Floral Induction and Development in *Myosotidium hortensia* and *Phormium cookianum*

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Abstract

Little is known of the stimuli needed for flowering in two New Zealand endemic plants, *Myosotidium hortensia* and *Phormium cookianum*. These plants are widely recognised by the horticulture sector and the concerns of this thesis were to aid understanding of floral induction and development in the two species. Environmental stimuli were investigated by growing plants under factorial combinations of daylength and temperature in controlled growth rooms. The two daylengths used, termed long days (LD) and short days (SD), consisted of night / day periods of 8 / 16 h and 16 / 8 h respectively. Two night / day temperature regimes of 4 / 7°C and 18 / 24°C referred to as Cold and Warm respectively, were combined with the daylengths to make four treatments.

Floral induction in both species was unaffected by temperature or daylength, with approximately 50% of the *P. cookianum* flowering under all environmental treatments. *M. hortensia* did not flower. The absence of flowering seen in half of the *P. cookianum* plants was associated with a small size (fewer nodes at the commencement of the environmental treatments). Floral development in those plants that did flower was accelerated in *P. cookianum* by eight weeks growth under Cold compared with Warm treatment. Floral development of *P. cookianum* was further enhanced by four weeks treatment at Cold temperatures followed by transfer for four weeks at Warm temperatures. Vegetative growth was enhanced under Warm temperatures compared with Cold, in both *P. cookianum* and *M. hortensia*.

Hormonal floral stimuli were investigated by application of the gibberellin A_3, followed by growth under Cold SD conditions. The proportion of plants flowering was increased by GA_3 in *P. cookianum*. GA_3-treated *P. cookianum* flowered with fewer nodes as GA_3 concentration increased. In *M. hortensia*, GA_3 application did not cause flowering although stem elongation was increased.

A region of the *P. cookianum FLORICAULA / LEAFY* (*FLO / LFY*) homologue (*PFL*) mRNA was isolated by reverse transcriptase-PCR and sequenced, and shown to share strong sequence identity with other *FLO / LFY*-like genes. *PFL* mRNA

expression was compared with levels of actin mRNA using Real Time reverse transcriptase-PCR, performed using a LightCycler and the double stranded DNA binding dye SYBR Green 1. Upregulation of *PFL* mRNA at the meristem occurred over time, and increases coincided with changes in morphology from vegetative to inflorescence development. As predicted, greater *PFL* expression was observed in fans of larger size, these being the fans with greater likelihood of flowering.

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Introduction

1.0 Introduction

Plants and their products are used for many widely different applications, but it is the reproductive phase and its products that are the focus of most research. It is due to the interesting and unique floral displays of the two New Zealand native species *Phormium cookianum* and *Myosotidium hortensia,* that floral induction and development in these species has been investigated. Knowledge enabling manipulation of flowering time for sale and display of these plants would be beneficial for horticulturists and enable them to use these plants profitably. For this cause the Public Good Science Fund provided incentive to establish the Native Ornamental Plants Programme. Under this initiative flowering time and post-harvest characteristics were to be investigated in selected New Zealand native species with potential for better use in the horticulture industry.

To elucidate flowering time and development in *P. cookianum* and *M. hortensia*, research initially focused on investigation of environmental and hormonal factors that might induce flowering. This was followed by an investigation into the expression of the homologue of the floral meristem identity gene, *FLORICAULA / LEAFY (FLO / LFY)*, in *P. cookianum*.

1.1 The inductive pathways to flowering

1.1.0 Introduction

The important role that products of plant reproduction have played in society has been an incentive for much research. According to Evans (1969), the induction of flowering in hops and hemp was one of the first investigations into floral induction. This was made by Julien Turnois in 1911, due to interest in the two crops shown by his father. In a final paper published in 1913, Turnois stated that 'precocious flowering in young

plants of hemp and hops occurs when, from germination, they are exposed to very short periods of daily illumination' (Evans 1969), suggesting that the plants were perceiving the length of day light hours as a cue for flowering. In order to appreciate the advances that have been made towards understanding the mechanisms of floral induction, the history behind elucidation of the stimuli will be presented followed briefly by information on the genes and gene products involved with the floral pathways.

1.1.1 Photoperiod

Garner and Allard confirmed, in the 1920's, that floral induction was related to daylength signals from the environment. They used many different plant species in a wide range of experiments, using dark houses to shorten light exposure and greenhouses with electric light bulbs to extend light periods. They found that daylength was the factor controlling floral induction in some plants, not the total amount of light received. Two classifications designated by Garner and Allard, short day plants (SDP) and long day plants (LDP), are now used to distinguish between plants that are induced to flower under daylengths shorter than a critical length (SDP) or longer than a critical length (LDP) (Garner and Allard 1920). Some plants require either of these daylengths in specific combinations, such as short-long day plants and long-short day plants.

In order to further their investigations, Garner and Allard (1925) exposed different portions of the SD plant *Cosmos bipinnatus* to LD and SD conditions and found that the response to the induction was localised within the plant. The inductive short light periods were presented to either the top or bottom half of individual plants by placing that region in a box where a door could be shut to restrict light access to less than 12 h (SD). It was discovered that only the induced region of the plant would flower while the rest remained in a vegetative growth phase. These very simple experiments demonstrated that the response to the induction was localised in *Cosmos* (Garner and Allard 1925).

After demonstration of this localisation, Knott (1934) attempted to determine where the action of an inductive photoperiod was perceived in the LD plant, spinach. The growth habit of spinach is of the rosette type, meaning it has one main axis of growth and

vegetative leaves are formed on a very short stem. In order to expose only the growing bud to a 15 h light period, a tube was placed over the bud and two metal shades, the lower inverted against the upper, with a light bulb, were used to provide light exclusively to the tube. The rest of the plant received a 10 h photoperiod and no flowering occurred. When a thimble was placed over the buds to induce a SD photoperiod (10 h) and the leaves of the spinach plants received 15 h of light, elongation of the seed stalk was evident (a florally-associated event in spinach). The results obtained showed that seed stalk formation was not associated with plants where the bud was the sole recipient of a LD photoperiod. However, in plants with leaves and not buds receiving LDs, there was floral formation. Knott also proposed the idea of a stimulus leaving the LD exposed leaves and being transported to the growing point (Knott 1934). This has been confirmed by grafting leaves from plants that received an inductive photoperiod onto plants without, the result being flowering in the uninduced stock. This graft transmissible flowering factor was first coined "florigen" and still has not been identified (Raven et al. 1999).

The mechanisms of the photoperiodic response will be discussed further at the molecular level in Section 1.2.2.

1.1.2 Vernalisation

The exposure of some biennial or winter annual plants to long periods of chilling can initiate a process that enables the shoot apical meristem (SAM) to respond to photoperiodic signals. The effects of seasonal changes in temperature on the induction of flowering were reported well before the 1850's (Evans 1969). An experimental comparison of chilling effects was first reported by Gustav Gassner in 1918, using spring and winter varieties of petkus rye and temperatures of 1 - 2°C, 5 - 6°C, 12°C and 24°C (Evans 1969). Other Russian plant physiologists soon undertook further research into this chilling phenomenon, and Lysenko was the first to name this inheritable character 'jarovizacija' (Evans 1969). The equivalent name, vernalisation, is derived from the Latin word *vernus*, meaning "of the spring" (Raven et al. 1999). After attempting to repeat Gassner's work with failed efforts, it was Lysenko who first

proposed that vernalisation needs to be followed by an inductive photoperiod (Evans 1969).

Once the effect of cold temperatures on flowering of cold requiring plants was realised, it was desirable to determine the effect of localised temperatures on different parts of the plant. Curtis and Chang (1930) encased the crown of celery plants in coils of rubber tubing, through which temperature controlled water could be passed. Celery would normally flower in cold temperatures but plants that were grown in a cool house with warm water flowing around the crown of the plants did not develop flower stalks. When the plants were grown in a warm house and just the crown was kept cool, flowering occurred. These results confirmed that the influence of chilling was acting at the SAM.

The molecular basis of the vernalisation pathway of floral induction will be discussed in section 1.2.1.

1.1.3 Gibberellic acid

The plant hormone group gibberellin (GA) is comprised of more than 120 different GAs. During the 1950's a large number of papers were published on the effects of GAs on flowering. Of the plants treated, *Hyoscyamus niger* was the first for which GAs were implicated in a flowering pathway (Zeevaart 1983). *H. niger* is an obligate LDP that requires vernalisation to induce flowering. When GA_3 was applied, flowering occurred in plants grown under LD conditions, but not SD. This indicated that GA can substitute for the cold requirement but not LDs that are still needed for flowering (Zeevaart 1983). Once the ability of GAs to induce flowering was realised, numerous reports on the effects of GA on numerous species were made. It was optimistically postulated that GA may be the stimulus "florigen" that was produced in the leaves and acted at the shoot apex to induce flowering. It was soon seen though, that the florally inducing effect of the GAs could not be extended to all plants. Numerous reviews have since reported on the widely varying effects of GA with many attempting to document the effects in different species. It is usual though, that GA will induce flowering in LDP under SD, long-short day plants in SD, short-long day plants in SD, or cold requiring plants at

warm temperatures under LD. The inability of GA to induce flowering in SDP is also commonly seen (Evans 1999).

Not only is the ability of GA to induce flowering in different species diverse, so too is the effect of the timing of application and different GA species. In *Lolium temulentum* and many LD rosette plants there is a coupling between stem elongation and flowering. *L. temulentum* is a prostrate grass with a growing point close to ground level. This growth habit protects the developing inflorescence from grazers until rapid stem elongation to expose the floral head to the environment. It was seen in *Lolium temulentum* that GA_3 could induce flowering with effects similar to those of exposure to LD (Evans 1999). However, GA_3 induced stem elongation that normally would not occur for at least three weeks under LD (Evans 1999). By application of natural and synthetically modified variants of GAs, antagonistic outcomes between enhanced vegetative growth and floral induction could be achieved to separate stem elongation from flowering (Evans 1990). This implied different roles for GAs during plant development at different times. That different GAs act antagonistically on flowering and vegetative growth to varying degrees, and development of an inflorescence temporally divides stem elongation and floral evocation, suggests different GAs may function at different times during flowering. This appears to be the case, at least in *L. temulentum*, where endogenous GAs with high florigenicity (GA_5 and GA_6) are seen in the apex upon LD induction, and growth-active GAs (GA_1 and GA_4) are denied access until stem elongation is to occur (King and Evans 2003).

In *Arabidopsis*, a plant with a rosette growth habit, stem elongation occurs synchronously with LD floral induction. A similar response to LD induction is seen when growth-active GA_4 is applied: there is a rapid increase in subapical cell division followed by stem bolting and flowering (King and Evans 2003).

In many woody species such as *Eucalyptus* and citrus, GA biosynthesis inhibitors, like chlormequat chloride and paclobutrazol, can induce flowering when applied exogenously (Zeevaart 1983). Correlating with floral induction in these experiments is the reduction of vegetative growth. This correlation is also seen in *Pharbitis nil* and *Fuschia hybrida* where growth active GAs inhibit flowering but their growth inactive derivatives permit flowering to occur (King et al. 2000). It would appear that stem

elongation diverts assimilate from the apex where flowering would occur. Clearly the response of a plant to GA involves several physiological steps involving varying degrees of stem elongation and flowering which are dependent on growth habit.

The role of GAs in flowering is also being uncovered at the molecular level. A mention of critical discoveries will be made later in Section 1.2.3.

1.2 Molecular mechanisms behind flowering: the interaction of the genome with the environment

1.2.0 Introduction

After germination from seed the vegetative growth habit of a plant is elaborated by the initiation of organs at meristems. Upon floral induction, changes occur at the SAM that see a vegetative meristem previously producing vegetative organs, change identity and turn into a floral meristem producing floral organs or into an inflorescence meristem (InfM) that generates secondary InfM and floral meristems (Weigel et al. 1992).

The molecular mechanisms involved in the switch from vegetative to floral meristem are currently being unveiled, thereby complementing the physiological and environmental work on floral induction and development. The model plant of choice used for studying the molecular genetics of flowering is *Arabidopsis thaliana* L. Heyn. The developmental genetics of this small plant are easy to study relative to many other plants as it typically takes about four to six weeks to obtain mature plants. It also has many other desirable features including easy transformation by *Agrobacterium tumefaciens*, a fully sequenced genome, its ability to grow in sterile media, a low abundance of repetitive DNA, and the ability to self-fertilise. Much of what we know about the interaction of flowering time and development genes is derived from work with *Arabidopsis* mutants having flowering phenotypes different from the wild type. A number of genes have been identified in this way and these have helped the

development of a map of the genetic interactions involved in the floral pathways (Fig. 1.1).

The purpose of flowering is to increase the chance of outcrossing by synchrony of reproductive development within a species. Different plants use many stimuli to achieve this synchrony but the conclusion is still the formation of reproductive structures. It is seen then that a hierarchy of control exists such that all stimuli, whether they be photoperiod or temperature etc., spur the action of a single factor that ultimately determines floral identity. Once the criteria for flowering time is met and floral identity determined, the formation of floral organs is finally undertaken and a flower formed. The flowering outcome is, therefore, determined by a hierarchy of control represented by flowering time genes, which stimulate floral identity genes, that drive expression of floral organ identity genes.

1.2.1 The molecular basis of the vernalisation pathway of floral induction

Arabidopsis was thought to originally be a vernalisation requiring, facultative long day plant. Naturally occurring ecotypes collected from different latitudes and altitudes have been classified as either late-flowering or early-flowering (Moon et al. 2003). Exposing late-flowering plants to cold temperatures (vernalisation) shortens flowering time. Initially it was found the vernalisation requirement of the late-flowering ecotypes mapped to the *FRIGIDA* (*FRI*) locus (Burn et al. 1993). Later, plants with an extreme late flowering phenotype were found to demonstrate the floral repressing action of a second locus acting additively with *FRI*, *FLOWERING LOCUS C* (*FLC*) (Koornneef et al. 1994). The *FRI* protein has two coiled coil domains that may have a role in positively up-regulating *FLC* expression. *FLC* codes for a protein with a MADS domain that inhibits flowering through the inactivation of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* 1 (*SOC1*) (Komeda 2004). Upstream of *FLC*, *VRN2* (the gene responsible for inhibiting *FLC* expression under cold conditions) is proposed to alter the physical conformation of the gene to make the *FLC* gene inaccessible to transcriptional machinery (Komeda 2004). The two genes, *FRI* and *FLC*,

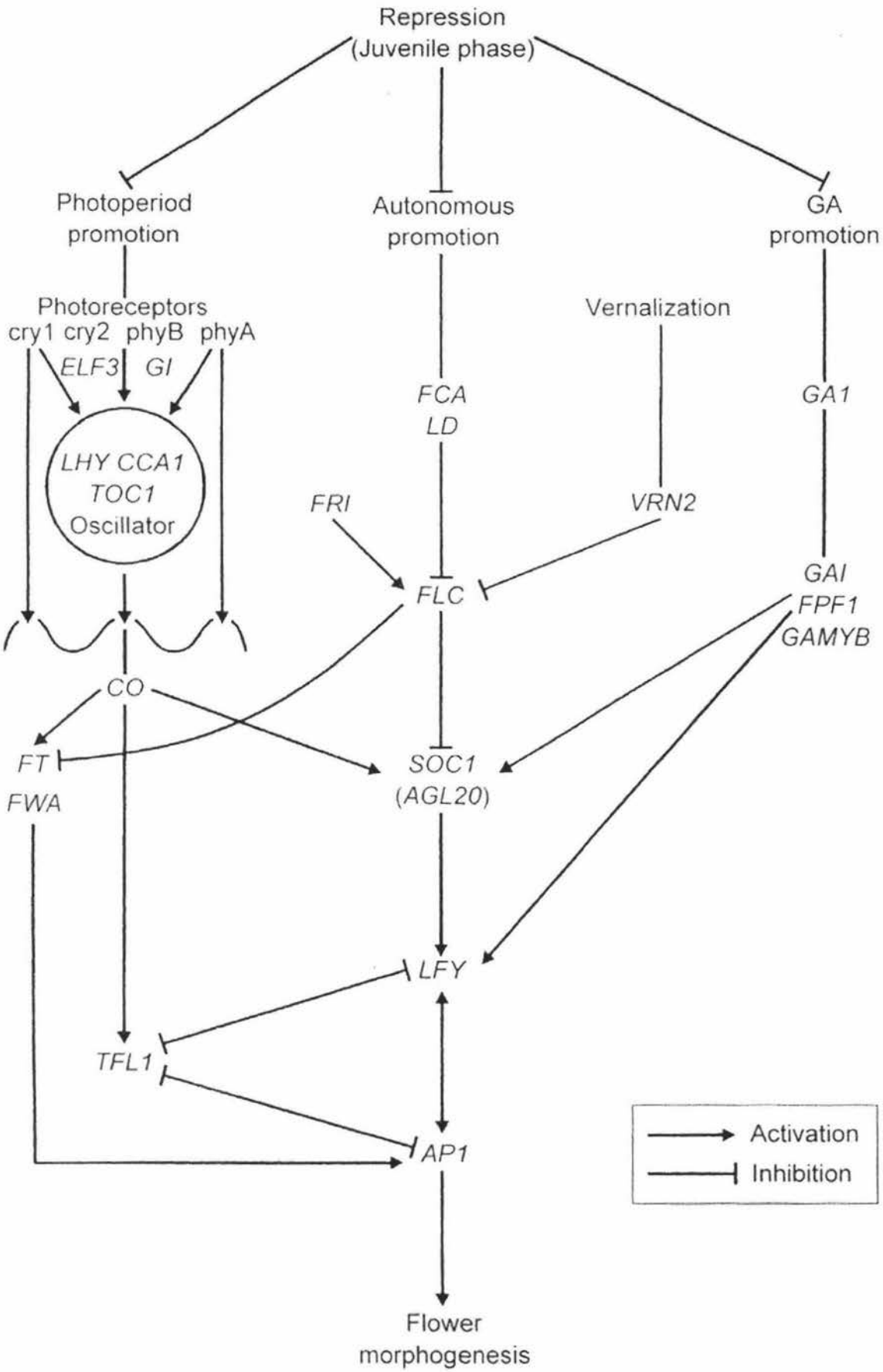


Figure 1.1: A simplified model of the possible interactions between genes and pathways controlling flowering time in *Arabidopsis* (Perilleux and Bernier 2002).

act in synergy to inhibit the actions of the photoperiod pathway and convey the late flowering phenotype (Fig. 1.1).

