD4
chilling	fixed	3	0, 10, 20

Analysis of Variance for Petiole length, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	1105.66	1165.06	388.35	9.72	0.000
V	2	1971.88	1955.00	977.50	24.46	0.000
DL*V	6	1238.11	1238.11	206.35	5.16	0.000
Error	918	36684.21	36684.21	39.96		
Total	929	40999.86				

Analysis of Variance for Blade length, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	3132.96	3228.83	1076.28	22.63	0.000
V	2	750.21	759.21	379.61	7.98	0.000
DL*V	6	550.60	550.60	91.77	1.93	0.073
Error	918	43652.89	43652.89	47.55		
Total	929	48086.66				

Analysis of Variance for Blade width, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	969.87	983.65	327.88	15.80	0.000
V	2	2.04	4.08	2.04	0.10	0.906
DL*V	6	168.61	168.61	28.10	1.35	0.230
Error	918	19049.56	19049.56	20.75		
Total	929	20190.07				

General Linear Model: silique, inflorescence branches versus Daylength (DL), Chilling (V)

Factor	Type	Levels	Values
DL	fixed	4	LD, SD/LD/SD1, SD/LD/SD4, SD
V	fixed	4	0, 10, 20, 30

Analysis of Variance for silique, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	7137.7	5766.8	1922.3	10.42	0.000
V	3	447.7	169.0	56.3	0.31	0.821
DL*V	9	3009.5	3009.5	334.4	1.81	0.070
Error	154	28397.4	28397.4	184.4		
Total	169	38992.2				

Analysis of Variance for inflorescence branches, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	5.139	4.639	1.546	0.96	0.411
V	3	9.305	7.916	2.639	1.65	0.181
DL*V	9	15.609	15.609	1.734	1.08	0.379
Error	154	246.907	246.907	1.603		
Total	169	276.960				

General Linear Model: Trichomes on abaxial, margin, adaxial, petiole versus daylength (DL), chilling (V)

Factor	Type	Levels	Values
DL	fixed	4	LD, SD, SD/LD/SD1, SD/LD/SD4
V	fixed	3	0, 10, 20

Analysis of Variance for abaxial, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	4941.7	4766.0	1588.7	4.42	0.004
V	2	11249.7	11289.8	5644.9	15.72	0.000
DL*V	6	7432.9	7432.9	1238.8	3.45	0.002
Error	1125	404029.0	404029.0	359.1		
Total	1136	427653.3				

Analysis of Variance for margin, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	3601.20	3625.71	1208.57	14.37	0.000
V	2	1361.82	1432.55	716.28	8.52	0.000
DL*V	6	997.46	997.46	166.24	1.98	0.066
Error	1125	94619.95	94619.95	84.11		
Total	1136	100580.43				

Analysis of Variance for adaxial, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	35.298	35.418	11.806	1.92	0.125
V	2	1.395	1.484	0.742	0.12	0.886
DL*V	6	22.355	22.355	3.726	0.61	0.726
Error	1125	6918.838	6918.838	6.150		
Total	1136	6977.886				

Analysis of Variance for petiole, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	2158.09	2112.77	704.26	9.82	0.000
V	2	2553.04	2563.89	1281.95	17.88	0.000
DL*V	6	676.95	676.95	112.82	1.57	0.151
Error	1125	80648.54	80648.54	71.69		
Total	1136	86036.62				

General Linear Model: Number of leaves to primary bolt versus daylength (DL), chilling (V)

Factor	Type	Levels	Values
DL	fixed	4	LD, SD/LD/SD1, SD/LD/SD4, SD
Vern	fixed	4	0, 10, 20, 30

Analysis of Variance for number of leaves to primary bolt, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DL	3	416.390	346.633	115.544	13.16	0.000
Vern	3	151.424	164.025	54.675	6.23	0.000
DL*Vern	9	117.511	117.511	13.057	1.49	0.155
Error	183	1606.202	1606.202	8.777		
Total	198	2291.528				

