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

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ANURADHA SOODA

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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, perenniality 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 adxial 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)

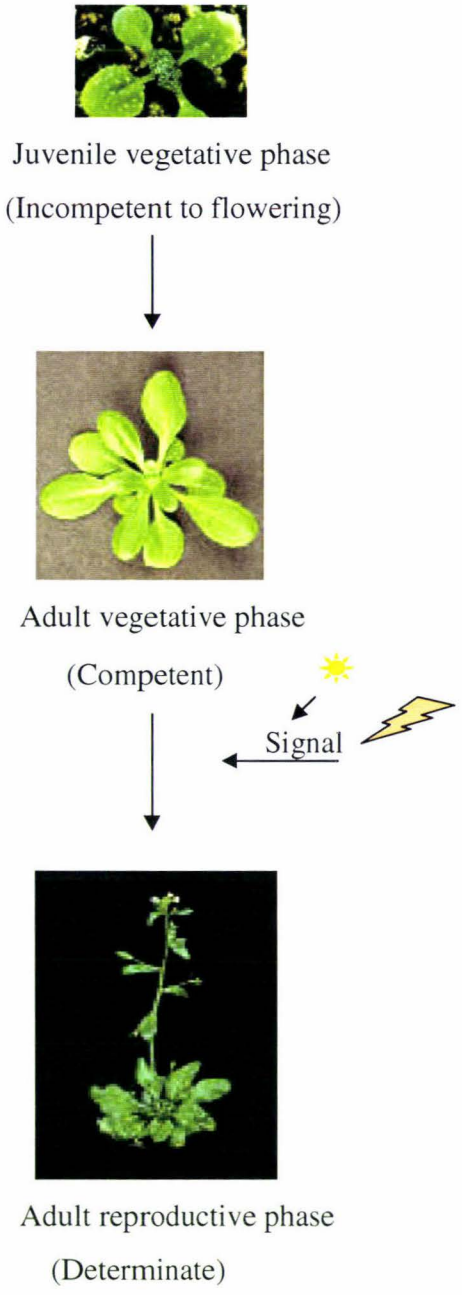

Figure 1.1 A simplified model of the phases of floral development in *Arabidopsis*. Based on McDaniel et al. (1992).

pathway (Fig. 1.2). In *Arabidopsis*, all four pathways converge to regulate the floral meristem identity genes *LEAFY (LFY)* and *APETALA1 (AP1)*, which cause floral induction. As the pathways are integrated by a common set of genes, this enables the plant to produce a synchronised flowering response under conditions in which multiple environmental factors are changing simultaneously (Mouradov et al., 2002).

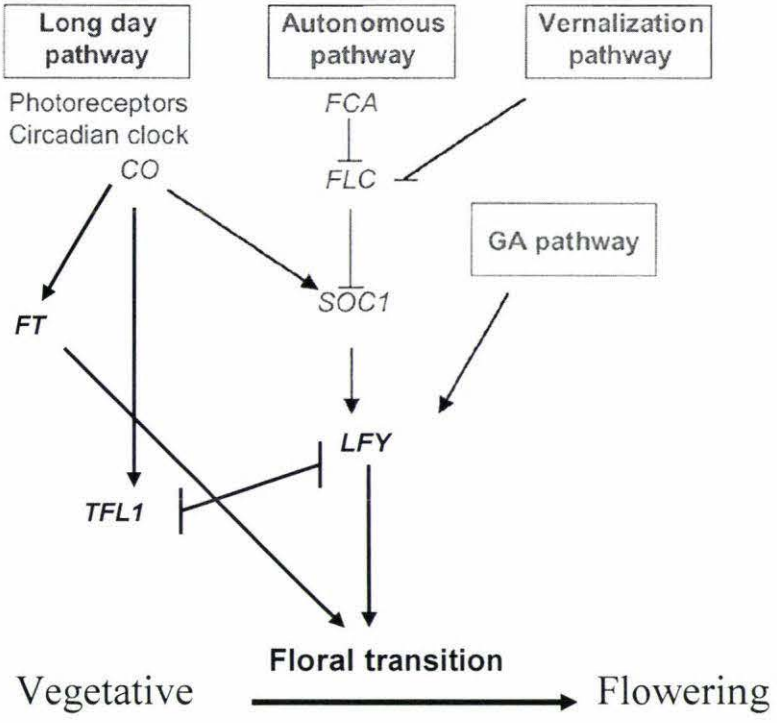

Figure 1.2 A simple model showing pathways controlling flowering time in *Arabidopsis*. →, Promotive effect; ⊥, Repressive effect. Adapted from Blazquez et al. (2001).