1.2.2 Molecular basis of the photoperiod pathway of floral induction

The photoperiodic pathway is initiated by the phytochrome (*PHYA/B*) and cryptochrome (*CRY1/2*) proteins that perceive red or blue light signals, respectively, to synchronise flowering. The effects of these proteins, under regulation of the biological clock, is to up-regulate *CONSTANS* (*CO*) expression under *LD* in *Arabidopsis* (Perilleux and Bernier 2002). *CO* encodes a zinc finger transcription factor that acts upstream of *SOC1* to up-regulate its expression (Komeda 2004). These gene products are considered part of a transcriptional cascade that ultimately triggers flowering by activating the promoter of the floral meristem identity gene *LEAFY* (*LFY*) (Perilleux and Bernier 2002). Vernalisation or chilling results in decreased levels of *FLC* which enable the photoperiod pathway to up-regulate *SOC1* and promote the change from vegetative to floral meristem by ultimately increasing *LFY* expression (Fig. 1.1).

1.2.3 Molecular basis of the gibberellin pathway of floral induction

The GA-dependent pathway acts independently of the photoperiod and vernalisation pathways under SDs. It is speculated that the GA pathway acts to ensure flowering when inductive conditions are not met after the plant has reached an adequate size. The gene product of *GIBBERELLIC ACID 1* is necessary for the very first committed step in GA biosynthesis (Komeda 2004). The sequential biosynthetic steps vary depending on the gibberellin generated. The synthesised GA molecules then act as signals for reception by downstream components that continue the signal cascade (Komeda 2004). Expression of the *Arabidopsis GAMYB*-like gene, *AtMYB33*, is up-regulated upon application of GA and binds directly to a *MYB*-binding motif found on the *LFY* promoter (Gocal et al. 2001). While the GA pathway may induce flowering if inductive conditions are not otherwise met, the *LFY*-link between the pathways is common to all.

The LD and GA pathways regulate *LFY* through separate *cis* elements in the *LFY* promoter (Moon et al. 2003).

1.3 *LEAFY*, the floral meristem identity gene

1.3.0 Introduction

LFY acts as a transcription factor and up-regulates transcription of floral organ-related genes and determines the transition from an inflorescence meristem (InfM) that produces floral meristems and secondary InfM, to a floral meristem that produces floral organs (Weigel et al. 1992). *LFY* homologues have been identified in many other species and are always intricately associated with flowering events. No similarities between *LFY* homologues and other plant protein families have been found (Kyozuka et al. 1998).

1.3.1 Mutant phenotype (*lfy*)

Strong *Arabidopsis LFY* mutants (*lfy*) that are induced to flower, display a phenotype quite different from the wild type (Weigel et al. 1992). Ordinarily, after floral induction, the vegetative SAM of wild type *Arabidopsis* changes into an indeterminate inflorescence meristem that initially produces secondary inflorescence meristems, then floral meristems. In plants harbouring a mutant *lfy* allele, the transition from vegetative meristem to inflorescence meristem still occurs, but the production of floral meristems occurs much later in development and flowers retain features of an inflorescence stem.

Flowers that are generated by *lfy* plants have characteristics typical of inflorescence development, such as a spiral pattern of organs instead of the concentric whorls typical of floral development. There are also bracts subtending flowers, that are absent in the wild type, which are similar to cauline leaves that normally subtend secondary inflorescences. Secondary flowers can also develop within the mutant flowers whose outermost floral organs can be leaf-like. The phenotype of *lfy* mutant flowers varies, but often they develop with an outer whorl of sepals, and inner whorls of organs with

sepaloid and carpelloid characters. The absence of petals seen in *lfy* flowers suggests an additional role for *LFY* in maintaining petal organ identity as well as defining floral meristem identities.

1.3.2 Expression of *LEAFY*

Expression of *LFY* in floral meristems is initially seen before the cells that define the floral meristem have even started to develop as buttresses on the sides of the InfM (Weigel et al. 1992). *LFY* is an activator of the homeotic genes involved in floral organ development and sepals, petals, stamens and carpels all show *LFY* expression at some stage during their development (Weigel et al. 1992). There is no expression seen in the InfM. *LFY* mRNA is also found in leaf primordia during vegetative growth, but surprisingly there is no visible change in the phenotype of *lfy* leaves (Weigel et al. 1992).

Ectopic expression of *LFY* under the viral 35S promoter leads to early flowering time in *Arabidopsis* and is consistent with the hypothesis that *LFY* controls the switch to floral meristem identity (Blazquez et al. 1998).

1.3.3 Dicotyledonous *LEAFY* homologues

LFY homologues have been isolated and sequenced from many dicotyledonous species and it is found that the sequences are highly conserved. The function and expression of the homologous genes are also very similar. *LFY* was isolated using a probe designed for *FLORICAULA* (*FLO*), the *Antirrhinum majus* homologue, which was the first floral meristem identity gene to be isolated (Weigel et al. 1992). The inflorescence phenotype displayed by *FLORICAULA* mutants (*flo*) is similar to that seen in *lfy* plants except that there is a complete conversion of all floral meristems into secondary InfM, and no flowers develop (Coen et al. 1990). This is an important difference between the two species, the switch to floral meristems normally maintained by *LFY* is a partially redundant pathway that another gene (*APETALA*) is able to substitute for in *lfy* plants. Many other homologous mutant phenotypes have been identified and show similar abnormalities in phenology and a delay in the time of flowering. It is from these first

two genes that the floral meristem identity gene has derived its name, *FLORICAULA / LEAFY* (*FLO / LFY*).

LFY has also been implicated in vegetative development as it is often expressed in vegetative tissue and mutants of other dicot species sometimes show changes in vegetative growth. When the *LFY*-like genes of tomato and pea, *FALSIFLORA* (*FALS*) and *UNIFOLIATA* (*UNI*) respectively, are mutated a change in leaf phenotype occurs. Pea plants normally develop compound leaves like tomato plants, but *UNI* mutant plants (*uni*) have simple leaves and tomato *FALS* mutant leaves (*fals*) have fewer small folioles (Molinero-Rosales et al. 1999). These observations suggest that *UNI* and *FALS* may have roles in leaf morphogenesis and development that are more pronounced in these plants than in *Arabidopsis*. Work on *Vitis vinifera* has also shown that *Vitis vinifera FLO / LFY* (*VFL*) is expressed in young leaf primordia and at leaf margins where proliferating cell growth continues, and may be involved in generating the palmate shape of the leaves (Carmona et al. 2002). As *VFL* is found in regions of cell-proliferation it is suggested that it may also function in maintaining indeterminacy before cells derived from the apical meristem differentiate (Carmona et al. 2002).

Work with woody perennials such as *Actinidia deliciosa*, *Vitis vinifera*, and *Metrosideros excelsa* revealed that *FLO / LFY* sequence homologies still exist in plants with extended floral development (Walton et al. 2001, Carmona et al. 2002, Sreekantan et al. 2004). Coinciding with extended floral development in the woody perennials, temporal expression patterns of floral identity homologues extend over many months. As seen in annuals, there is an upregulation of *FLO / LFY* upon floral induction at meristems which are destined to become inflorescences. There is, however, a delay in floral organogenesis (spanning 10 months in *A. deliciosa*) in these woody perennials, and *FLO / LFY* levels drop in dormant floral buds until floral organogenesis is to occur, when it is once again upregulated.

1.3.4 Monocotyledonous *FLO/LFY* homologues

The similarities within the dicots do not appear to apply to all of the monocot species investigated, which are mostly grasses and considered a highly diverged group of

angiosperms. These include *Lolium temulentum*, *Zea mays* and *Oryza sativa*. *In situ* hybridisation of floral meristems and tissues revealed divergent expression patterns of *FLO / LFY* compared to dicots and even within the grasses. Although the grasses have floral organs diverged from those of the dicots, the sequence, expression, and function of floral organ genes are moderately conserved. In the dicots *FLO / LFY* acts as a transcriptional activator of floral organ genes. Its role in the grasses has yet to be confirmed.

1.3.4.1 *Oryza sativa FLORICAULA / LEAFY (OSL, RFL)* (Kyozuka et al. 1998)

Upon floral induction in *O. sativa*, the SAM changes identity and becomes a young panicle apex. The apex continues generating organs in the form of panicle bracts until it eventually degenerates then terminates leaving a scar on the main rachis. From the axils of the panicle bracts primary branches develop that generate secondary branches which terminate in a single-flowered spikelet.

The *O. sativa FLO / LFY* homologue, *RFL*, was expressed in vegetative tissue in the epidermal cells of very young leaves at the margins but not in stems. The SAM shows *RFL* expression once the change to early panicle has occurred. Once panicle development was underway *RFL* expression was seen throughout the panicle except where primary branches are differentiating. *RFL* levels started to decrease in the main panicle axis as development approached the middle stage of primary branch differentiation. After all primary branch primordia were formed RFL expression ceased in the main panicle axis. As the primary branches developed, *RFL* was expressed in all cell layers of the branches except the apical meristem which would terminate in a floret. Down-regulation of *RFL* expression was seen in the primordia of developing secondary branches and was entirely absent from spikelet tissue. After the branch formation stage, no *RFL* was expressed at detectable levels in the panicle.

The appearance of *RFL* before the development of any floral meristems and its absence from any region destined to produce a floret demonstrated that *RFL* was not needed for floral meristem identity. It was more likely that *RFL* was required for maintenance of the proliferating branching pattern of the inflorescence.

As *RFL* was found in the epidermal cells of young leaf margins it is possible that, like *FLO / LFY*, *RFL* plays a role in vegetative development.

1.3.4.2 *Lolium temulentum FLORICAULA / LEAFY (LtLFY)* (Gocal et al. 2001)

Like the expression of *RFL* seen in *O. sativa*, *LtLFY*, the *L. temulentum FLO / LFY* homologue showed divergence from the typical dicot expression pattern of *FLO / LFY* homologues. *LtLFY*, was not associated with floret meristem initiation. *In situ* hybridisation revealed that *LtLFY* was expressed transiently after 12 days of inductive LD treatment, instead of being closely linked to the floral meristem transition as in dicots. Expression was localised to spikelet meristems and also glume and lemma primordia.

The expression of two floral organ homologues (*LtMADS1* and *LtMADS2*) was also tracked (Gocal et al. 2001) and found to be consistent with the dicot pattern of floral development where they were expressed in perianth-like organs. It was suggested that, as *LtMADS1* and *LtMADS2* are expressed before *LtLFY*, they may play an initial role independent of, and then interact with, *LtLFY* (Gocal et al. 2001). This suggestion discounted *LtLFY* as having a key role in determining floral meristem identity in *L. temulentum*.

1.3.4.3 *Zea mays FLORICAULA / LEAFY (ZFL)* (Bomblies et al. 2003)

While *RFL* and *LtLFY* appeared to play a different role in flowering than in the dicots, work with the *Z. mays FLO / LFY* homologue (*ZFL*) has revealed similarities. Expression studies using RT-PCR found that *ZFL* was expressed in vegetative apices and was strongly up-regulated during reproductive development.

In situ hybridisation showed the localisation of *ZFL* expression during floral development and similar temporal and spatial patterns of *ZFL* expression were observed between the male and female florets. As the inflorescence structure took shape, *ZFL* expression was detected at the sites where the spikelet-pair meristems were to develop.

As the spikelet pairs started to differentiate, *ZFL* expression was up-regulated and was sustained as the two floret meristems developed. Expression was seen in the upper half of developing floret meristems and was absent from tissue between the two florets. Expression was detected in developing floret organs. The temporal and spatial pattern of expression was similar to that of the dicots.

It seems that while the dicot flowering model may be applied to *Z. mays*, a new model may need to be formulated to explain floral organ initiation in the other two grasses, *O. sativa* and *L. temulentum*.

1.4 Species description

1.4.1 *Myosotidium hortensia*

Myosotidium hortensia is known as the Chatham Island forget-me-not and is closely related to other forget-me-nots of the genus *Myosotis* (Clough 1997). Like *Myosotis, M. hortensia* has five yellow scales at the mouth of the flower, which lend the genera their similarities in the family Boraginaceae (Clough 1997). The fruit, having the appearance of four broadly winged nuts, distinguishes the monotypic genus *Myosotidium* (Clough 1997).

M. hortensia produces large (>30 cm wide), fleshy, deeply grooved leaves that initiate at a central growing point below the soil surface (Fig. 1.2). Flowering occurs in October/November when dense heads of flowers are produced (Fig. 1.3) and elevated well above the foliage (Clough 1997).

M. hortensia is native to the Chatham Islands (44°S, 176°30'W) where it was originally found growing in coastal regions. The Chathams are located 860 km east of Christchurch and have a cool maritime climate with an average 4.2 h of sunlight per day and very high (>84%) average humidity. Annually, the average rainfall is 850 mm and the temperature 11°C with extremes at - 3°C and 23°C (Clough 1997).

1.4.2 *Phormium cookianum*

Phormium is a genus widespread in New Zealand comprised of only two recognised species, *P. tenax* and the species of interest in this study *P. cookianum*. *P. cookianum* consists of two subspecies: *P. cookianum.* ssp. *cookianum* is the Southern mountain form and *P. cookianum.* ssp. *hookeri* the Northern lowland variety (Beckett 1992).

P. cookianum is a perenniating, herbaceous, monocot with long strap-like leaves arranged in a distichous manner (Fig. 1.4) (Beckett 1992). Flowering occurs in summer (Fig. 1.5), after which the main axis of growth dies leaving axillary fans to continue the perennial habit.

P. cookianum is found occupying a range of habitats, from sea level to about 1,500 m, growing anywhere from coastal cliffs to mountain slopes (Cheek 1979).

1.5 Floral Induction In Related Species

Myosotis scorpioides has been classified as a day-neutral plant with a very strong flowering response to cooling treatments (Armitage and Garner 1999). Flowering in *Myosotis alpestris* was induced by application of GA_7, while GA_3 caused only stem elongation (Zevaart 1983). Observations on the effect of gibberellins and daylength on *Cordyline terminalis*, a plant closely related to *Phormium cookianum*, showed that it flowered in response to exogenous GA_3, and GA_{4+7} and this GA-induced flowering was day-neutral (Fisher, 1980). Fisher also speculated that flowering may occur in response to seasonal changes in temperature but not daylength.

1.6 Aims And Objectives

The aim of the first year's research was to define factors that would enable the control of flowering time in two species, *Myosotidium hortensia* and *Phormium cookianum*, and to monitor changes in vegetative growth associated with flowering. Environmental factors were manipulated in growth rooms at the Controlled Environment Laboratory

Figure 1.2: **An experimental subject (*Myosotidium hortensia)***

Figure 1.3: **Flowering *Myosotidium hortensia* (picture sourced from www.liddlewonder.co.nz)**

Figure 1.4: **An experimental subject (*Phormium cookianum)***

Figure 1.5: **Flowering *Phormium cookianum* (Experimental subject)**

(Palmerston North) and trials with application of GA_3 were undertaken. This would enable the hypothesis that both *M. hortensia* and *P. cookianum* are daylength neutral but will flower in response to cold temperatures to be tested. In addition, the suggestion that GA_3 might enhance the flowering response was also tested.

Flowering in *Phormium cookianum* was examined more closely in the second year. The objectives of this work were to isolate a *FLO / LFY* homologue (*PFL*) and to investigate the effects of plant size, as expressed by node number, and GA_3 application on *PFL* expression as meristems transformed from vegetative to floral. These objectives were established in order to test the hypothesis that GA_3 and increasing node count will increase *PFL* expression in the meristem. These objectives were attempted using a Real Time Reverse Transcriptase-PCR approach to monitor temporal changes in mRNA transcript levels, and *in-situ* hybridisation to observe spatial differences in gene expression.

Chapter 2

Floral induction and development in *Myosotidium hortensia* and *Phormium cookianum*

2.0 Introduction

The principles of photoperiod and vernalisation applied by early researchers to identify the many exogenous and endogenous signals that induce flowering in a wide range of plants are still being used today. Gibberellins (GAs) are being used with varying effects on floral induction in different species dependent on the timing of application and the species of GA used.

Based on the observations (see Chapter 1) made by Zeevaart (1983), Fisher (1980), and Armitage and Garner (1999) it was hypothesised:

> that both *Myosotidium hortensia* and *Phormium cookianum* would show no floral response due to different daylengths, but would flower in response to chilling, and that application of GA_3 would enhance this floral response.

To witness the effects of environmental stimuli on floral development in *Myosotidium hortensia* and *Phormium cookianum* and to test the hypothesis, four rooms at the Controlled Environment Laboratory (CEL), HortResearch, Palmerston North were utilised to manipulate photoperiod and temperature. The rooms were used to create four artificial environments for the plants as combinations of Short (SD) and Long (LD) daylengths and Warm and Cold temperatures (detailed below). After this treatment, all plants were moved to a forcing house with Warm temperatures and daylength extension. The growth and development of the plants were monitored for the time of the trial. Plants were also taken for sectioning of shoot apical meristems (SAMs) after 4 and 8 weeks of treatment in the CEL.