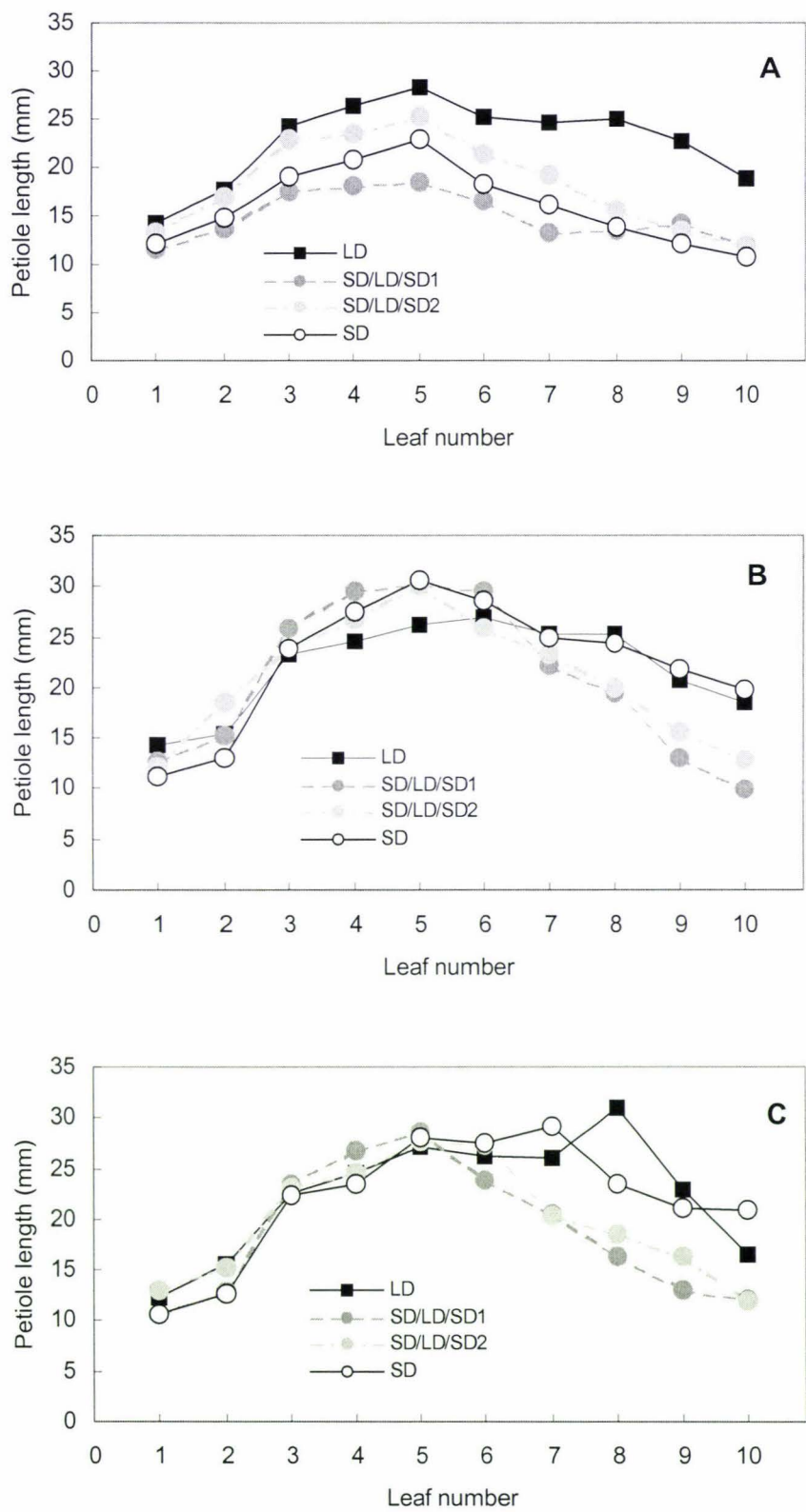


Figure 3.6 Effect of daylength on petiole length in Leaves 1-10. A, 0 d chilling; B, 10 d chilling; C, 20 d chilling.