1.3.1 Light dependent pathway

The use of the daylength to measure the time of the year and to regulate physiological processes is called photoperiodism (Taiz & Zeiger, 1998). Plants generally fall into one of three groups depending on their responsiveness to daylength: Long Day Plants (flowering is initiated when the daylength is above a critical value), Short Day Plants

(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 *ga1-3* contains a deletion in *KAURENE SYNTHASE*, an enzyme that catalyses the first step of GA biosynthesis and thus disrupts GA production in *ga1-3* mutants (Sun et al., 1992). Under LD, *ga1-3* mutants exhibit a short delay in flowering, whereas under SD (8 h) conditions, *ga1-3* mutants do not flower even after vernalisation. They remain vegetative and eventually undergo senescence (Wilson et al., 1992). The *ga1-3* mutation converts *Arabidopsis* from a facultative to an obligate LD plant (Michael & Amasino, 1999).

Michaels & Amasino (1999) demonstrated that the *ga1-3* 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 GA1-3 (RGA)*, and *RGA-LIKE 1(RGL1)*. Mutations in *RGA* and *GAI* rescued the phenotype of *ga1* 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 *ga1-3* 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 *ga1-3* 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 *AP1*, *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).

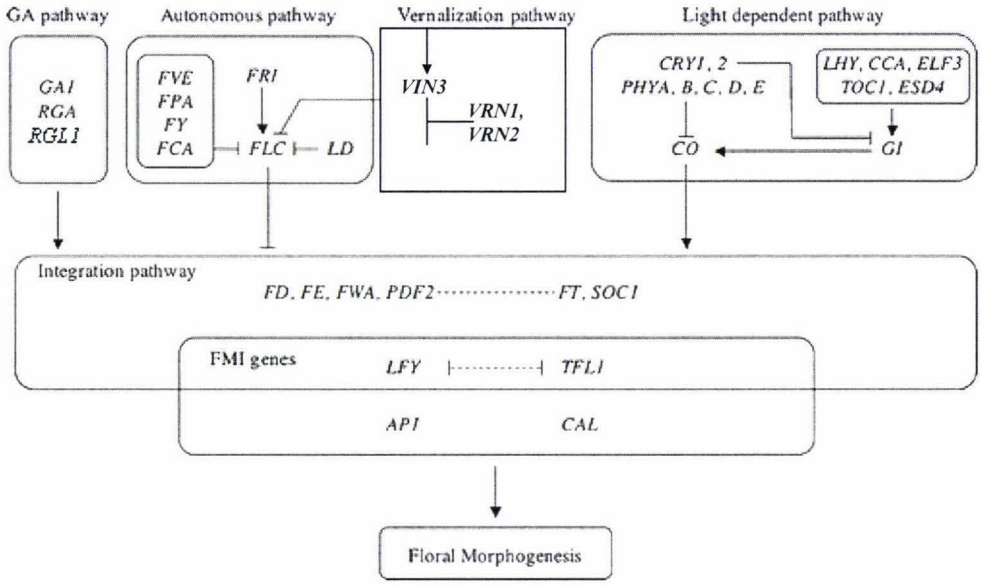

Figure 1.3 The Genetic pathways of flowering in *A. thaliana*. Arrows indicate positive interaction and T lines show negative interaction. Dotted lines show an undescribed interaction. Modified from Amasino (2004) & Komeda (2004).

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 *AP1* 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; Parcy 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 *AP1* (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 *AP1* 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 *AP1* acts downstream of *LFY* in floral transition.

LFY controls the transition to the reproductive stage by inducing *AP1* 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 *AP1*. This *AP1* induction was independent of protein synthesis and occurred specifically in the development stage tissues in which floral fate are assumed. Later expression of *AP1* appears to be only indirectly affected by *LFY*. In addition, it has been shown that *LFY* binds to cis-regulatory elements that control *AP1* expression (Parcy et al., 1998).

LFY null mutations cause severe defects in the transition to floral development. However, the strongest available *AP1* 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 *AP1* 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 *AP1*, 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.