Gibberellic acid (GA_3) was also applied to a subset of plants of the two species that were grown under Cold SD conditions before being moved to the forcing house.

As a comparison a third set of experimental plants was held under ambient conditions, half of which was moved into the forcing house with the plants of the GA_3 and controlled environment experiments.

2.1 Materials and methods

2.1.1 Plant material

Phormium cookianum plants were obtained from New Zealand Flax Hybridisers (Tauranga) in 1.5 L planter bags in the form of mature rooted divisions. They were received on 29 Nov 2001 and grown in a tunnel house at the Massey University Plant Growth Unit on raised tables. They received water twice daily from timed overhead sprinklers. On 1 Dec 2001 the plants were repotted into 15 cm pots with a controlled release fertiliser and grown outdoors, where they were watered twice daily from overhead sprinklers. Plants remained here until the start of experimental treatments on 22 April 2002.

Myosotidium hortensia seedlings were delivered from a private grower in Ohakune in 10 cm pots. Plants were grown in the same tunnel house as that used for the *P. cookianum* plants, on raised tables under the same watering regime. On 1 Dec 2001 the plants were repotted into 15 cm pots with the same fertiliser mix as for *P. cookianum.* Plants were returned to the tunnel house and grown under shade cloth. They remained under the shade cloth until commencement of the experiments on 22 April 2002.

2.1.2 Photoperiod and temperature treatments

Four climate rooms at the CEL were used to investigate the effects of Short and Long daylengths and Cold and Warm temperatures on floral initiation and subsequent development. A Short day photoperiod (SD) was applied between 10:00 and 18:00

using four metal halide 1.0 kW bulbs and four tungsten halogen 1.0 kW bulbs, supplying a photon flux density of 500 µmol. $m.^{-2}$ s^{-1}. The Long day photoperiod (LD) was achieved by additional lighting from 06:00 to 22:00 with six tungsten 150 W bulbs, providing a photon flux density of 7µmol. $m.^{-2}$ s^{-1}. Two different temperature regimes were used to provide Cold and Warm environments with night / day temperatures of 4 / 7°C and 18 / 24°C, respectively. The two photoperiods and two temperature regimes applied provided a combination of four different environmental conditions, a Cold SD room, a Warm SD room, a Cold LD room, and a Warm LD room.

On 22 April 2002, *P. cookianum* and *M. hortensia* plants were randomly assigned to each room. Within each room there were five rectangular tables, three on one side and two on the other. Six positions closest to the centre of the room on each table were assigned for the *M. hortensia* blocks, and the six positions next to these for the *P. cookianum* blocks.

Two different experiments were run concurrently. In the first, 80 plants of each species, 20 per room, were assigned positions in the five blocks, four to a block. Plants were grown continuously under each of the four room environments. One plant per block was removed for sectioning and microscopy at four weeks and at eight weeks (randomly assigned per block). In the second experiment, plants were only assigned positions in the two Cold rooms. Ten plants of each species were assigned positions in the five blocks in each Cold room, two to a block. After four weeks in the Cold they were transferred to the Warm room with the corresponding photoperiod. After eight weeks, on 17 June 2002, all plants were moved to a forcing greenhouse with natural lighting that was extended to 12 h by twelve 40 W incandescent light bulbs. Temperatures in the forcing house were typically maintained between 11°C and 15°C.

To track the vegetative growth of both species, leaf emergence was recorded and the youngest and subsequently appearing leaves were measured from the start of the experimental treatments in the climate rooms and throughout their growth in the forcing house. Leaf length in *M. hortensia* was measured from the soil surface to the leaf tip, and in *P. cookianum* from the tip of the lamina to the axis at the stem.

To observe qualitative and quantitative effects of temperature and daylength on floral development the number of plants flowering, the days to anthesis, the number of flowers, and the number of secondary floral axes on the main bolt were measured. The number of leaves each plant had before treatments and the number of leaves generated from Day 1 of treatment, until the node subtending the first floral branch, were also counted.

A separate set of plants were left to grow under ambient conditions, the *P. cookianum* in the courtyard and the *M. hortensia* in the tunnel house, when the CEL experiments began. Half of each were moved into the forcing house on 17 June 2002 with the plants from the CEL trials. Developmental characters were tracked as for the CEL plants mentioned above.

2.1.3 Gibberellin applications

Gibberellin A_3 (GA_3, GibcoBRL, Grand Island, N. Y.) was dissolved in ethanol (EtOH, 95%) to obtain concentrations of 0, 1, 10, and 100 µg. plant^{-1} and applied randomly to 40 *M. hortensia* plants, on 31 January. The GA_3 solutions were applied by pipetting 10 µl along the adaxial side of the midrib of the youngest visible leaf. Plants were then grown in a glass/shade house in random blocks on raised tables and watered by capillary mats twice a day until 22 April 2002. The rate of leaf growth of the treated plants was observed by measuring the distance from the soil surface to the tip of the treated leaf, and leaves that developed after GA_3 treatment. On 22 April 2002 plants were transferred to the CEL and grown for 8 weeks under an 8h photoperiod and a day / night temperature of 7 / 4°C (Cold SD) until 17 June, when they were transferred to the forcing house.

On February 5, 8, and 11, GA_3 was applied to 40 randomly selected *P. cookianum* plants with the assistance of fellow student Grant Irving. Each plant consisted of several fans per pot. Of these fans the two largest were treated and observed, the largest fan being Fan1 and the smaller Fan2. GA_3 was dissolved in 95% EtOH and applied to the fans in a 25 µl aliquot so that each fan received an amount of either 0, 25, 250, or 2500 µg. fan^{-1}. Until the commencement of the photoperiod and temperature experiments at

the CEL on the 22 April 2002 the plants were grown as for *M. hortensia*. At the CEL the plants received an 8hr photoperiod and a day/night temperature of 4/7°C (Cold SD) until 17 June. After this date they were transferred to the forcing greenhouse. The proportion of plants flowering was recorded for each level of GA_3 treatment.

2.1.4 Sectioning of shoot apical meristems

On 20 May 2002, the fourth week of the CEL experiment, 5 plants of both species were taken from each of the two Cold rooms for sectioning, one plant that had been randomly assigned from each table. No plants were taken from the Warm rooms at this time as numbers were reduced by an aphid infestation. On the eighth week (17 June) all the plants designated for sectioning were removed from the Cold and Warm rooms. All plants being sectioned were treated as follows. The larger Fan1 and smaller Fan2 of the *P. cookianum* plants were excised from the lower root tissue below the apical meristem with a scalpel. The cuttings were wrapped in wet paper towels and placed in a bucket of water for transport. The *M. hortensia* were removed from the soil and washed. Most lateral roots were removed from the large primary root and also some older leaves. The remainder was wrapped in a wet paper towel and placed into a bucket of water for transport.

The remaining leaves of both plants were removed in the laboratory down to approximately two or three very young leaves. Most of the tissue below the suspected meristem of each plant was excised to leave a plug of vascular tissue approximately 5 mm long. The tissue was vacuum infiltrated in fixative (FAA; 90 ml 70% ethanol, 5 ml glacial acetic acid, and 5 ml 37% formalin) for 2 h then held at room temperature for 24 h. The tissue was rinsed and stored at 4°C in 70% ethanol to be embedded in wax at a later date. The tissue was dehydrated using an EtOH gradient with changes every 2 h starting at 15% EtOH through to 30%, 50%, and 70% and left overnight at 4°C. The following morning two hourly changes were made starting at 85% EtOH through to 95%, 100% plus erythrosine dye, and another 100% change to be left overnight again at 4°C. The next morning a final 100% EtOH change was made for 2 h followed by hourly changes of EtOH / Histo-Clear II (National Diagnositics, USA, Atlanta Georgia) solutions. The gradient started at 75% EtOH / 25% Histo-Clear II and was followed by

changes of 50% / 50%, 25% / 75% and three 100% Histo-Clear II changes. Wax infiltration was started immediately with the addition of molten Paraplast (Tyco Healthcare Group, USA, Mansfield, MA) at ¼ the volume of Histo-Clear II incubated at 42°C. Wax was added three more times at half hourly intervals until the volume of Histo-Clear II was doubled, and then left at 60°C. After 2 h the Histo-Clear II / Paraplast solution was poured off and replaced with molten Paraplast and left at 60°C. Two hours later the Paraplast was changed and the tissue left overnight at 60°C. Paraplast changes were made twice daily for three more days. Tissue was then embedded in wax (Paraplast) using an embedder (Leica EG 1160) and stored at 4°C for sectioning at a later date. The tissue was sectioned into 10 µm sections using a microtome (Leica RM 2145) and the sections floated in a water bath (42°C) for easier placement onto Polysine microscope slides (BDH Laboratory Supplies). Sections were stained with Toluidene Blue following hydration / stain / dehydration gradients. Slides were first placed in Histo-Clear II for 10 min then a 50 / 50 Histo-Clear II / EtOH solution for 5 min. The following EtOH changes were made every 5 min, 100% EtOH x 2, 95%, 85%, 70%, 50%, 25%, and a final 10% change. The hydration was completed by soaking the sections for 5 min in milliQ H_2O and then staining in Toluidene Blue for 10 min. The slides were then dehydrated back up an EtOH gradient that is the inverse of the hydration gradient starting at 10% EtOH through to two changes of 100% EtOH. The slides were then soaked in a 50 / 50 Histo-Clear II / EtOH solution for 5 min and rinsed in Histo-Clear II twice for 5 sec. Slides were allowed to dry for several minutes then coverslips were mounted using DPX. Sections were viewed and photographed using an Olympus BX51 microscope, an Optronics digital camera and MagnaFire software.

2.1.5 Statistical analyses

The effects of the four treatments on vegetative growth were analysed using orthogonal polynomial linear contrasts in SASv8.2 with a repeated measures model (Appendix A). In order to construct an analysis of variance (ANOVA), certain assumptions were required that needed to be tested to validate any inferences made about the data (Appendix B).

Tests of significance were performed on frequencies of flowering plants and days to anthesis, using chi-squares in SASv8.2. Fisher's exact test was also used where frequencies less than five occurred.

One way tests were performed using the t-test procedure in SASv8.2. Where variances tested different, the Satterthwaite Method was used, otherwise the Pooled Means Method was used to compare two groups of means.

2.2 Results

2.2.1 *Phormium cookianum*

2.2.1.1 Photoperiod and temperature experiments

2.2.1.1.1 Vegetative growth

The environmental treatments applied to the *P. cookianum* plants had different effects on the growth of the youngest visible leaf from Day 1 of treatment through to Day 174. These differences between the plants were significant ($p=0.0007$, Appendix A: Table A.1), indicating differences in growth between plants grown under different conditions. The repeated measures factor, day, also had a significant effect ($p<0.0001$, Appendix A: Table A.2), indicating differences in the length of the leaves over the 174 days. In addition, the interaction effect, treatment*day, was significant ($p<0.0001$Appendix A: Table A.2) indicating the treatments had different effects on growth over time.

Specific comparisons (Appendix A: Table A.3) between the different treatments over time showed significant differences in growth between the Warm and Cold rooms ($p<0.0001$), with growth being greatly accelerated under Warm temperatures when compared with growth in the Cold rooms (Fig. 2.1). A plateau of leaf growth was reached by plants grown under Warm conditions before transfer to the forcing greenhouse on Day 56, whereas maximum leaf growth was not reached by plants in the Cold treatments until approximately 100 days after transfer. In the contrast of the

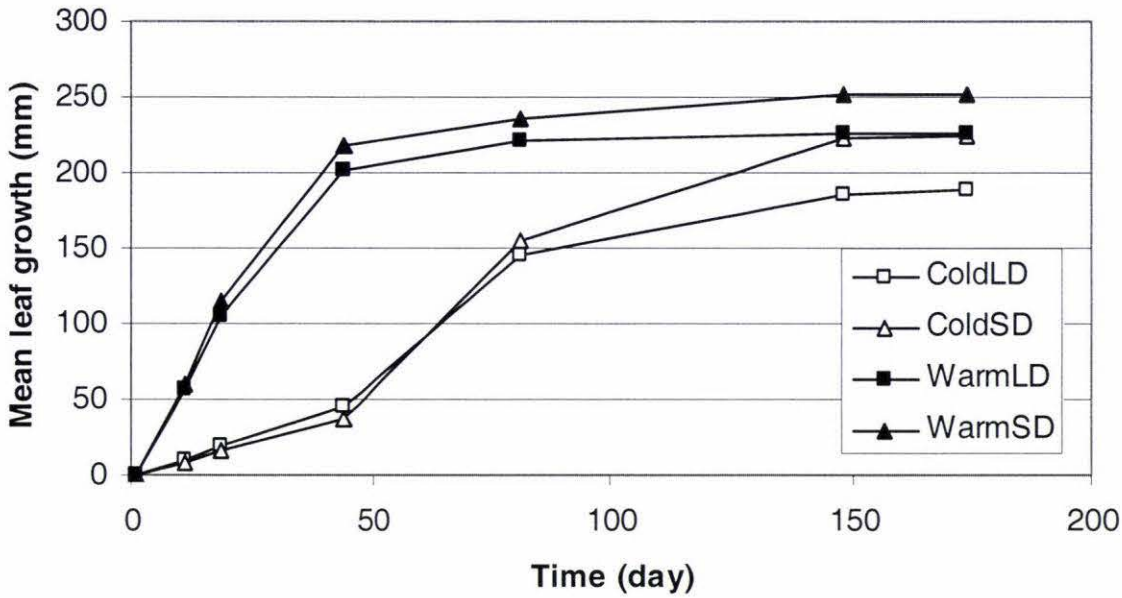


Figure 2.1: **Effect of daylength and temperature treatments applied for 56 days on leaf growth in *Phormium cookianum*. Plants were transferred to a warm greenhouse on Day 56. Refer to Materials and Methods for treatment specifications.**

grouped means for LD and SD under both temperatures, leaf growth in SD plants was significantly higher (p=0.0132) than in LD plants.

The difference between growth under LD and SD in the Cold environments was not significant (Appendix A: Table A.3), although mean leaf length was higher in Cold SD plants than in Cold LD plants at Day 174. Within the Warm treatment, daylength had a marginally significant effect on leaf growth, being consistently lower under LD than SD conditions (p=0.0419).

2.2.1.1.2 Morphological changes at the meristem

To observe any developmental changes occurring in the meristem upon floral induction in *P. cookianum*, plants were taken from the CEL rooms after four weeks and eight weeks of growth and the meristems extracted for fixation. After four weeks of Cold treatment, meristems of *P. cookianum* were at different stages of floral development with varying degrees of elongation (Fig. 2.2 A-C) or still in a vegetative state (Fig. 2.2 D). Large axillary structures in the axils of leaves/bracts of induced meristems were likely developing floral branches. Very little axillary development was associated with vegetative shoot apices. The large apical dome of induced meristems was typical of primary inflorescence meristems compared with the small collection of cells that made up the vegetative meristems. No sections were taken from Warm rooms after 4 weeks due to an aphid infestation.