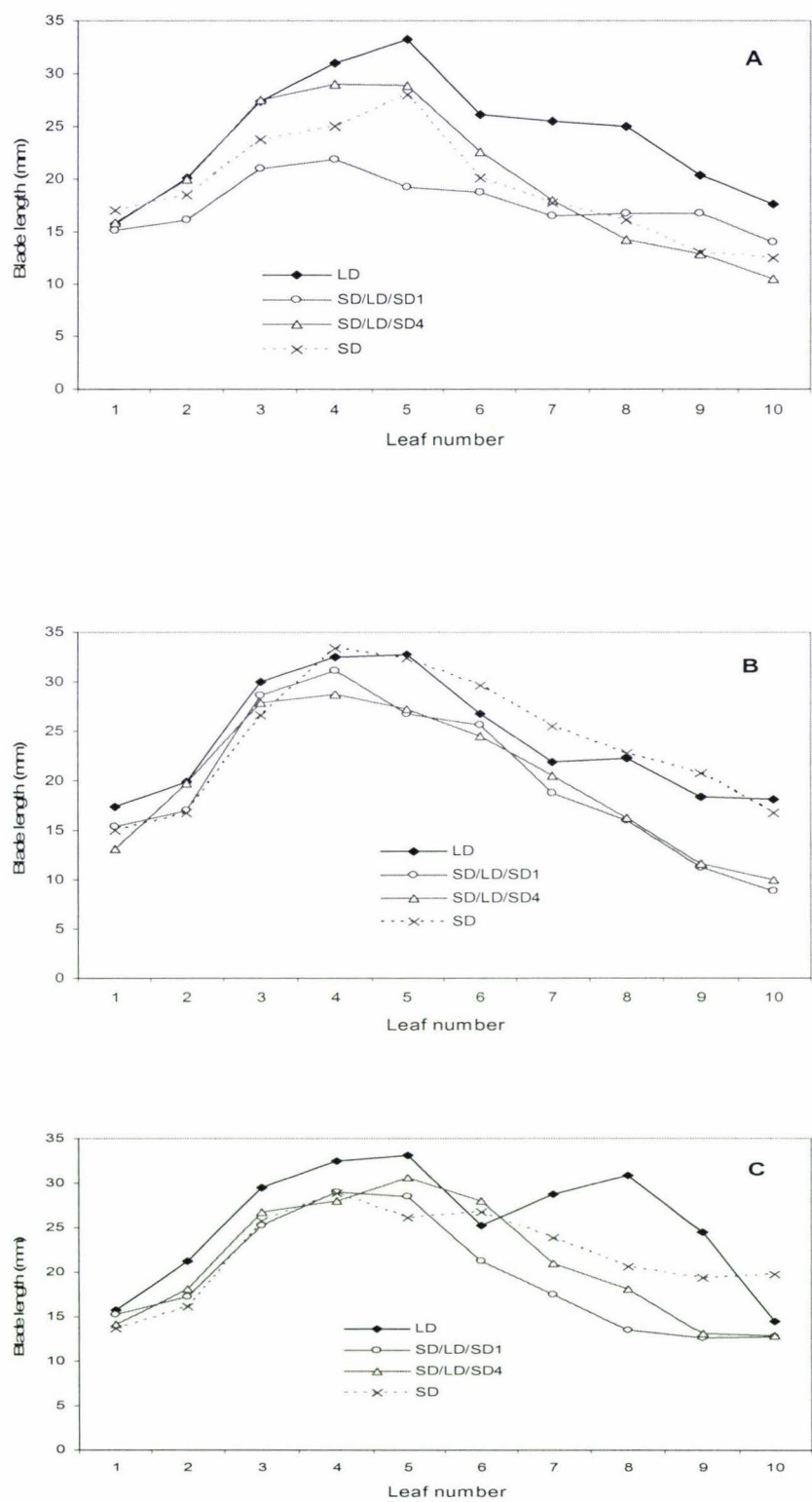


Figure 3.7 Effect of daylength on blade length in leaves 1-10. A, 0 d chilling; B, 10 d chilling; C, 20 d chilling.

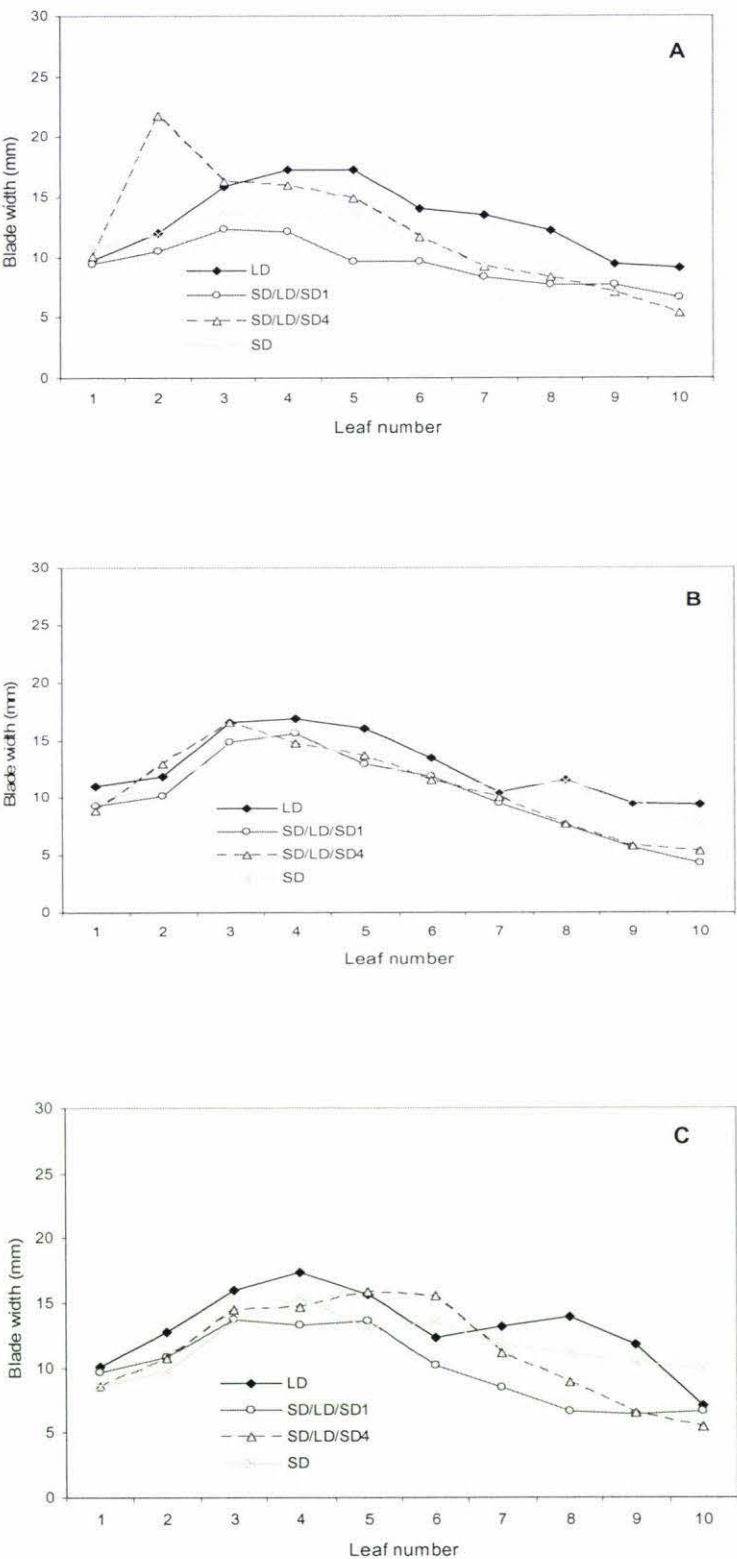


Figure 3.8 Effect of daylength on blade width in leaves 1-10. A, 0 d chilling; B, 10 d chilling; C, 20 d chilling.

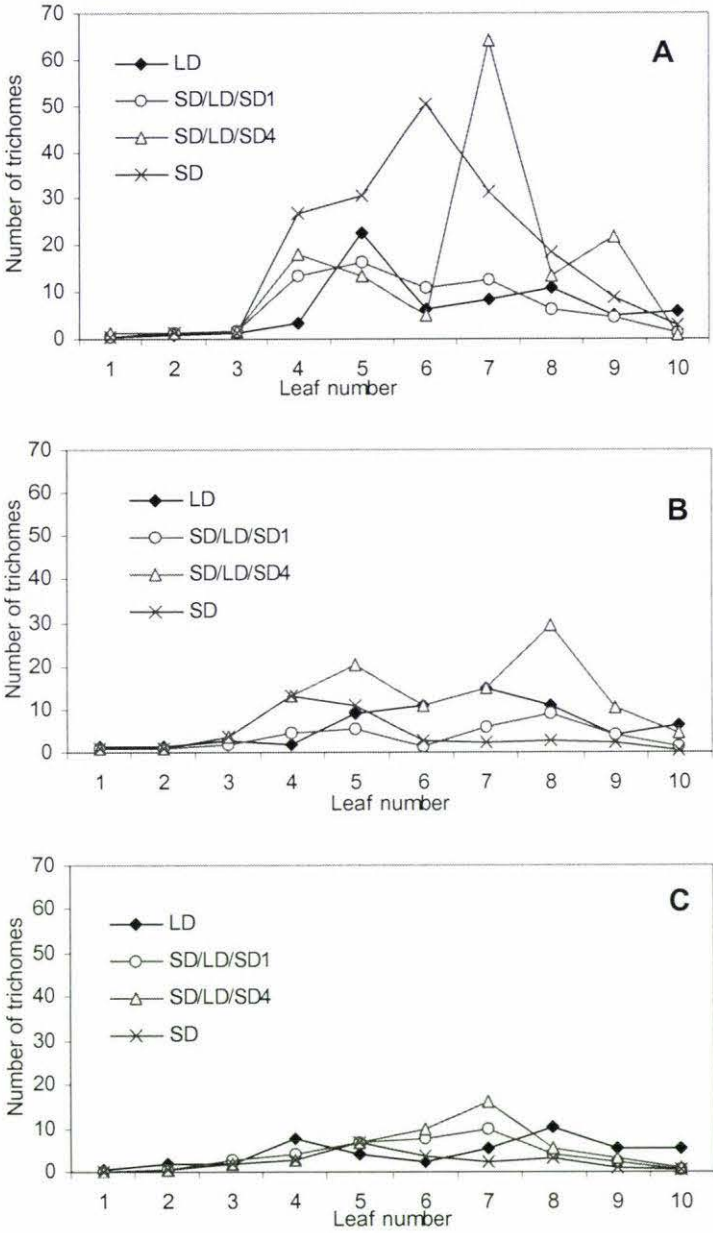


Figure 3.9 Number of trichomes on abaxial surface in leaves 1-10. A, 0 d chilling; B, 10 d chilling; C, 20 d chilling.

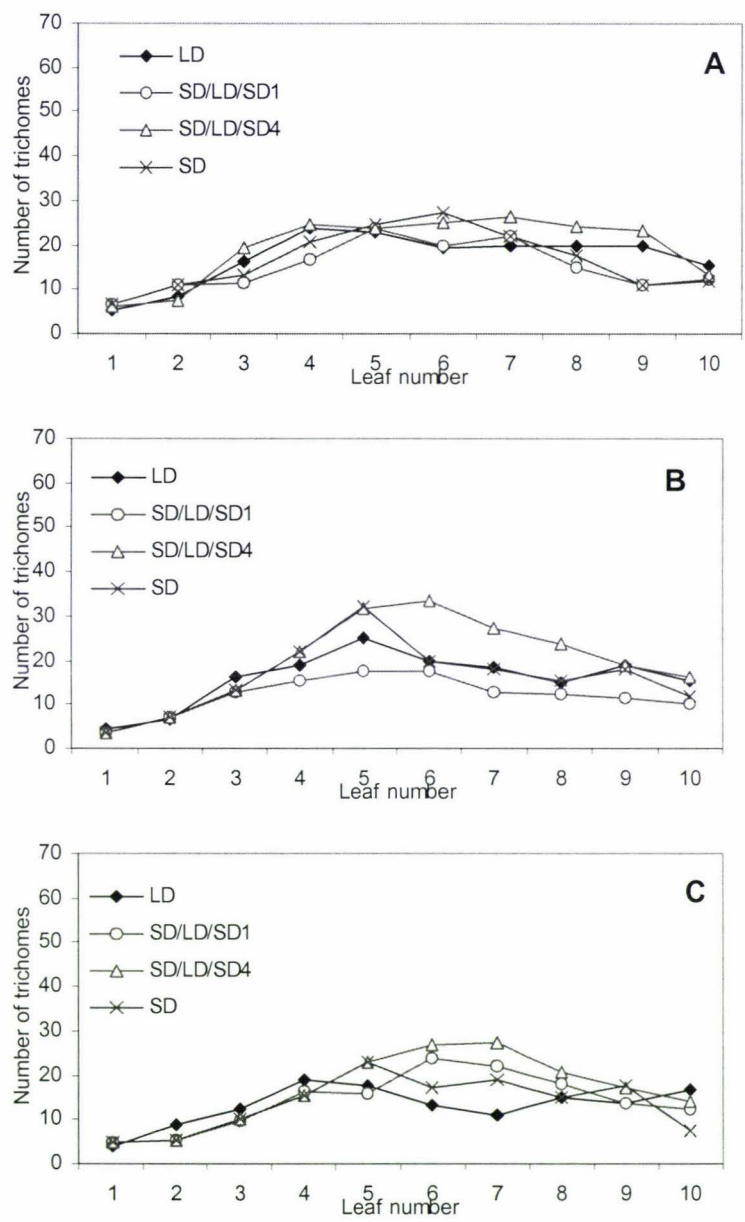


Figure 3.10 Number of trichomes on marginal surface in leaves 1-10. A, 0 d chilling; B, 10 d chilling; C, 20 d chilling.