At eight weeks of Cold treatment (Fig. 2.3 A,B) there was still a large range of developmental differences seen in floral meristems. A meristem at a very early stage of floral development was very elongated, little could be seen of the axillary buds as the section was taken perpendicular to the leaf/bract axils (Fig. 2.3 A). A meristem at an advanced stage of floral development was very elongated and had very large axillary structures (Fig. 2.3 B). The development of axillary meristems has still occurred in Warm grown floral meristems, but with little elongation between the nodes (Fig. 2.3 C,D). The large dome shape of the primary inflorescence meristem was still characteristic of all floral meristems, whether grown under Cold or Warm temperatures. Meristems in a vegetative state after eight weeks treatment showed characters similar to

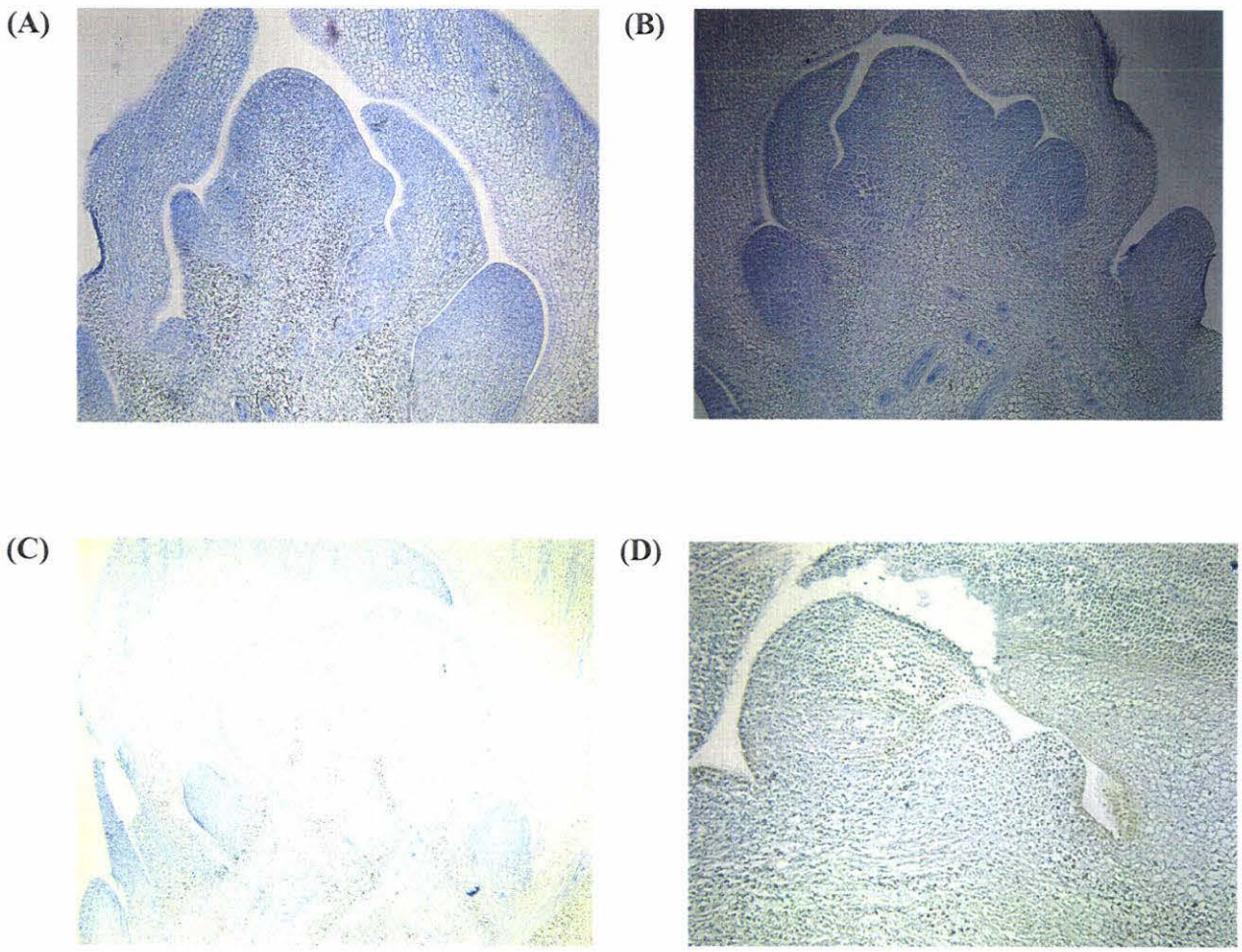


Figure 2.2: **Microscopic study of the shoot apical meristem of *P. cookianum* plants after four weeks of daylength and temperature treatment. Subjects were chosen to display the range in developmental stages and are not representative of all meristems under a particular treatment. (A & B) Cold LD grown meristems. (C & D) Cold SD grown meristems.**

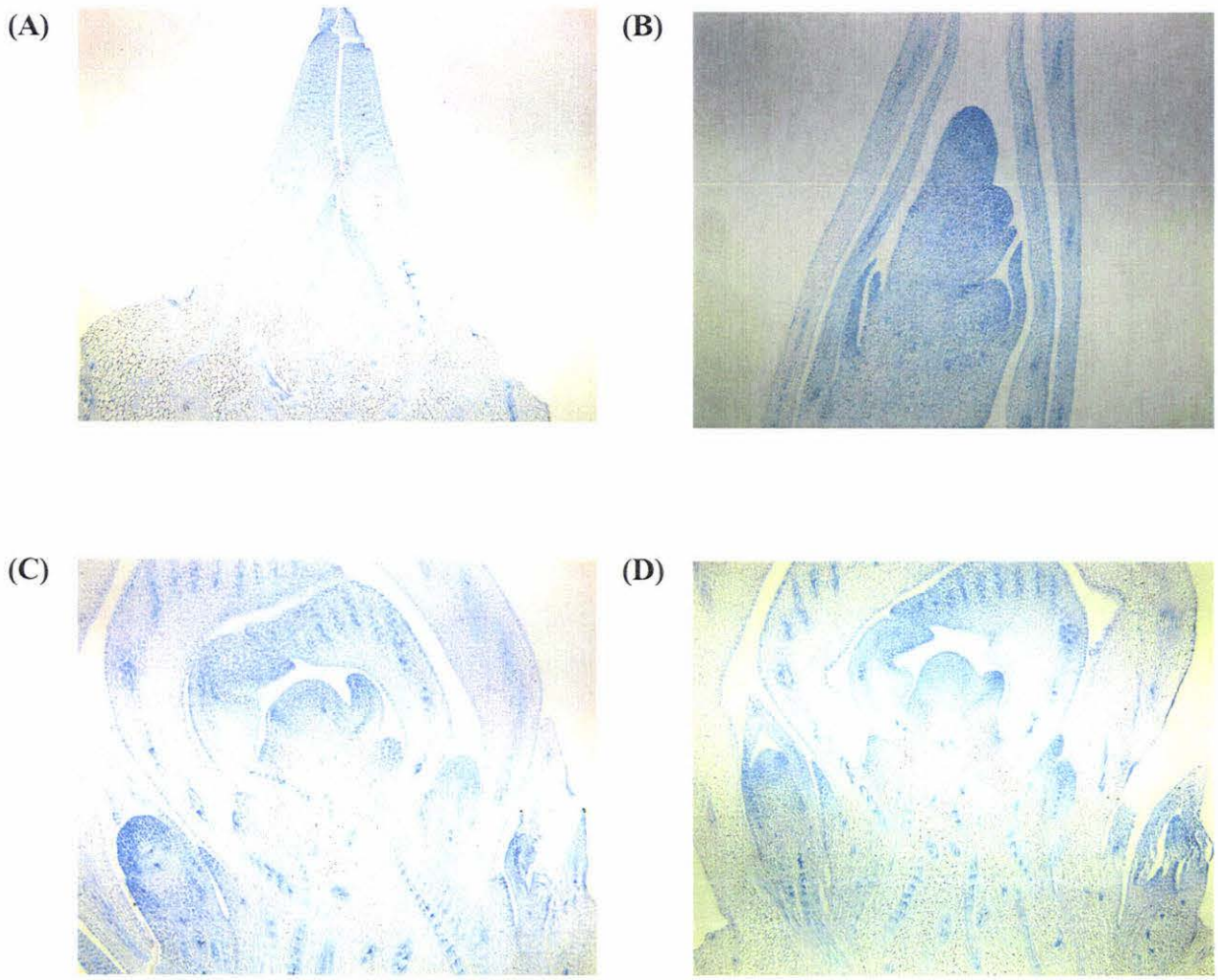


Figure 2.3: **Microscopic study of the shoot apical meristem of *P. cookianum* plants after eight weeks of daylength and temperature treatment. Subjects were chosen to display the range in developmental stages and are not representative of all meristems under a particular treatment. (A) Cold LD grown meristem. (B) Cold SD grown meristem. (C) Warm LD grown meristem. (D) Warm SD grown meristem.**

vegetative meristems of four weeks treatment. The SAMs were small and there was little axillary growth (picture not shown).

2.2.1.1.3 Floral induction

The temperature treatments applied, Warm and Cold, had no effect on the number of plants flowering. Ten plants flowered (F) and ten did not flower (NF) under both temperatures (Appendix A: Table A.4). The null hypothesis that there would be no difference between the number of flowering plants under the two treatments, was confirmed by the chi-square test (p=1.00, Appendix A: Table A.5). The observed frequency of plants flowering under the two daylength treatments was slightly different to the expected frequency (Appendix A: Table A.6). The observed frequency of flowering plants under LD and SD were 11 and 9, respectively. However, this difference was not significant, as indicated by the chi-square value of 52.71% (Appendix A: Table A.7), and the null hypothesis was confirmed.

2.2.1.1.4 Floral development

Although there was no effect of temperature or daylength on the frequency of plants flowering, time to anthesis was dramatically reduced for plants that did flower and had been exposed to the Cold room treatments. After 210 days, flowering Cold treated plants had all reached anthesis, whereas only 20% of Warm treated plants had reached this stage of development. A chi-square test performed on the frequencies found in the contingency table of the proportion of plants reaching anthesis on Day 210 (Appendix A: Table A.8) showed that there was a strong dependency on temperature (p=0.0003 Appendix A: Table A.9).

In a t-test for flowering plants on the comparison of the number of flowers per plant under Cold and Warm treatments there was no significant difference between the means (p=0.4904, Appendix A: Table A.10). The Pooled Means Method was used as the variances had tested not significantly different (p=0.2821).

Again using a t-test for comparison of the mean number of nodes produced from Day 1 of treatment to the first floral branch, there was a significant differences between Warm

and Cold treatments. The Cold treated plants produced fewer nodes (after Day 1) before the first flowering branch ($p<0.0001$, Appendix A: Table A.11). The mean most proximal flowering node was 6.7 and 9.5 in Cold and Warm treated plants, respectively.

No difference was found by the t-test in the number of floral branches under Cold and Warm temperatures ($p=0.1328$, Appendix A: Table A.12).

There was a significant difference between the height of the inflorescences of plants grown under the two different temperatures ($p=0.05$, Appendix A: Table A.13). The Cold grown plants were taller at 540.2 mm compared with 418.7 mm for Warm grown plants.

Tests were performed using daylength as the distinguishing treatment to investigate differences in the same developmental factors above. There was no significant differences found between plants grown under LD or SD for the number of flowers, the node with the first floral branch, the number of floral branches, or the height of the inflorescence (data not shown).

2.2.1.1.5 Floral induction and vegetative growth

The change in vegetative growth associated with flowering could be used to identify flowering plants before the obvious appearance of floral tissues and help map floral development. It was thus decided that further investigation of the effect of floral induction on vegetative growth would be beneficial. As the four treatment combinations had no distinct effect on floral induction, and all treatment effects were found to significantly affect vegetative growth, eight new groups were formed based on treatment and flowering (treatflow). These groups were defined as Cold LD-flowering (Cold LD F), Cold LD-non-flowering (Cold LD NF), Warm LD-flowering (Warm LD F), and Warm LD-non-flowering (Warm LD NF), Cold SD-flowering (Cold SD F), Cold SD-non-flowering (Cold SD NF), Warm SD-flowering (Warm SD F), and Warm SD-non-flowering (Warm SD NF).

Between the groups there was a significant difference in leaf growth ($p<0.0001$, Appendix A: Table A.14). The factor day had a significant effect on leaf length and the

interaction between day and the treatflow groups also had a critically significant effect on growth ($p<0.0001$, Appendix A: Table A.15). These results indicated differences in leaf length over time being found within the eight groups.

Using linear contrasts (Appendix A: Table A.16) to specifically investigate an effect of floral induction on vegetative growth showed significant differences between all contrasts. Leaf lengths in flowering and non-flowering plants under Cold SD and Warm SD conditions were both significantly different ($p<0.0001$). There were also significant differences between the leaf lengths of flowering and non-flowering plants in the Cold LD treatment ($p=0.0008$) and in the Warm LD treatment ($p=0.03$).

Examination of the means (Fig. 2.4) showed that until Day 81, the leaf growth of the non-flowering and flowering plants under the Cold conditions was much lower than that of the plants under Warm conditions, as noted above (Fig. 2.1) when no distinction between flowering and non-flowering plants was made. After this measurement (Day 81) the rate of leaf growth of the Cold NF plants continued rapidly while the Cold F plants had reached a plateau that remains much lower than the leaf growth in all other groups. The Cold NF plants reached a maximum leaf length similar to that of the Warm grown plants.

The Warm SD plants showed a similar trend to that of Cold grown plants, with the flowering plants achieving a much lower plateau than non-flowering plants. The distinct change in growth between non-flowering and flowering plants was seen much earlier in Warm SD grown plants than Cold, by Day 44 compared with Day 148. The Warm LD plants also showed a difference in leaf growth rate by Day 44, but in contrast to other comparisons, flowering plants reached a slightly higher leaf length plateau than the non-flowering plants.

2.2.1.1.6 Crossover experiment

To witness any facultative effects of temperatures on floral development a set of ten plants was transferred after four weeks from the Cold rooms into the Warm rooms with the same daylength factor. As differences in floral development were only found to be significantly different between the two temperature treatments above, t-tests comparing

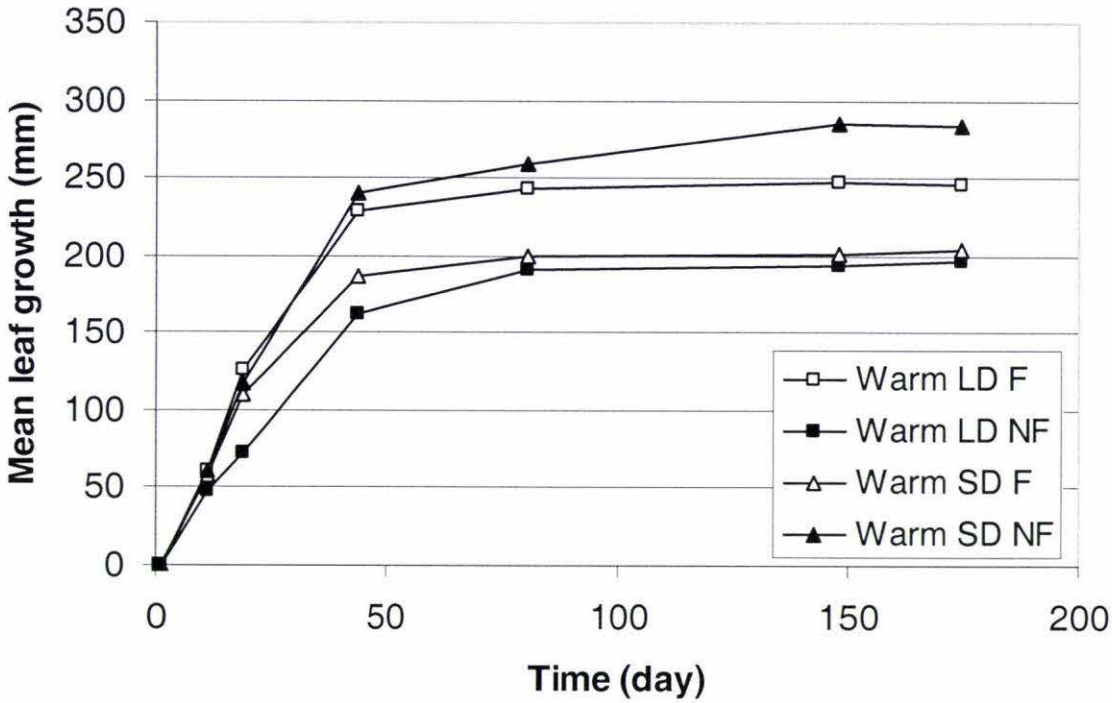


Figure 2.4: **Effect of daylength within Warm temperature treatments applied for 56 days on leaf growth in flowering (F) and non-flowering (NF) plants of *Phormium cookianum*. Plants were transferred to a warm greenhouse on Day 56. Refer to Materials and Methods for treatment specifications.**

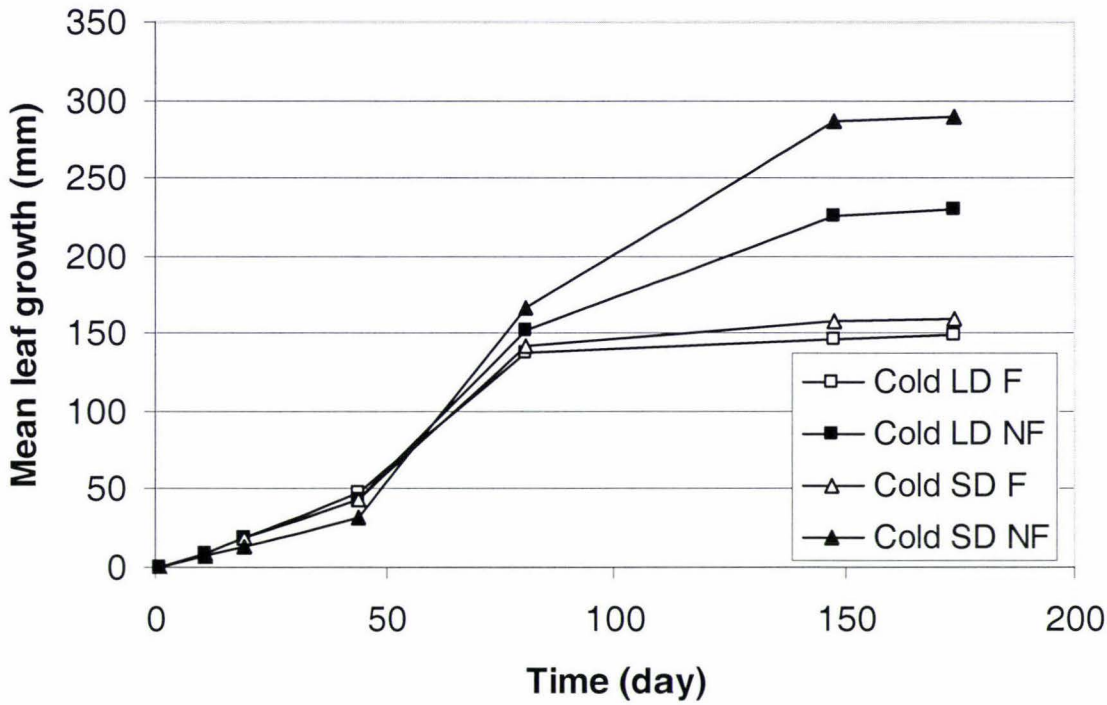


Figure 2.5: **Effect of daylength within Cold temperature treatments applied for 56 days on leaf growth in flowering (F) and non-flowering (NF) plants of *Phormium cookianum*. Plants were transferred to a warm greenhouse on Day 56. Refer to Materials and Methods for treatment specifications.**

the means of these transferred plants and the means of the Cold grown plants (of the photoperiod and temperature experiment above) were performed.