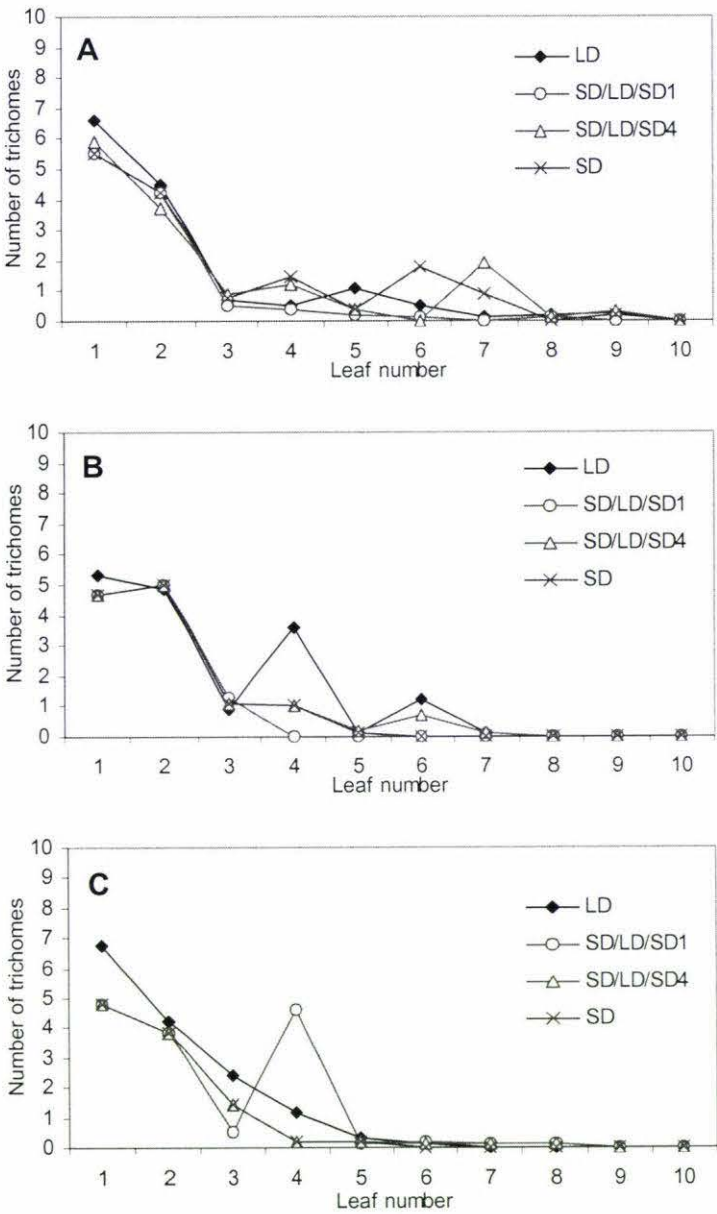


Figure 3.11 Number of trichomes on adaxial surface in leaves 1-10. A, 0 d chilling; B, 10 d chilling; C, 20 d chilling.