The t-test used to test the null hypothesis, that there was no difference between the mean number of flowers on Cold grown (eight weeks) and transferred plants (four weeks), revealed no significant difference ($p=0.5097$, Appendix A: Table A.17). The variances, however, tested significantly different ($p=0.0621$, Appendix A: Table A.17) so the Satterthwaite method was used to determine the degrees of freedom to be used with the t-test.

The mean node with the first floral branch was found significantly different after performing a t-test ($p=0.0020$, Appendix A: Table A.18). The plants that were transferred after four weeks of Cold treatment flowered on average at node 5 and the plants with eight weeks growth under Cold conditions at node 6.7.

Another t-test revealed that the number of floral branches on the inflorescences of transferred plants (mean 10.3) was significantly higher than the mean 7.9 branches found for plants grown for eight weeks under Cold conditions ($p<0.03$, Appendix A: Table A.19).

The mean height of the inflorescences was not significantly affected between the transferred plants and the Cold grown plants. A t-test demonstrated that the null hypothesis is true ($p=0.4999$, Appendix A: Table A.20).

2.2.1.1.7 Outdoor plants

Upon commencement of the CEL experiments, another group of plants was left to grow outside under the original conditions (refer to Section 2.1.1). These plants were exposed to the conditions of winter. After eight weeks half of the plants were moved into the forcing house (simulating Warm LD of summer) along with the plants of the CEL experiments.

Of the 26 plants that were placed outside, 13 were transferred into the forcing house. Four of the 13 plants that remained outside flowered compared with five of the 13

plants that were transferred to the forcing house. All five of the flowering plants that had been transferred to the forcing house had reached anthesis by 210 days, as had the Cold treated plants of the CEL experiment. None of the flowering plants that remained outside had reached anthesis at this time.

2.2.1.2 Gibberellin experiment

A contingency table (Appendix A: Table A.21) was constructed to depict the actual frequencies of flowering (F) and non-flowering (NF) plants of the largest fans treated with GA_3. The expected frequencies were those that would be observed if the null hypothesis were true, that the GA treatments had no effect on floral induction. As suggested by the contingency table, a chi-square test shows there was very strong evidence against the null hypothesis (p=0.0059, Appendix A: Table A.22).

Application of GA enhanced flowering, from 20% to more than 80%, when applied in amounts greater than 250 $\mu g.\ fan^{-1}$. There also appeared to be an effect of GA concentration on the number of flowers per inflorescence (Fig. 2.6). However, the number of flowers at each concentration was not significantly different (p=0.1603, Appendix A: Table A.23).

In each pot there were several fans, some often growing from the axils of the large main fan that has been the subject of the GA investigation. Only one of all the smaller axillary fans flowered, and this had grown in a pot treated with the highest GA concentration of 2500 $\mu g.\ fan^{-1}$.

2.2.1.3 Fan size and floral induction

The results of the photoperiod and temperature experiments suggested the treatments had subtle effects on floral induction in *P. cookianum*. Approximately 50% of the plants flowered under all four conditions, and alternatively 50% of the plants did not flower under conditions that were potentially florally inductive. The nodes of the fans under investigation in the photoperiod and temperature experiment and the GA experiment were counted. Of the 20 plants that flowered under the four environments of the CEL experiment, all had between eight and twelve nodes counting the youngest visible leaf

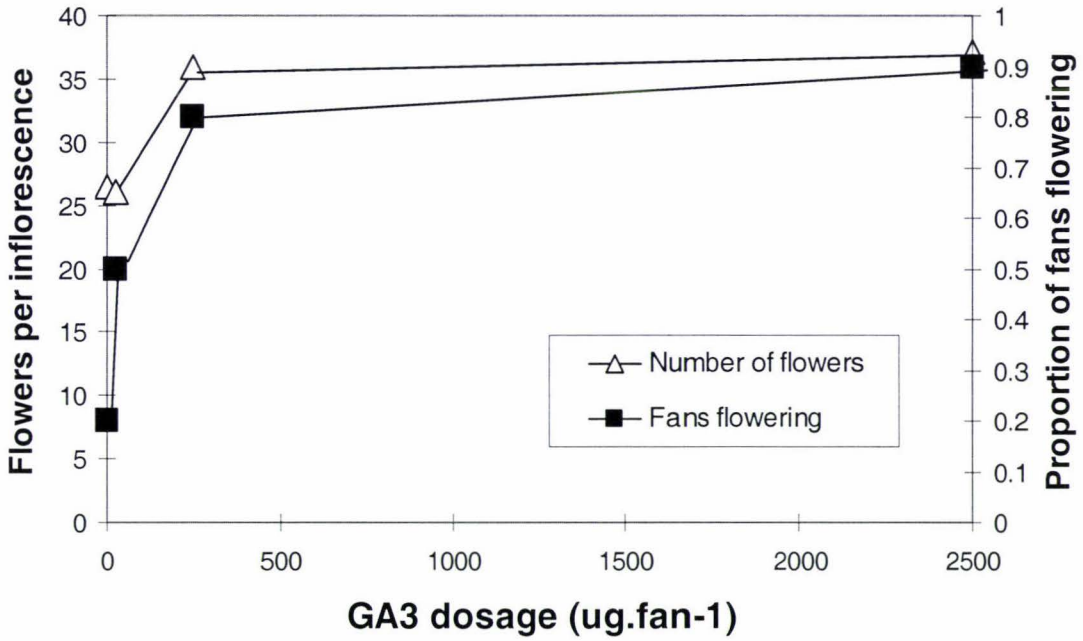


Figure 2.6: **Effect of GA_3 on the proportion of fans of *P. cookianum* flowering, and the number of flowers per inflorescence.**

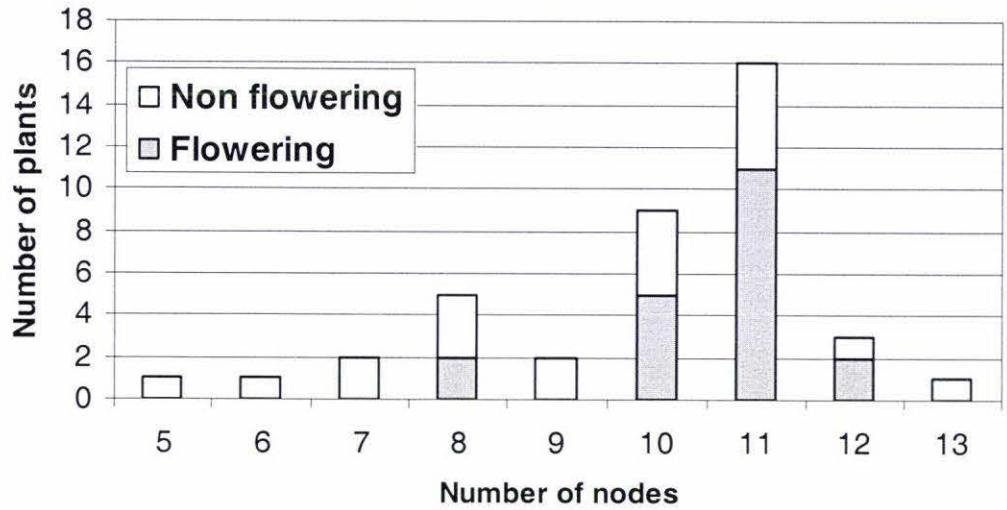


Figure 2.7: **The influence of node number on floral induction in *Phormium cookianum* plants grown under the four different environments of the photoperiod and temperature experiment (Section 2.2.1.1.1). See section 2.1.2 Photoperiod and temperature treatments, for conditions of treatment and growth.**

Table 2.1: **The effect of GA_3 applied to *P. cookianum* and node number on the proportion of plants flowering (Section 2.2.1.2.1). See section 2.1.3 Gibberellin applications, for conditions of treatment and growth.**

	GA (µg.fan-1)			
Nodes	**0**	**25**	**250**	**2500**
5-7	-/-	1/3	-/-	1/1
8-10	1/9	2/5	5/7	5/6
11-13	1/1	2/2	3/3	3/3

at Day 1 (Fig. 2.7). Sixteen plants with eight to 13 nodes did not flower and neither did the four plants with fewer than eight nodes. As node number increased in *P. cookianum* it appeared a plant was more likely to flower when exposed to florally inductive signals. A similar trend was seen in the fans treated with 0 and 25 µg of GA (Table 2.1). Smaller plants with fewer nodes were less likely to flower compared with larger plants that had more nodes. However, as the GA concentration was increased, to 250 µg and 2500 µg. fan^{-1}, the permissive node number of flowering plants was reduced (Table 2.1).

2.2.2 *Myosotidium hortensia*

2.2.2.1 Photoperiod and temperature experiments

2.2.2.1.1 Vegetative growth

The treatments applied to the plants in the four different rooms made critically significant differences to the mean lengths of the leaves ($p=0.0004$, Appendix A: Table A.24). The repeated measures factor Day was also critically significant ($p<0.0001$, Appendix A: Table A.25) indicating differences in the lengths of the leaves at the different times of measurement. A significant interaction effect between treatment and Days implied the different treatments were having different effects on leaf length over time ($p<0.0001$, Appendix A: Table A.25).

Using linear contrasts to specifically identify differences the treatments were having over time, direct comparisons could be made (Appendix A: Table A.26). All differences between Cold rooms and Warm rooms were critically significant over both daylengths ($p<0.0001$). The comparison grouping Cold and Warm rooms by daylength revealed significant differences between LD and SD grown plants ($p=0.0096$). The mean growth when contrasted within the Cold rooms was not significantly different ($p=0.06$), but that within the Warm rooms strongly significant ($p<0.0001$).

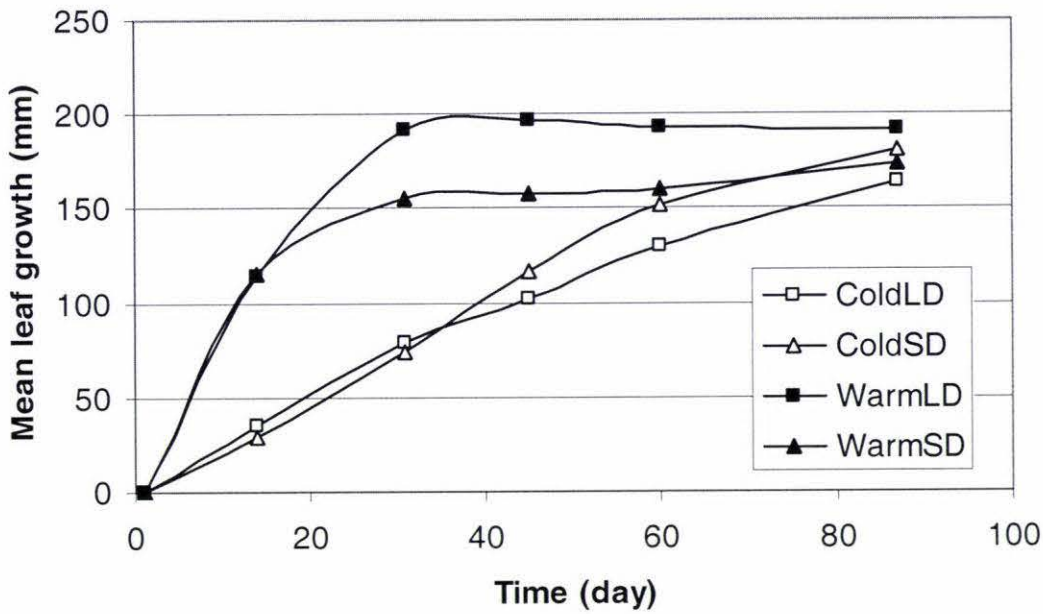


Figure 2.8: **Effect of daylength and temperature treatments applied for 56 days on leaf growth in *Myosotidium hortensia*. Plants were transferred to a warm greenhouse on Day 56. Refer to Materials and Methods for treatment specifications.**

All treatments allowed positive growth over time, the two Cold rooms initially to a much lesser extent than the Warm (Fig. 2.8). The growth of the Warm treated plants was very similar up to Day 14, but diverged by the next measurement at Day 31. By the measurement made on day 31 the Warm SD rate of growth was dramatically lower than that of the Warm LD plants, and remained lower until day 87.

All leaves reached a similar average length by the last measurement on Day 87, although this may not have been a plateau for the Cold grown plants as it was for the Warm treated.

No signs of floral development were seen in *M. hortensia* through October and November when plants would normally be flowering.

2.2.2.1.2 Morphological changes at the meristem

Meristems were extracted from *M. hortensia* plants that had been randomly selected before growth in the CEL rooms. All meristems were vegetative with no floral characters (Figs. 2.9 A, B) regardless of which treatment was received.

2.2.2.2 Gibberellin experiment

2.2.2.2.1 Vegetative growth

During normal vegetative growth, nodes and internodes were very close to or beneath the soil surface. After GA_3 application extension between the nodes at the base of the shoot resulted in an elongated stem as seen during bolting (Fig. 2.10).

No flowering occurred in the *M. hortensia* after application of GA_3.

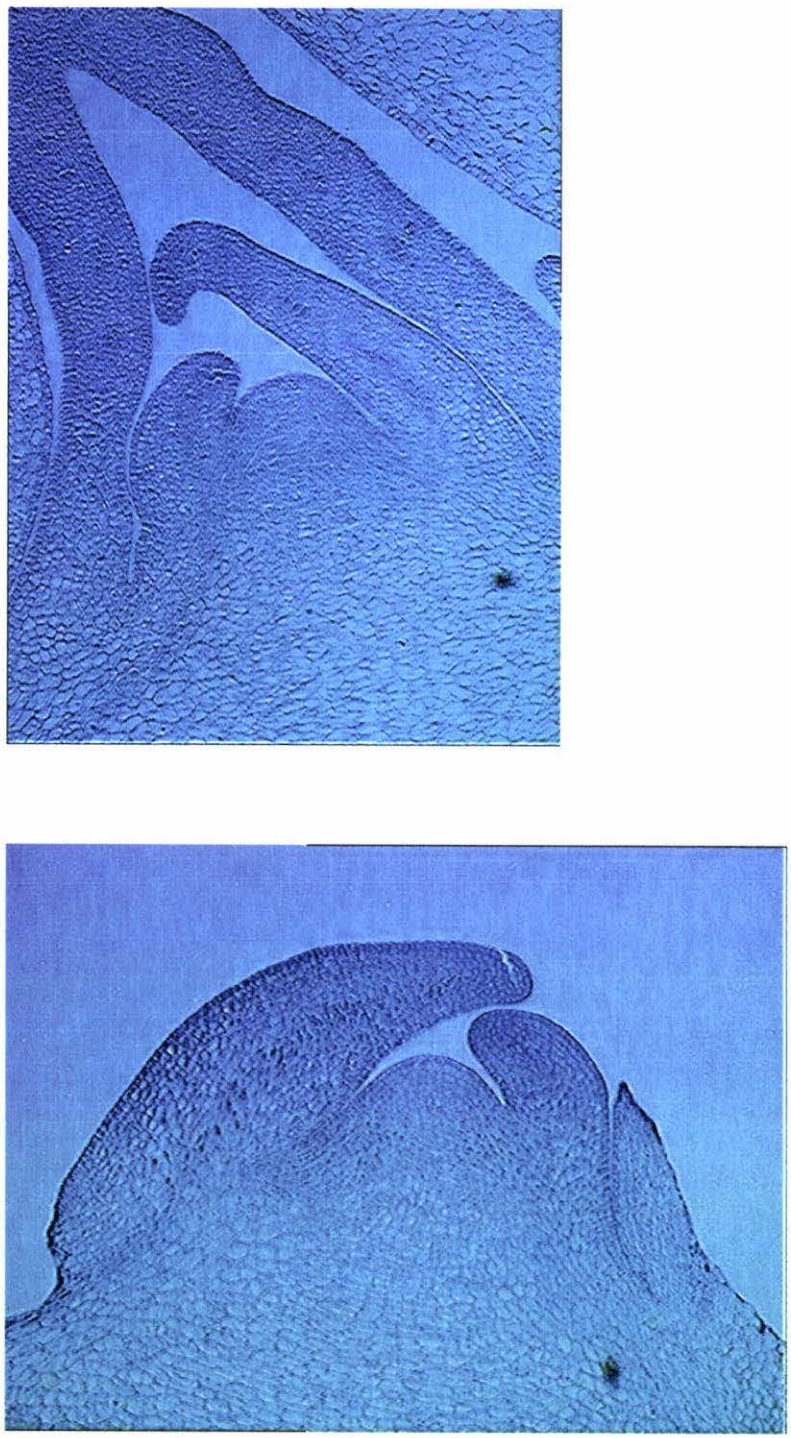


Figure 2.9: **Microscopic study of the shoot apical meristem of *M. hortensia* plants after eight weeks of daylength and temperature treatment.**

Figure 2.10: Internode elongation of *Myosotidium hortensia* treated with 100 µg GA_3 (Extended internodes shown by arrows).

2.4 Discussion

The four temperature and daylength combinations affected the rates of vegetative growth in *P. cookianum* differently. Cold temperatures provided less favourable circumstances for vegetative growth than Warm, as indicated by the slower rates of leaf growth. Constrained vegetative growth has been seen in *Parthenium hysterophorus* at low winter temperatures (7°C), where node production was reduced (Pandey et al. 2003), and in bean cultivars where the relative growth rate was reduced at lower temperatures (10.5 °C, 12 °C, 15 °C, 18 °C, and 20°C) (Kapitsimadi 1988).

During growth, the expansion and elongation of newly formed organs requires the carbohydrate a plant generates in photosynthetic tissues (Wardlaw 1990). For example, Berman and DeJong (2003) showed that growing fruit in *Prunus persica* became competitive sinks for photo-assimilate and suppressed vegetative growth, and while developing flowers generally do not pose as major competitors for resources (Wardlaw 1990), inflorescence emergence in *Lolium italicum* was associated with impeded root growth (Troughton 1956). Therefore it could be expected that environmental treatments that promoted flowering would bring about a measurable reduction in vegetative growth in *P. cookianum*. Although the same proportion of plants flowered across all four treatments, when a comparison was made between flowering and non-flowering plants, in general a decrease was seen in the maximum leaf growth of plants that were to flower. This was a highly significant effect under Cold LD, Cold SD, and Warm SD treatments. This suggests the development of the inflorescence has altered carbon partitioning and withdrew resources away from vegetative growth, promoting its own growth. Plants that flowered after Warm LD treatment had a greater maximum leaf growth than non-flowering plants, although this effect was only marginally significant.

The photoperiod and temperature treatments used to induce flowering in *Phormium cookianum* made little difference to the numbers of plants that flowered. Transferring plants from Cold to Warm rooms also made little difference to floral induction. Half of the plants of the CEL experiments flowered regardless of the treatment applied, suggesting that induction had possibly taken place prior to the start of treatment on 22 April. It is highly unlikely these plants had been responding to temperature or daylength

signals in autumn because the remaining half did not flower in response to such potentially inductive conditions.

Using retrospect to investigate this lack of floral response, it was found that the size of a fan with respect to the number of nodes, was correlated with floral induction, smaller fans being less likely to flower. A larger plant has increased fitness, and increased fecundity, so flowering may be inhibited in smaller plants if increased size improves the viability of offspring (Rees et al. 1999). Supporting this idea, a higher node count in *P. cookianum* was correlated with an increased chance of flowering. Smaller plants with fewer nodes were presumably less able to provide the beneficial resources required for growth of the large inflorescence and fruit set.

A study of periodic flowering in New Zealand flax has implicated high air temperatures in late summer or autumn as promoting flowering the following year in *Phormium tenax* (Brockie 1986). It was seen here that the rate of vegetative growth was temperature dependent in *P. cookianum*, plants at warmer temperatures grew faster. If this was also the case in *P. tenax* then increased vegetative growth the summer before would increase the size of plants, promoting flowering the following spring.

Whereas no significant effect of the different photoperiods and temperatures on floral induction were determined, differences in the rate and intensity of floral development occurred under different temperatures. That the Warm grown plants reached anthesis later, had shorter inflorescences, and bolted at a later node than Cold grown plants suggests *P. cookianum* has a facultative cold temperature requirement for organogenesis. Increased floral stem elongation in cultivars of *Aquilegia* has been demonstrated by Garner and Armitage (1998) after plants were exposed to cold temperatures. Increasing the duration of exposure to cold temperatures increased the length of stems in a quantitative manner (Garner and Armitage 1998). Plants of *P. cookianum* grown under Cold conditions for four weeks and then transferred to Warm conditions, however, displayed floral development that was further enhanced compared with plants grown for eight weeks under Cold conditions. These transferred plants had more floral branches and flowered at an even earlier node than plants grown in the Cold rooms for eight weeks.

The sections of meristems harvested after four weeks treatment revealed development of the inflorescence was well advanced. The Cold response had already made its impact on enhancing floral development by four weeks, and subsequent warm temperatures acted to further enhance the floral response. The Warm temperatures increased flowering in the axils of phytomers that were already formed by four weeks, reducing the number of nodes before flowering. The days to anthesis were not recorded day by day so whether the transferred plants flowered before Cold grown plants was not known. If days to anthesis were reduced, it could be that Warm temperatures played a role in cueing anthesis. Alternatively, the advancement of floral development under subsequent Warm temperatures could have been a result of accelerated growth as seen in vegetative tissues, rather than a specific physiological response. That anthesis occurred sooner in the transferred plants than the Cold grown plants could be expected as accelerated anthesis was seen in the outdoor grown plants that were transferred to the forcing house compared with plants that remained outside. King et al. (1992) demonstrated hastened flowering in *Pimelea ferruginea* by growth and induction at cool temperatures (10 / 15°C) for 7 weeks followed by subsequent growth at warm temperatures (19 / 24°C), while plants induced and left to grow at cold temperatures showed delayed floral development.

The monocot *Cordyline terminalis* flowered upon application of GA_3, and GA_{4+7}, but not GA_{13} (Fisher 1980). Fisher (1980) made GA applications at different times of the year, exposing *C. terminalis* to different ambient daylengths. Despite this change in daylength, flowering always occurred four to six weeks after application, suggesting the response to GA in this species was day neutral. In the current study, GA_3 induced flowering in *P. cookianum*, the proportion of plants flowering increasing with GA_3 concentration. GA_3 treated *P. cookianum* flowered (reached anthesis) at similar times to control plants (observational), suggesting promotion of flowering in *P. cookianum* by GA_3 still required other external or endogenous cueing signals.

The size of the fan (node number) was positively correlated with an increase in flowering in the experimental plants. Upon application of increasing GA_3 concentration, fans with fewer nodes than the smallest flowering in the temperature and daylength experiment flowered, suggesting GA_3 over-rode an inhibition maintained by smaller plants. Precocious flowering induced by GA_3 or GA_{4+7} application was noted by Fisher

(1980) when newly rooted cuttings, and even seedlings flowered after GA treatment. GA_3 may be inhibiting a floral repressing factor in smaller plants of *P. cookianum* or acting downstream of repression and interacting with another inductive pathway.

The effects of temperature on vegetative growth in *M. hortensia* were similar to those seen in *P. cookianum*. Under Cold temperatures plants grew more slowly but leaves eventually reached the same final size as Warm grown plants. No effects of daylength were recognised. No flowering occurred in *M. hortensia* under any of the four potentially inductive conditions. It could be that smaller size played a role in inhibiting flowering and that the plants were too small and repressed the outcome of inductive signals (Rees et al. 1999).

In many plants a certain developmental age must be attained before floral induction can occur (Telfer and Poethig 1998). This change in competency to flower is often associated with changes in vegetative characteristics that define a juvenile vegetative phase, where flowering is inhibited, and an adult vegetative phase, where competency to flower is achieved (Telfer and Poethig 1998). Growth under LDs induces flowering in wild-type *Arabidopsis*, but only once the change to adult phase has been accomplished. Many genes have been identified that repress this change to adult vegetative or reproductive phases and mutations in these genes result in early flowering time (Telfer and Poethig 1998). Just as there are default developmental age factors that inhibit flowering in *Arabidopsis*, the lack of any floral response in *M. hortensia* may be due to similar endogenous factors.

The pronounced stem elongation observed in *M. hortensia* after application of GA_3 was also seen in *Myosotis alpestris* (Zeevaart 1983) where no resultant flowering occurred. The application of a different species of GA may have a different effect on vegetative growth and promote flowering. GA_7 is known to induce flowering in *M. alpestris* and it is seen that there are different roles for different GAs in vegetative and floral growth (King et al. 2000). Often a GA species that promotes stem elongation (vegetative growth) will inhibit flowering, and vice versa (King and Evans 2003).

In conclusion, results obtained here demonstrate that floral development in *Phormium cookianum* was accelerated by Cold temperatures, although not supporting the

hypothesis that Cold temperatures were a requirement for floral induction, and intensified by subsequent Warm temperatures. The lack of flowering in *M. hortensia* under Cold temperatures also disagrees with the hypothesis. Both species displayed no response to either of the daylengths, implying they are day neutral, supporting the hypothesis. GA_3-promoted floral induction in *P. cookianum* supported the hypothesis, that GA_3 would enhance the floral response, but the lack of flowering in *M. hortensia* in response to GA_3 treatment did not. Flowering was positively correlated with fan size in *P. cookianum* and the inhibition of flowering seen in smaller plants could be overcome by application of GA_3. While floral induction was promoted by GA_3, there was still cueing of floral induction by unknown exogenous or endogenous factors. Also, the inductive treatments applied to *Myosotidium hortensia* were not conducive to floral induction, possibly due to the developmental age of the plants.

Chapter 3

Effects of gibberellin A_3 and size/age factors on *FLORICAULA/LEAFY* expression in *Phormium cookianum*

3.0 Introduction

Based on the results described in Chapter 2 it was decided to investigate further the effect that applied GA_3 and size/age factors had on floral induction in *P. cookianum*. As the homologues *FLORICAULA / LEAFY* (*FLO / LFY*) act as floral identity genes in other species, the two factors, GA_3 and size/age, were tested to find if differences in expression of *Phormium cookianum FLO / LFY* (*PFL*) could be observed in the SAM over time. It was hypothesised that *PFL* would be most highly expressed in the largest fans, then the medium, and least in the small fans. A higher level of expression would be seen amongst GA_3 treated samples, since GA_3 promoted flowering in plants with a greater number of nodes.

Phormium cookianum is a New Zealand native species and cloning of genes is not permitted without extensive consultation with relevant iwi and approval by ERMA. In order to isolate expressed *P. cookianum FLORICAULA / LEAFY* without cloning, another technique using a reverse transcriptase-polymerase chain reaction and degenerate primers was used.

It is often advantageous to quantify and compare expression of a gene in different organ or tissue types or to witness the effects of time, or of a treatment over time, in the same tissue or organ type. Upregulation of *FLO / LFY* expression is an event known to be associated with floral induction and development and if followed closely could be used to carefully dissect floral induction and organogenesis in *P. cookianum*. There are many examples where gels or blots of mRNA or cDNA are used to compare the relative expression of a target gene to a housekeeping gene with (theoretically) constant

expression over all treatments, tissues, and times. For example, Bomblies et al. (2003) used an agarose gel and polymerase chain reaction (PCR) products after different cycles of a PCR reaction to compare *FLO / LFY* levels with actin in different tissue types in *Zea mays*. Mellerowicz et al. (1998) presented a northern blot to compare *PRFLL* levels, the *Pinus radiata FLO / LFY* homologue, to 26S ribosomal RNA over different ontogenetic stages. These methods involved staining and comparisons of the relative amounts visualised by eye. More recently an alternative method, Real Time reverse transcriptase-PCR , has been used to measure the exponential increase in levels of PCR products during a reaction to quantify gene expression. It is easier, more accurate, and reproducible. This method involves a PCR reaction and a dye that binds dsDNA enabling monitoring of the products during the reaction by a quantitative PCR machine. Using quantitative PCR and the dsDNA binding SYBR Green, Czechowski et al. (2004) monitored expression levels of over 1400 *Arabidopsis* transcription factors in the roots and shoots of young plants. They reported sensitivity levels of 1 – 100 copies of various transcription factors per cell. Expression levels of salt stress-induced transcripts in *Populus euphratica* were compared to β-actin levels by Gu et al. (2004) also using Real Time quantitative PCR.

In this thesis, *PFL* expression in meristems at different developmental stages was compared with actin levels, by pooling extracted total RNA from shoot apical meristems, generating cDNA and using a Roche Light Cycler and the double strand DNA binding dye SYBR Green 1 (Roche).

3.1 Materials and methods

3.1.1 *PFL* isolation and sequencing

3.1.1.1 RNA extraction

To obtain *PFL* mRNA, shoot apical meristems of large plants expected to flower and express *PFL* were dissected. The plants (made up of many fans) were removed from their pots with any excess soil removed. The root systems were then placed in a bucket of water until each fan was processed. Fans were removed one at a time with a scalpel

and the majority of leaves were quickly removed until the tissue was small enough to work with. Once the meristem plus a few small leaves (<10 mm) were left, the tissue was placed in a Falcon tube cooled in liquid N_2, with several meristems to a tube. Meristems were then stored at -80°C until further processing.

Total RNA was extracted using TRI Reagent and a protocol modified from the supplier's (ProGENZ Limited, Auckland, New Zealand) for a mini-preparation using around 100 mg of tissue instead of 1 g. Six meristems per extraction (<100 mg) were ground into a powder in liquid N_2 using a cooled mortar and pestle that had been baked at 180°C overnight. The powder was transferred with a baked cooled spatula to a 2 ml micro tube containing 1.3 ml of TRI reagent. The solution was vortexed to homogenise and centrifuged for 2 min at 12000rpm at 4°C in an Eppendorf Centrifuge 5417R. The supernatant was transferred to a new tube and stored at room temperature for 5 min. Chloroform (260 µl) was added and the tube was shaken vigorously for 15 s. After standing at room temperature for a further 10 min, the solution was centrifuged for 15 min at 12000 rpm at 4°C and the aqueous phase was transferred to a new tube by pipetting. After adding 320 µl of isopropanol and 320 µl of salt precipitation solution (0.8 M Na citrate and 1.2 M NaCl), the solution was mixed well and stored for 8 min. The solution was centrifuged at 10,000 rpm for 8 min at 4°C and the supernatant decanted. The RNA pellet was washed by vortexing with 1.3 ml of 75% EtOH and centrifuged at 7000 rpm for 5 min at 4°C. The EtOH was removed and the pellet air dried for 3 - 5 min. The RNA was dissolved in 30 µl RNase-free water (diethyl pyrocarbonate, DEPC, treated) and 1.2 µl of 25 x RNAsecure (Ambion) was added. The RNA was heated at 65°C for 2 min to denature any RNases and stored at -20°C.

Extractions typically yielded 2 - 4 µg RNA $.\mu l^{-1}$ with an absorbance 360/380 purity ratio of 1.79 - 1.9, determined by spectrophotometry.

3.1.1.2 Reverse transcriptase reaction

Total RNA was used in a reverse transcriptase reaction using Expand Reverse Transcriptase (Roche) and a Roche protocol using an oligo $(dT)_{15}$ to prime the reaction. Total RNA (1µg) was added to 1 µl of oligo $(dT)_{15}$ and DEPC treated water to make a total volume of 10.2 µl. The initial reaction mix was heated at 65°C for 10 min to

denature the RNA and primers then cooled on ice. 4 µl of 5 x expand reverse transcriptase buffer, 2 µl of 100 mM DTT, 2 µl of 10 mM dNTPs, 0.8 µl of 25 x RNA Secure, and 1 µl of expand Reverse transcriptase was added. The final reaction mix (20 µl) was incubated at 43°C for 45-60 min then placed on ice to stop the reaction.

3.1.1.3 Primer design

FLORICAULA/LEAFY sequences for many species (monocots and dicots) were retrieved from the NCBI GenBank and aligned using ClustalX 1.8. Primers for cDNA (JHLFYF1, and JHLFYR, Appendix D: Fig D.1) were designed for conserved regions, paying particular attention to the monocot sequences of orchid (*Orchis italica*, accession AB088851), rice (*Oryza sativa*, accessions AB005620 and AF065992) and *Lolium temulentum* (accession AF321273), which were available at the time.

Based on the experience of co-workers with a number of other species, specific and degenerate primers designed by others were also used (Appendix D: Fig. D.1). Jien Chen Song designed primers: LFYF1B, LFYR1, LFYF2, and LFYR2 and Lekha Sreekantan designed primers: LFYLF and LFYLR. The nucleotide sequences the primers coded for were compared with the *L. temulentum* sequence to determine the size of fragments expected to be amplified (Table 3.1). Using this information the positions of the primers along the mRNA sequence were visualised (Fig. 3.1).

3.1.1.4 PCR reactions

cDNA (2µl) from the reverse transcriptase reaction was used in a first round PCR with a non-specific low annealing temperature of 48°C (due to use of degenerate primers), and the following reaction mix and PCR program with the combinations of primers in Table 3.1:

> 10.3 µl of DEPC water, 2.5 µl of 2 mM dNTPs, 2 µl of 10 X Taq buffer, 1 µl of 25 mM $MgCl_2$, 1 µl each of 10 pmol. μl^{-1} forward and reverse primers, 2 µl of cDNA, and 0.2 µl of 5 U. μl^{-1} Taq polymerase was mixed in a 0.2 ml PCR tube (20 µl reaction mix).

The reaction was incubated at 94°C for 5 min, 40°C for 5 min, and 72°C for 5 min for synthesis of the first strand of cDNA. Then at 94°C for 1 min, 48°C for 1 min, and 72°C

Table 3.1: Expected sizes of amplified *PFL* fragments (bp) from using different primer pair combinations.

	LFYR1	LFYR2	LFYLR	JHLFYR
LFYF1B	820	630	780	600
LFYF2	410	220	375	190
LFYLF	230	40	200	15
JHLFYF1	930	738	890	710

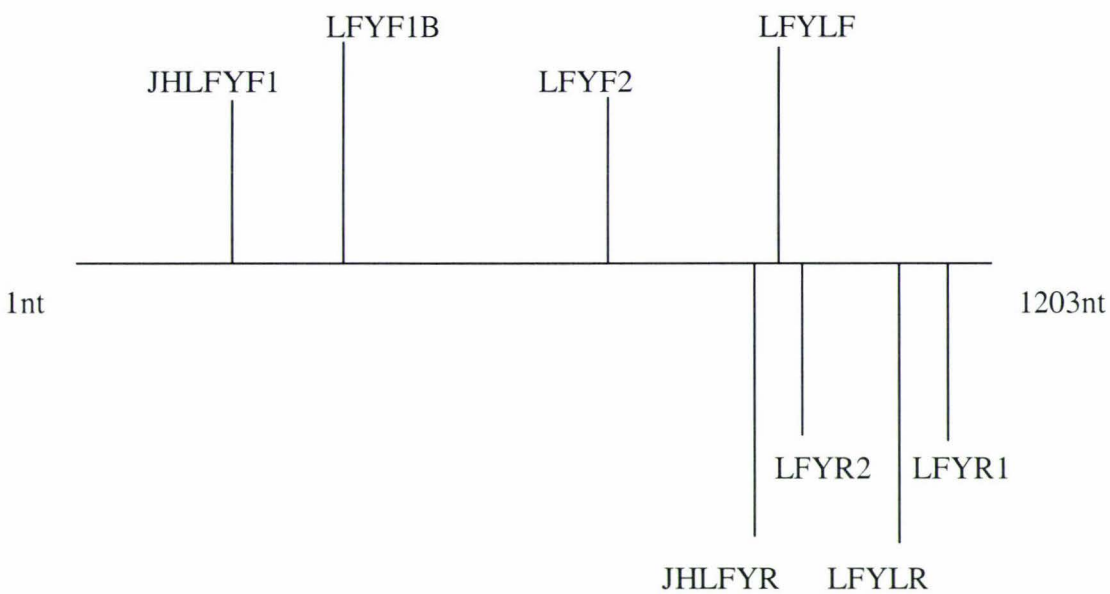


Figure 3.1: Position of primers on the *Lolium temulentum* mRNA *LFY* sequence.

for 2 min, cycled 30 times for amplification of the PCR product. A final 5 min elongation step of 72°C was used.