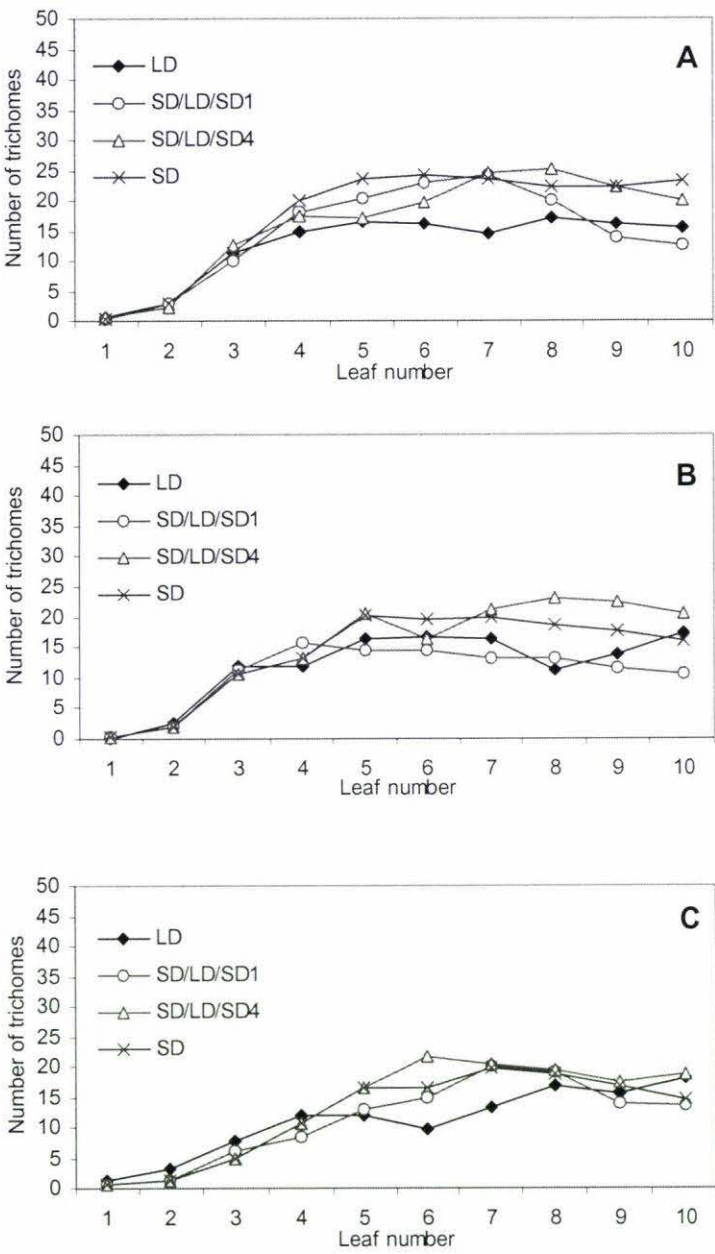


Figure 3.12 Number of trichomes on petiole in leaves 1-10. A, 0 d chilling; B, 10 d chilling; C, 20 d chilling.

Appendix III

Alignment of amino acid sequences used for tree building

Alignment of LFY-like sequences

<i>A. lyrata</i>	(1)	PFIVTEPGEVARGKKNGLDYLFHLYEQCREFLIQVQTIAKDRGEKCPTKV
<i>P. exilis</i>	(1)	PFMVTEPGEVARGKKNGLDYLFHLYEQCREFLIQVQTIAKDRGEKCPTKV
<i>A. thaliana</i>	(1)	PFIVTEPGEVARGKKNGLDYLFHLYEQCREFLIQVQTIAKDRGEKCPTKV
<i>B. oleraceae</i>	(1)	PFIVTEPGEVARGKKNGLDYLFHLYEQCREFLIQVQTIAKDRGEKCPTKV
<i>P. communis</i>	(1)	PFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQNIAKERGEKCPTKV
<i>P. pyrifolia</i>	(1)	PFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQNIAKERGEKCPTKV
<i>P. sativum</i>	(1)	PFIVTEPGEVARGKKNGLDYLFHLYEQCREFLIQVQAIAKERGEKCPTKV
<i>A. majus</i>	(1)	PFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQTIAKERGEKCPTKV
<i>L. esculentum</i>	(1)	PFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQTIAKERGEKCPTKV
<i>V. vinifera</i>	(1)	PFIVTEPGEVARGKKNGLDYLFHLYEQCRDFLIQVQNIAKERGEKCPTKV

<i>A. lyrata</i>	(51)	TNQVFRYAKKSGASYINKPKMRHYVHCYALHCLDEEDASNALRRAFKERGE
<i>P. exilis</i>	(51)	TNQVFRYAKKSGASYINKPKMRHYVHCYALHCLDEEDASNALRRAFKERGE
<i>A. thaliana</i>	(51)	TNQVFRYAKKSGASYINKPKMRHYVHCYALHCLDEEDASNALRRAFKERGE
<i>B. oleraceae</i>	(51)	TNQVFRYAKKSGANYINKPKMRHYVHCYALHCLDEEDASNALRRSAFKVERGE
<i>P. communis</i>	(51)	TNQVFRYAKKSGASYINKPKMRHYVHCYALHCLDVEASNVLRRAFKETGE
<i>P. pyrifolia</i>	(51)	TNQVFRYAKKSGASYINKPKMRHYVHCYALHCLDEEDASNALRRAFKERGE
<i>P. sativum</i>	(51)	TNQVFRYAKKAGASYINKPKMRHYVHCYALHCLDEEVSNELRRGFKERGE
<i>A. majus</i>	(51)	TNQVFRYAKKAGANYINKPKMRHYVHCYALHCLDEEDASNALRRAFKERGE
<i>L. esculentum</i>	(51)	TNQVFRYAKKAGASYINKPKMRHYVHCYALHCLDEEDASNALRRAFKERGE
<i>V. vinifera</i>	(51)	TNQVFRYAKKAGASYINKPKMRHYVHCYALHCLDEEDASNALRRAFKERGE

<i>A. lyrata</i>	(101)	NVGSWRQACYKPLVNIACRHGWDID
<i>P. exilis</i>	(101)	NVGSWRQACYRPLVNIACRHGWDID
<i>A. thaliana</i>	(101)	NVGSWRQACYKPLVNIACRHGWDID
<i>B. oleraceae</i>	(101)	NVGSWRQACYKPLVDIACRHGWDID
<i>P. communis</i>	(101)	NVGSWRQACYKPLVVIAAGRGWDID
<i>P. pyrifolia</i>	(101)	NVGAWRQACYKPLVAIAAGRGWDID
<i>P. sativum</i>	(101)	NVGAWRQACYKPLVAIAARQGWDID
<i>A. majus</i>	(101)	NVGAWRQACYKPLVAIAARQGWDID
<i>L. esculentum</i>	(101)	NVGAWRQACYKPLVAIAARQGWDID
<i>V. vinifera</i>	(101)	NVGAWRQACYKPLVALAARQGWDID

GenBank accession numbers – *Arabidopsis thaliana* (NM_125579); *Pisum sativum* (AF010190); *Arabidopsis lyrata* (AF466802); *Vitis vinifera* (AF450278); *Lycopersicon esculentum* (AF197934); *pyrus communis* (AB162030); *Brassica oleraceae* (Z18362); *Pyrus pyrifolia* (AB162029); *Antirrhinum majus* (M55525).