The PCR product from the first round was then used in a second PCR with the same primers as above, a higher more specific annealing temp of 55°C, and the following reaction mix and program:

> 10.3 µl of DEPC water, 2.5 µl of 2 mM dNTPs, 2 µl of 10 X Taq buffer, 1 µl of 25 mM $MgCl_2$, 1 µl each of 10 pmol. μl^{-1} forward and reverse primers, 2 µl of first round PCR product, and 0.2 µl of 5 U. μl^{-1} Taq polymerase was added to a 0.2 ml PCR tube (20 µl reaction mix).

The reaction was incubated at 94°C for 5 min to denature primers and template then at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, cycled 30 times to amplify the DNA. A final elongation step of 72°C was held for 5 min.

3.1.1.5 Visualisation of PCR products and gel extraction

The PCR products were run on a large 2% agarose gel at 40V to resolve the bands. Bands of the expected size compared with the *Lolium* sequence (Table. 3.1 & Fig. 3.1) were excised under UV, and the cDNA extracted from the gel segment using a Quiagen Gel Extraction kit. Modifications were made to the protocol of the manufacturer to remove excess ethanol contained in the reagents. The DNA fragment was excised from the agarose with a scalpel blade and weighed. Gel slices were incubated at 50°C for 10 min with 3 µl of Buffer QG for each 1 µg of gel. To help dissolve gel slices they were vortexed every 2 - 3 min. Once dissolved, the pH was checked by making sure the solution was yellow. Isopropanol (1 gel volume) was added to the sample and the solution was mixed. The sample was applied to the QIAquick column and centrifuged for 1 min at 13000 rpm. The flow through was discarded and 0.5 ml of Buffer QG was added to the QIAquick column. The column was then centrifuged for 1 min at 13000 rpm. The flow through was discarded and 0.5 ml of Buffer PE was added to the QIAquick column and then centrifuged for 1 min at 13000 rpm. Again flow through was discarded and 0.25 ml of Buffer PE was added to the QIAquick column. The column was then centrifuged for 1 min at 13000 rpm. The same QIAquick column was then placed in a clean 1.7 ml centrifuge tube and centrifuged for another min at 13000 rpm. The column was then placed in another clean 1.7 ml centrifuge tube with the lid open and left to air for 5 - 10 min. cDNA was eluted by pipetting 30 µl of pH 7.0 - 8.5

milliQ water directly onto the centre of the membrane and allowed to stand for one min and centrifuged for 1 min at 13000 rpm.

3.1.1.6 Sequencing of products

An amount of isolated cDNA (Section 3.1.1.5) was quantified on an agarose gel using a low DNA mass ladder (Roche). DNA and primers were diluted as required for sequencing, and sequenced using the Big Dye Terminator Reaction V 3.0 and capillary separation in the Allan Wilson Centre sequencing facility.

3.1.1.7 Actin as a positive control

To act as a positive PCR control, degenerate actin primers previously designed for *Metrosideros excelsa* by Lekha Sreekantan (Appendix D: D.2) were used to isolate the *P. cookianum* actin sequence. Reverse transcriptase-PCR, first and second round PCR, gel extraction and sequencing were done using the same procedures as for *PFL*.

3.1.2 *PFL* expression during floral induction and organogenesis following GA_3 application

3.1.2.1 Experimental treatments

P. cookianum were grown under the conditions of the tunnel house stated in Section 2.1.1. Plants of *Phormium cookianum* (135) were allocated a random number generated in MS Excel and assigned a treatment to be applied. Treatments included application of EtOH or GA_3 dissolved in EtOH and five different sample times. All except Time 0 plants were treated two times with either EtOH or 2000 µg GA_3 / EtOH per fan, on 25 and 31 March 2003, by the method reported in Section 2.1.3. To test the effect of fan size on flowering, fans of three size categories were identified within both the GA_3-treated and control plants by counting all the leaves of the fans in each pot. Small fans were defined as having 1 - 7 nodes, medium fans had 8 - 10 nodes, and large fans had 11+ nodes. Fans were categorised on the basis of size and separated at the time of sampling. Meristems were taken for RNA extraction or sectioning at the following

times: Time 0 samples were taken on 1 April. Harvests for GA_3 / EtOH and EtOH (control) treated meristems were taken seven days after treatment (Time 1, 2 / 3 April), and after two and four months (Time 2, 28 / 29 May and Time 3, 1 / 2 July, respectively). Time 4 plants were not harvested in order to monitor the proportion of treated fans that flowered under the two treatments. Fifteen plants, each with different numbers and sizes of fans, were allocated to each time and hormone treatment.

3.1.2.2 RNA extraction

TRI Reagent was used to extract RNA from the meristems using the protocol described in section 3.1.1.1.

3.1.2.3 *PFL* and actin specific primers

Using Primer Premier 5.0 and the resultant *PFL* and actin sequences specific primers were designed for use in the Real Time reverse transcriptase-PCR application to amplify *PFL* and actin (Appendix D: Fig.3.2).

3.1.2.4 Deoxynuclease treatment of RNA extractions and reverse transcriptase reaction

As genomic *PFL* would also be amplified during Real Time reverse transcriptase-PCR if it were present in the RNA extraction, a Real-Time PCR was performed using only RNA and the primers shown in Section 3.1.2.3. No bands were detected by gel analysis (not shown), but peaks were detected in the melting curve (Fig. 3.2). Further RNA extractions were deoxynuclease (DNase) treated using the following protocol as recommended by Sigma: 1 µg of total RNA was diluted in 8 µl of DEPC treated water and 1 µl each of 10 x reaction buffer and DNase 1 was added. The reaction was left at room temperature for 15 min then 1µl of stop solution (EDTA) was added. The reaction was incubated at 70°C for 10 min and then chilled on ice.

cDNA was made using the total DNase reaction mix and a protocol altered from that recommended by Roche to compensate for the DNase reaction: 1 µl of 50 pmol. μl^{-1} $oligo(dT)_{15}$ was added to the DNase treated RNA (1 µg RNA in the 11 µl from the

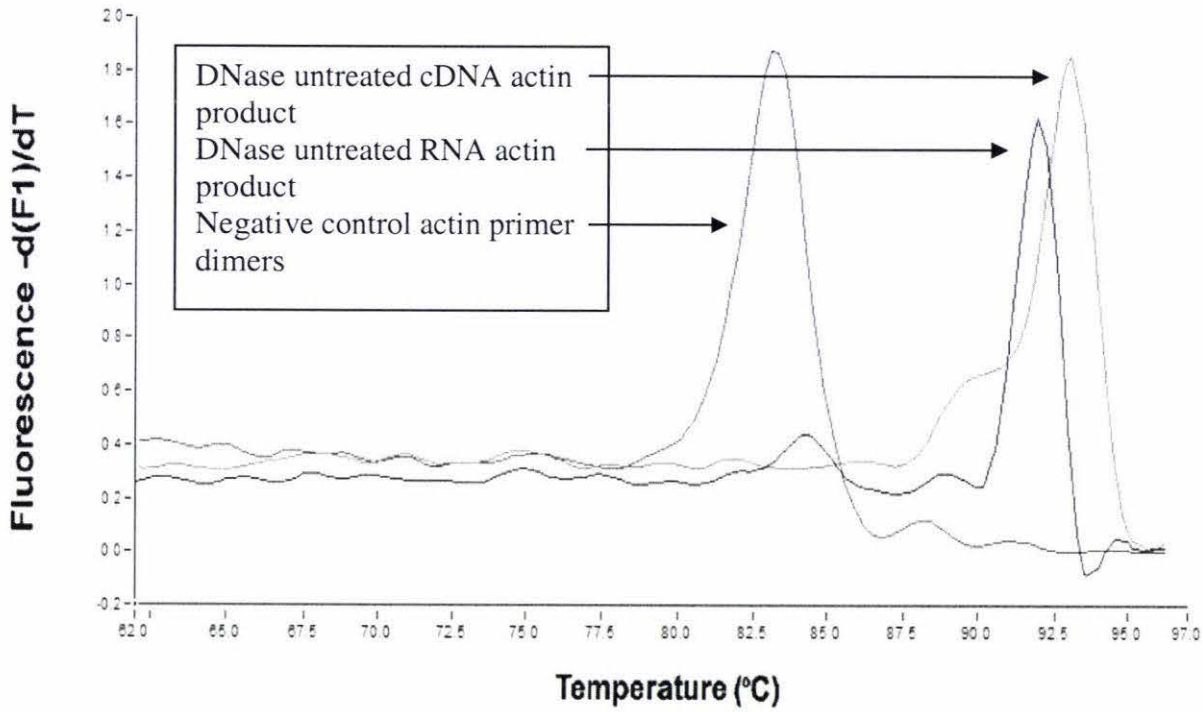


Figure 3.2: **Melting curve of actin and *PFL* products generated from genomic DNA contamination of RNA extractions. The peaks represent distinct drops in fluorescence as double stranded DNA products separate (or melt) at particular temperatures (Tm). The peak at 83°C represents the Tm of actin primer dimers, 92°C the Tm of product generated from RNA using actin specific primers, and 93°C the Tm of product generated using cDNA and actin specific primers.**

DNase reaction). The mix was heated at 65°C for 10 min to denature RNA and primer and then cooled on ice. 4 µl of 5 x RT buffer, 2 µl of 100 mM DTT, 2 µl of 10 mM dNTPs, 0.8 µl of RNA Secure, and 1 µl of Reverse Transcriptase (Roche) was added and the 22 µl reaction mix was incubated for 45 - 60 min at 43°C then put on ice to stop the reaction.

3.1.2.5 Real Time PCR reaction

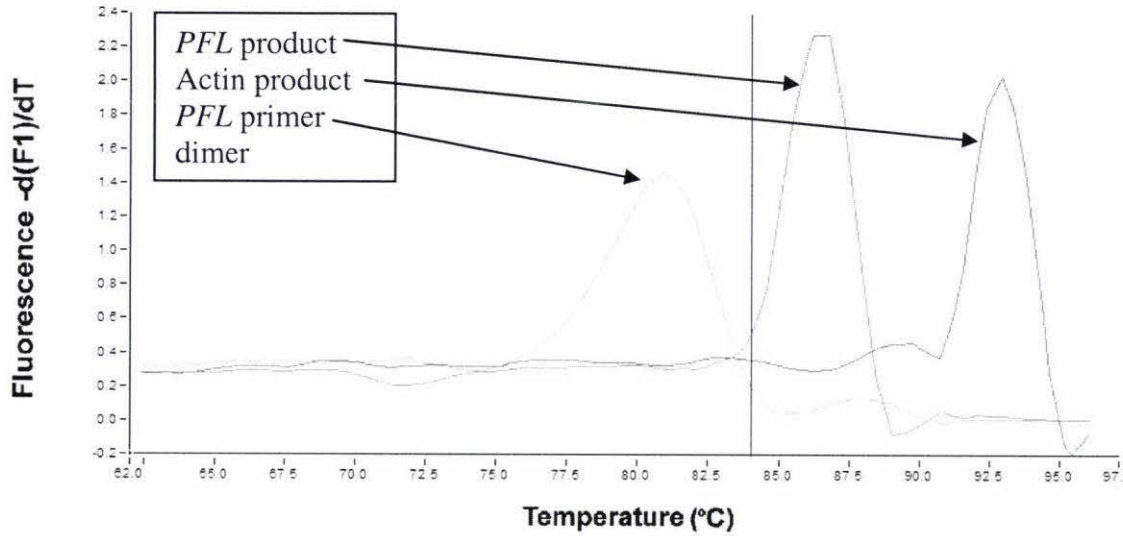
Real Time PCR reactions were carried out using a LightCycler instrument (Roche) and software Version 3.5. Analysis was achieved using the RelQuant program (Roche). Reactions were initially done in triplicate until it was found adequate mixing of cDNA removed variation. The rest of the reactions were performed as single reactions with a calibrator being used between the two runs necessary to complete all 42 samples. Samples consisted of cDNA generated from RNA of fans of 3 sizes (large, medium, and small), treated with EtOH or EtOH / GA_3, at 3 different times (Time 1, Time 2, and Time 3), plus RNA of 3 sizes of untreated fans (Time 0). Each run included triplicate reactions with calibrator cDNA and specific actin and *PFL* primers. Two rounds were necessary to complete all reactions. The experiment was duplicated, firstly Time 0 and Time 1 reactions and then Time 2 and Time 3 reactions were run together followed by runs with Time 0 and Time3 reactions and then Time 1 and Time 2 together. Similar results were found between duplicates.

The 20 µl PCR mixes contained:

> 10.8 µl of PCR grade water, 2.0 µl of 10 x SYBR buffer, 3.2 µl of 25 mM $MgCl_2$, 1 µl each of 10 pmol.µl^{-1} forward and reverse primers, and 2 µl of reverse transcriptase cDNA product diluted 1:10.

The PCR program had an initial step to activate Faststart Taq DNA Polymerase by heating at 95°C for 10 min. This was followed by an amplification cycle repeated 45 times: 95°C for 10 s, 56°C for 5 s, 72°C for 12 s. Temperature changes were made at a rate of 20°C. s^{-1}. The melting temperature of the primer dimers was below 84°C so data was acquired at 84°C to negate effects of primer dimer accumulation on fluorescence levels (Fig. 3.3).

(A)



(B)

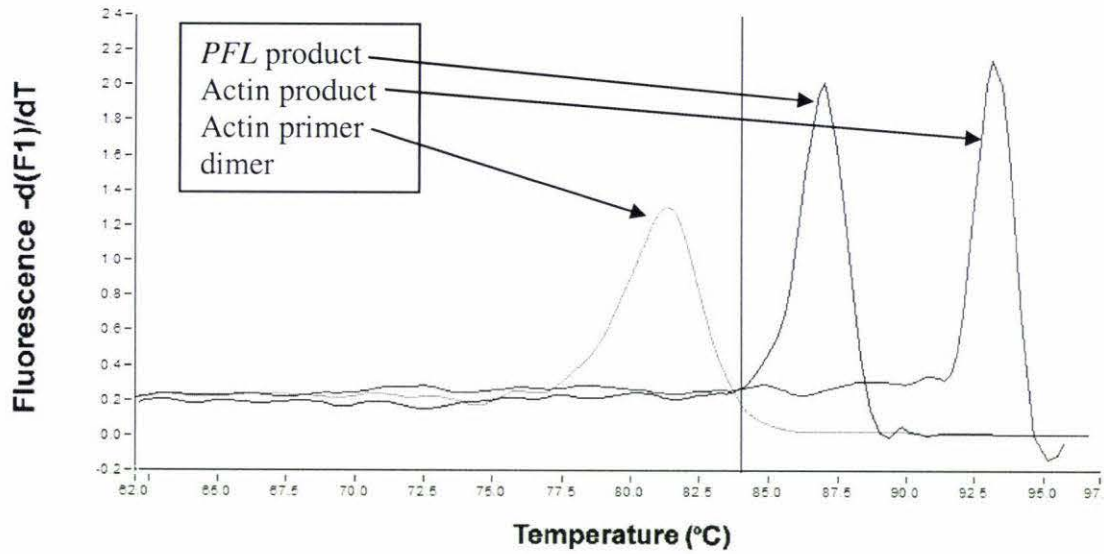


Figure 3.3: **Melting curve of *PFL* and actin primer dimers and PCR products generated from genomic DNA contamination in RNA samples. A) Melting peak of *PFL* primer dimers and *PFL* and actin product. B) Melting peak of actin primer dimers and *PFL* and actin product. Data was acquired at 84°C (vertical line) to avoid primer dimer fluorescence, as dimers melt below this temperature.**

The melting curve program used to identify the final products generated during the PCR reaction. was as follows: cDNA was initially heated to 95°C at 20°C. s^{-1} for 0 s, followed by a decrease to 60°C at 20°C. s^{-1} for 30 seconds and then a gradual increase in temperature of 0.1°C. s^{-1} to 95°C.

Products of the Real Time reverse transcriptase-PCRs gave bands of the expected size when visualised by agarose gel electrophoresis (Fig. 3.4). This confirmed the sequences being amplified were the desired products. Once sizes of the products were confirmed, the melting curve generated during a run on the LightCycler could be used to confirm the same products were being monitored negating the necessity of a gel (Fig. 3.3).

The Roche Light Cycler Data analysis program was used to export the crossing points (cycle number where fluorescence levels rise above background) of the PCR reactions (Fig. 3.5) to the RelQuant program (Roche) to determine the relative levels of *PFL*.