Alignment of TFL1-like sequences

<i>A. lyrata</i>	(1)	IDPDVPGPSDFFLKEHLHWIVTNIPGTTDATFGKEVVS	YELPRPSIGIHR
<i>A. thaliana</i>	(1)	IDPDVPGPSDFFLKEHLHWIVTNIPGTTDATFGKEVVS	YELPRPSIGIHR
<i>P. exilis</i>	(1)	IDPDVPGPSDFFLKEHLHWIVTNIPGTTDATFGKEVVS	YELPRPSIGIHR
<i>B. oleraceae</i>	(1)	TDPDVFNPSDFFLKERLHRLVMNIPGTTDATLGKEVVS	YELPKPNIGIHR
<i>Pisum sativum</i>	(1)	TDPDVPGPSDPYLREHLHWIVTDIPGTTDATFGKEIVS	YELPKPNIGIHR
<i>P. pyrifolia</i>	(1)	TDPDFPGPSDPYLREHLHWIVTDIPGTTDATFGREVV	SYEMPKPNIGIHR
<i>Pyrus communis</i>	(1)	TDPDFPGPSDPYLREHLHWIVTDIPGTTDATFGREVV	SYEMPKPNIGIHR
<i>A. majus</i>	(1)	TDPDVPGPSDPYLREHLHWIVTDIPGTTDSSFGKEVVS	YEMPRPNIGIHR
<i>L. esculentum</i>	(1)	IDPDVPGPSDPYLREHLHWIVTDIPGTTDCSFGREVV	GYEMPRPNIGIHR
<i>Vitis vinifera</i>	(1)	TDPDVPGPSDPYLREHLHWIVTDIPGTTDSTFGREIVN	YEMPRPNIGIHR
<i>A. lyrata</i>	(51)	FV	FV
<i>A. thaliana</i>	(51)	FV	FV
<i>P. exilis</i>	(51)	FV	FK
<i>B. oleraceae</i>	(51)	YV	FV
<i>Pisum sativum</i>	(51)	FV	FV
<i>P. pyrifolia</i>	(51)	FV	FV
<i>Pyrus communis</i>	(51)	FV	FV
<i>A. majus</i>	(51)	FV	FL
<i>L. esculentum</i>	(51)	FV	FL
<i>Vitis vinifera</i>	(51)	FV	FL

GenBank accession numbers - *Arabidopsis thaliana* (NM_120465); *Arabidopsis lyrata* (AF466817); *Pyrus communis* (AB162042); *Pisum sativum* (AY340579); *Brassica oleraceae* (AB017530); *Lycopersicon esculentum* (U84140); *Pyrus pyrifolia* (AB162041); *Antirrhinum majus* (AJ251994); *Vitis vinifera* (AF378127).

Alignment of Actin-like sequences

<i>B. oleracea</i> ACT1	EIVRDIKEKLSFVAVDYEQEMETSKTSSSIEKNYELPAGQVITIGAERFR
<i>A. thaliana</i> ACT2	EIVRDIKEKLSFVAVDYEQEMETSKTSSSIEKNYELPDGQVITIGAERFR
<i>P. exilis</i>	EIVRDIKEKLSFVAVDYEQEMETSKTSSSIEKNYELPDGQVITIGAERFR
<i>A. thaliana</i> ACT8	EIVRDIKEKLSFVAVDYEQEMETSKTSSSIEKNYELPDGQVITIGAERFR
<i>G. hirsutum</i> ACT2	EIVRDMKEKLAYVALDYEQELETAKSSSSVEKNYELPDGQVITIGAERFR
<i>B. napus</i>	EIVRDIKEKLAYVALDYEQELETAKSSSSVEKNYELPDGQVITIGAERFR
<i>P. sativum</i> ACT2	EIVRDIKEKLAYVALDYEQELETAKSSSSIEKNYELPDGQVITIGAERFR
<i>T. pratense</i>	EIVRDIKEKLAYVAVDYEQELETAKSSSSIEKNYELPDGQVITIGAERFR
<i>S. asiatica</i> ACT2	EIVRDIKEKLAYIALDYEQEMETAKTSSAVEKNYELPDGQVITIGAERFR
<i>A. thaliana</i> ACT1	EIVRDIKEKLAYIALDYEQELETAKSSSVEKNYELPDGQVITIGSERFR
<i>A. thaliana</i> ACT11	EIVRDVKEKLAYIALDYEQEMETANTSSSVEKSYELPDGQVITIGGERFR
<i>S. tuberosum</i>	EIVRDMKEKLAYVALDYEQELETAKSSSSAVEKSYELPDGQVITIGAERFR

B. oleracea ACT1	CPEVLFQPSFVVGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
A. thaliana ACT2	CPEVLFQPSFVVGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
P. exilis	CPEVLFQPSFVVGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
A. thaliana ACT8	CPEVLFQPSFVVGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
G. hirsutum ACT2	CPEVLFQPSFIVGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
B. napus	CPEVLFQPSLVVGMEAPGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
P. sativum ACT2	CPEVLFQPSMIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
T. pratense	CPEVLFQPSMIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
S. asiatica ACT2	CPEVLFQPSMIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
A. thaliana ACT1	CPEVLYQPSMIGMENAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
A. thalianaACT	CPEVLFQPSLVGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF
S. tuberosum	CPEVLFQPSLVGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGTTFMF

B. oleracea ACT1	SGIADRMSKEITALAPSSMKIKVV
A. thaliana ACT2	SGIADRMSKEITALAPSSMKIKVV
P. exilis	SGIADRMSKEITALAPSSMKIKVV
A. thaliana ACT8	LGIADRMSKEITALAPSSMKIKVV
G. hirsutum ACT2	FGIADRMSKEITALAPSSMKIKVV
B. napus	FGIADRMSKEITALAPSSMKIKVV
P. sativum ACT2	FGIADRMSKEITALAPSSMKIKVV
T. pratense	FGIADRMSKEITALAPSSMKIKVV
S. asiatica ACT2	FGIADRMSKEITALAPSSMKIKVV
A. thaliana ACT1	FGIADRMSKEITALAPSSMKIKVV
A. thalianaACT11	FGIADRMSKEITALAPSSMKIKVV
S. tuberosum	FGIADRMSKEITALAPSSMKIKVV

GenBank accession numbers - *B. oleracea* ACT1 (AAD02328); *A. thaliana* ACT2 (NP_188508.1); *A. thaliana* ACT8 (NP_175350.1); *G. hirsutum* ACT2 (AAP73449); *B. napus* (AAD03741); *P. sativum* ACT2 (P30165); *T. pratense* (AAQ74875.1); *S. asiatica* ACT2 (T51178); *A. thaliana* ACT1 (NP_850284); *A. thaliana* ACT11 (AAM65277); *S. tuberosum* (AAB40096.1).

Appendix IV

Primer positions

LFY specific primer positions

CCGTTTATGGTGACGGAGCCGGTGGAAGTGGCACGTGGCAAAAAGAACGGT
 ATAGATTATCTGTTCCATTTGTACGAACAATGCCGTGAGTTCCTTCTTC
 AGGTTCAGACTATAGCTAAAGACCGTGGCGAAAAATGCCCCACCAAGGTGA
CGAACCAAGT → GTTTAGGTACGCGAAGAAATCTGGAGCGAGTTACATAAACA
 AGCCCCAAAATGCGCCACTACGTCGGCAGTTATGCTCTCCACTGCCTAGACGA
 AGACGCTT CAAACGCTCTCCGAAAAGCGTTTAAAGAACGCGG
 TGAGAACGTTGGCTCGTGGCGTCAGGCTTGTTACAGGCCACTTGTTAACATC
 GCTTGTCGTCATGGCTGGGATATAGACGCCGTCTTAACGCTCACCCTCGCTC
 T

PeLFY Forward: ACC AAG GTG ACG AAC CAA GT

PeLFY Reverse: GCT TTT CGG AGA GCG TTT GA

TFL1 specific primer positions

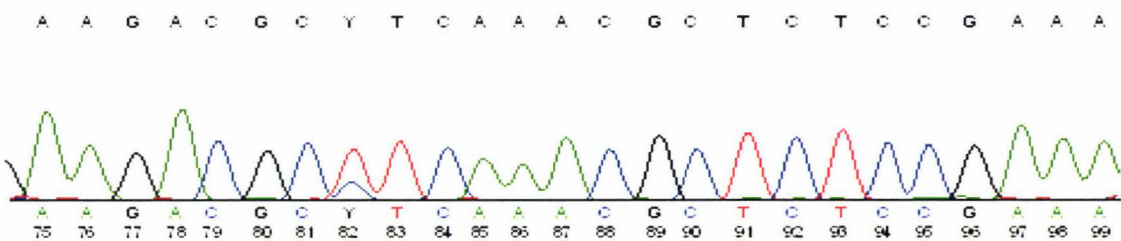
TGATAGACCCAGATGTTCC → AGGTCCTAGTGATCCCTTTCTAAAAGAACACC
 TGCCTGGATCGTTACAAACATTCCCGGTACAACAGATGCT TACATTTGGAA
AAGAGGTGGT GAGCTATGAATTGCCAAGGCCAAGCATAGGGAT

PeTFL Forward: TGA TAG ACC CAG ATG TTC CA

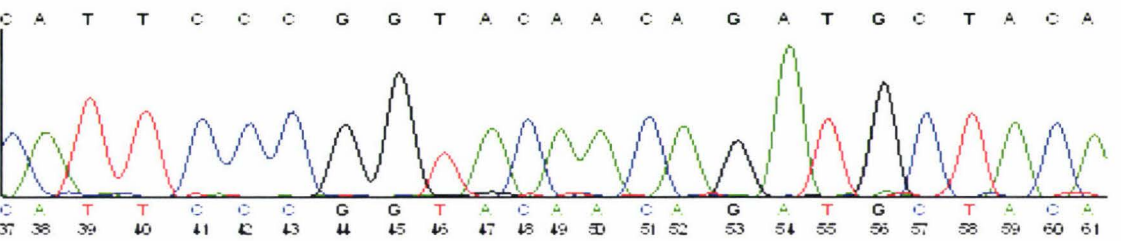
PeTFL Reverse: CCA CCT CTT TTC CAA ATG TA

Appendix V

Sequence segments of *PeLFY* and *PeTFL1* using specific primers



Fragment of *LFY* sequence using specific primers in *P. exilis*



Fragment of *TFL1* sequence using specific primers in *P. exilis*