A PCR efficiency curve was generated from a relative standard curve with the RelQuant program to make a co-efficient file for use when analysing Real Time reverse transcriptase-PCR reactions. To generate the co-efficient file, serial dilutions of a cDNA sample were made of 1 x 10^{-1}, 1 x 10^{-10}, 1 x 10^{-100}, 1 x 10^{-1000}, and 1 x $10^{-10,000}$. The diluted cDNA was then used in a Real Time PCR with the same reaction mix and program as above, for both *PFL* and actin. The target gene, *PFL*, demonstrated a typical effect on PCR efficiency as the concentration of initial template decreased (Fig. 3.6 A). The fitted line had two sections, the first linear part of the fitted curve described the upper concentration where strong signals were displayed; the curved section described the lower concentrations where signals are displayed that exaggerate lower cDNA concentrations if not corrected for by the co-efficient file. The reference gene, actin, on the other hand, has a linear PCR efficiency over the five different concentrations (Fig. 3.6 B).

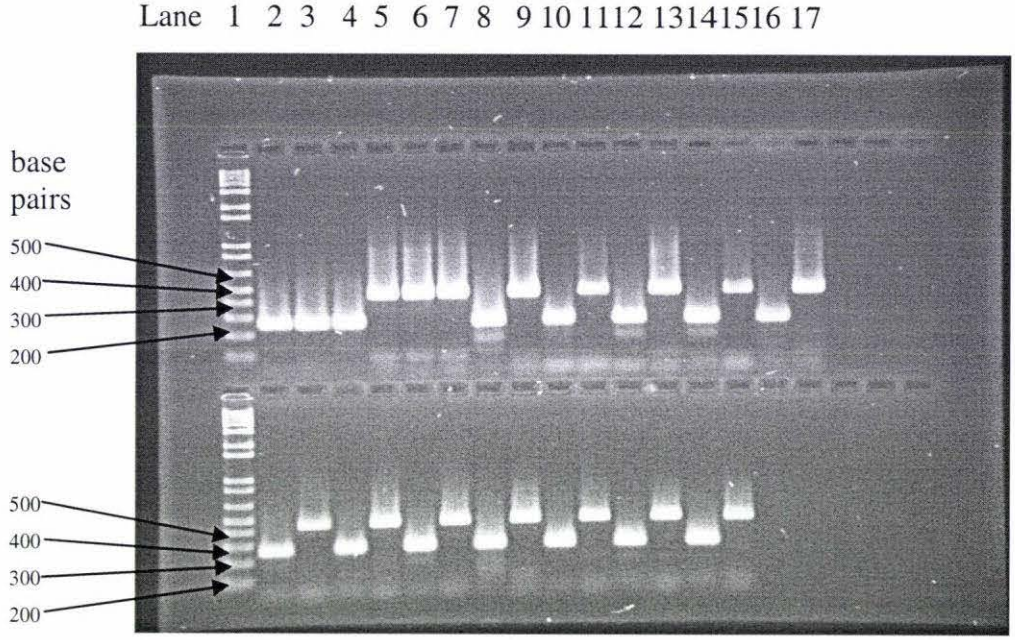


Figure 3.4: Gel analysis of actin (435 base pairs) and *PFL* (262 base pairs) products. Top lanes are, lane 1: 1 Kb^+ ladder, lanes 2 – 4: calibrator *PFL* product, lanes 5 – 7: calibrator actin product, lanes 8 – 17: examples of paired *PFL* and actin products of cDNA samples from different RNA extractions. Bottom lanes are, lane 1: 1 Kb^+ ladder, lanes 2 – 14 examples of pairs of alternating *PFL* and actin cDNA products.

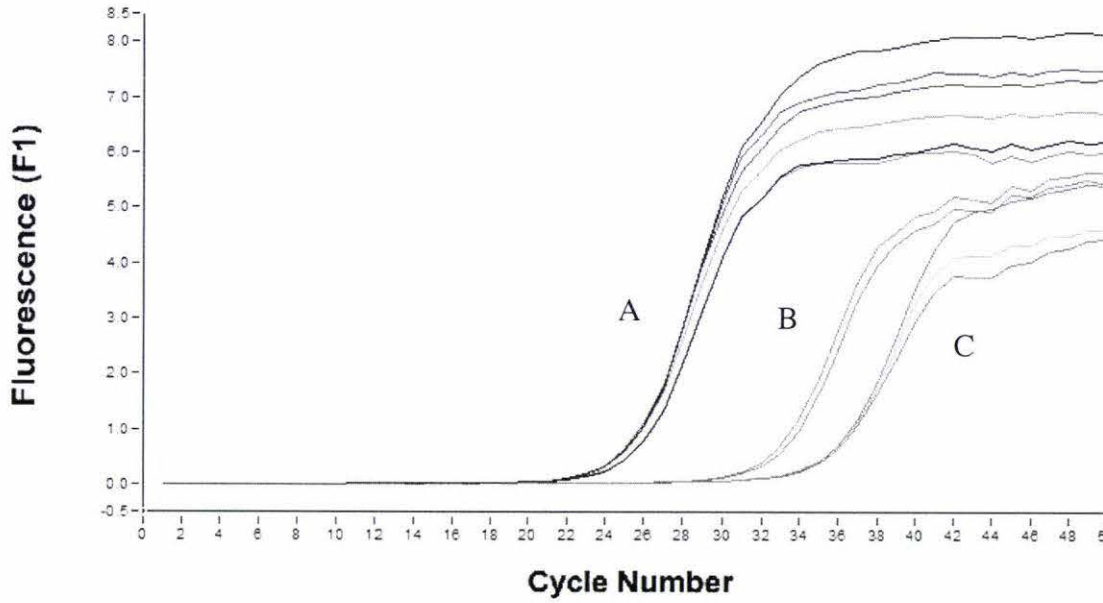
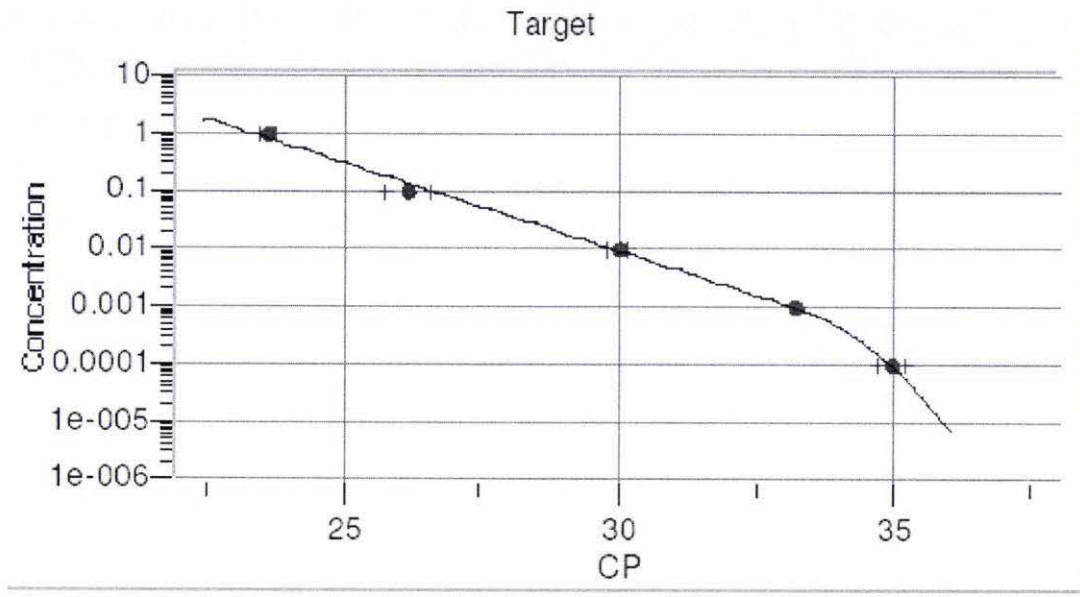


Figure 3.5: **The increase in fluorescence as cDNA products are amplified exponentially used to determine the cycle number where fluorescence rises above background levels. Overlapping fluorescence levels are seen for *PFL* and actin products generated in triplicate from calibrator cDNA (A), cDNA generated from Time 3, large, GA_3-treated RNA samples amplified with actin (B) or *PFL* (C) specific primers was also performed in triplicate.**

(A)



(B)

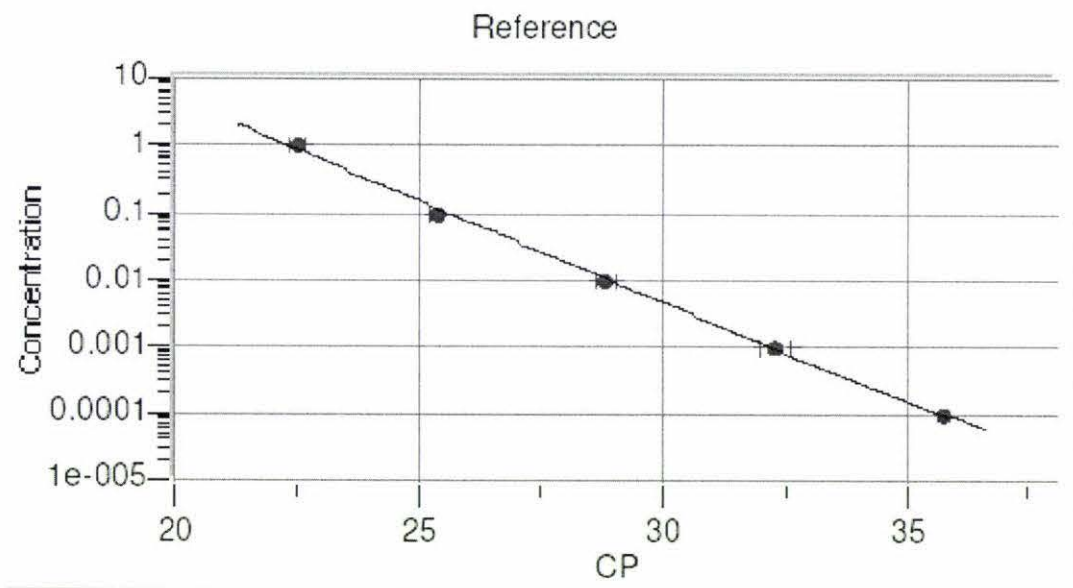


Figure 3.6: Efficiency of the Real Time PCR reactions for *PFL* (A) and actin (B) over a dilution series (CP = crossing point, cycle number)

3.2 Results

3.2.1 *PFL* and actin isolation and sequencing

DNA isolated using the monocot specific primers JHLFYF1 and JHLFYR gave a band of approximately 310 bp, instead of the predicted 710 bp, and a sequence closely related to rRNA of several plant species determined by BLAST search (Appendix C: Fig. C.1). *Oryza sativa*, and *Triticum aestivum* mitochondrial DNA shared 94% similarity with regions of the isolated *P. cookianum* sequence. *Zea mays*, *Beta vulgaris*, and *Oenothera berteriana* rRNA shared 93%, 95%, and 93% sequence similarity over shorter regions of the isolated cDNA.

A band of 400 bp was isolated and sequenced using the LFYF2 / LFYR1 primer pair. The BLAST revealed the cDNA had strong sequence identity with many other *FLO / LFY* homologues at the nucleotide level (Appendix C: Fig. C.2). *Oryza sativa OSL* and *RFL* genes had similarities of 86% over a 103 nucleotide region, and *Lolium temulentum LtLFY* was 86% similar over 85 nucleotides. *Hyacinthus orientalis* floral meristem identity protein (*HLY*, Accession number: AY520841.1) mRNA shared 80% similarity over 351 nucleotides, the longest sequence reported in a BLAST search. Similarities were also found for the coded amino acids (Appendix C: Fig. C.3). *OSL* shared 77% identity with *PFL* over 95 amino acids.

The DNA amplified using the degenerate actin primers gave an expected band of approximately 450 bp. A BLAST search confirmed the *P. cookianum* actin sequence was being amplified (Appendix C Fig. C.4).

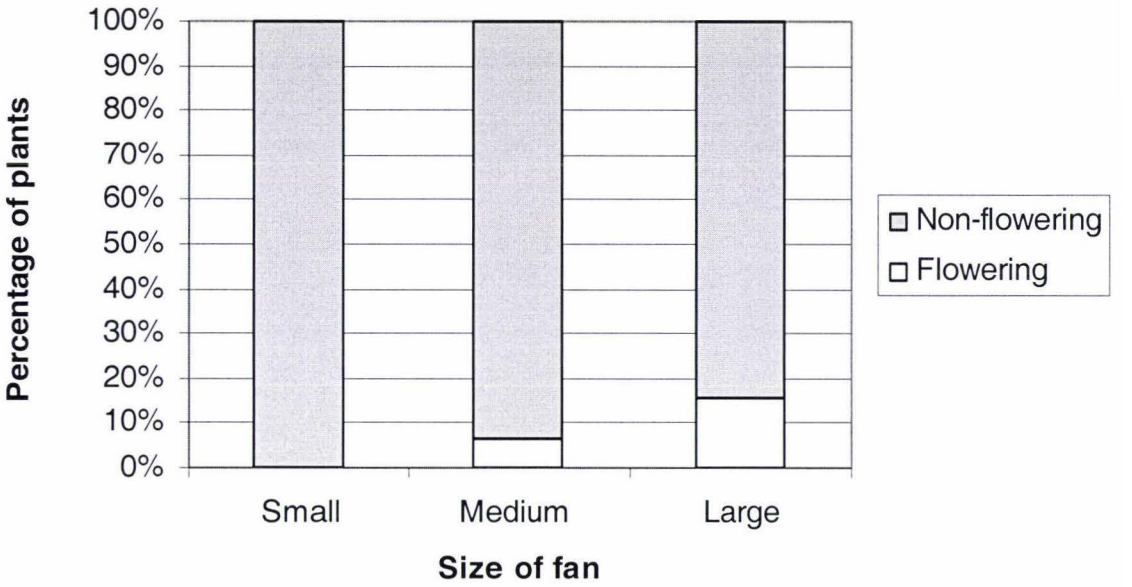
3.2.2 Effects of GA_3 on floral induction

To ensure GA_3 was promoting floral induction as it had the previous year, Time 4 plants were not harvested to allow them to flower. As the size (node number) of the fans increased so did the proportion of fans flowering (Fig. 3.7). The application of GA dramatically increased the percentage of fans that flowered compared with EtOH-treated control plants across all size categories. None of the small EtOH-treated fans (fewer than 8 nodes) flowered compared with 47.1% of the small GA_3 fans. While some EtOH treated medium fans (8 – 10 nodes) flowered (6.6%), 71.49% of the GA_3-treated medium fans flowered. The large fans (11 + nodes) had the highest flowering percentages with the GA_3 treatment increasing flowering to 94.1% from the 15.4% of control plants.

3.2.3 Expression levels of *PFL* during inflorescence development

PFL mRNA levels at the meristem had increased, relative to actin (Fig. 3.8), as developmental changes from vegetative to floral were taking place. The *PFL* levels under all treatments were uniformly low at Time 0 and Time 1. On 1 April (Time 1) *PFL* mRNA levels were the lowest seen and coincided with vegetative meristems of untreated plants giving rise to leaf primordia (Fig. 3.9). By 1 June (Time 2) *PFL* mRNA was at a medium level and untreated meristems with different developmental states (Fig. 3.10), i.e. floral and vegetative, were emerging. The highest *PFL* levels of control (EtOH) plants were measured in harvests taken on 1 July (Fig. 3.8 A) when advanced inflorescence development was seen (Fig. 3.11). By Time 3, *PFL* levels of GA-treated plants had started to decrease in the meristems of large and small fans, but not in meristems of medium fans which increased to a level comparable with those of meristems of large fans at Time 2 (Fig. 3.8 B).

(A)



(B)

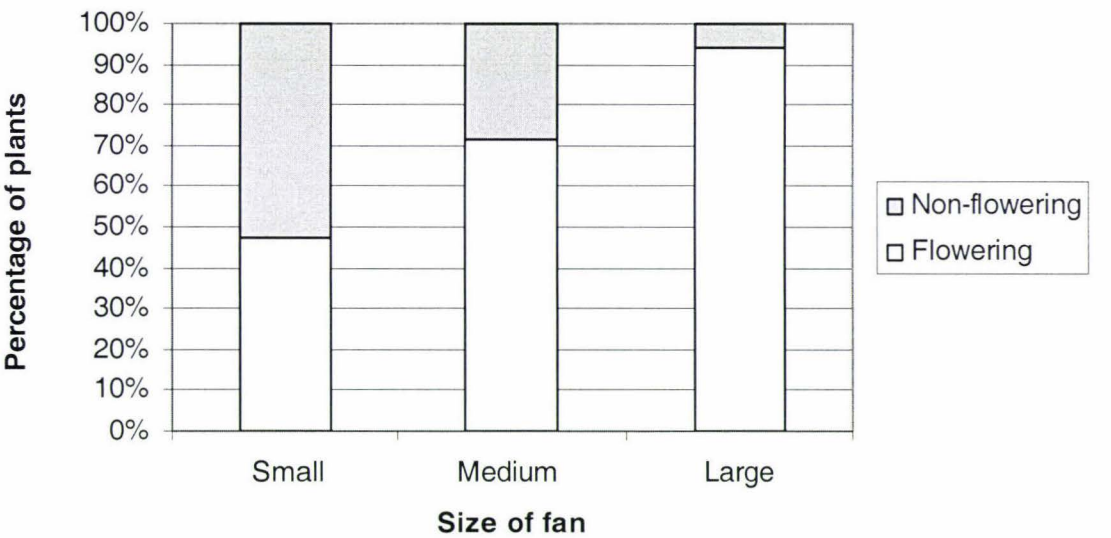
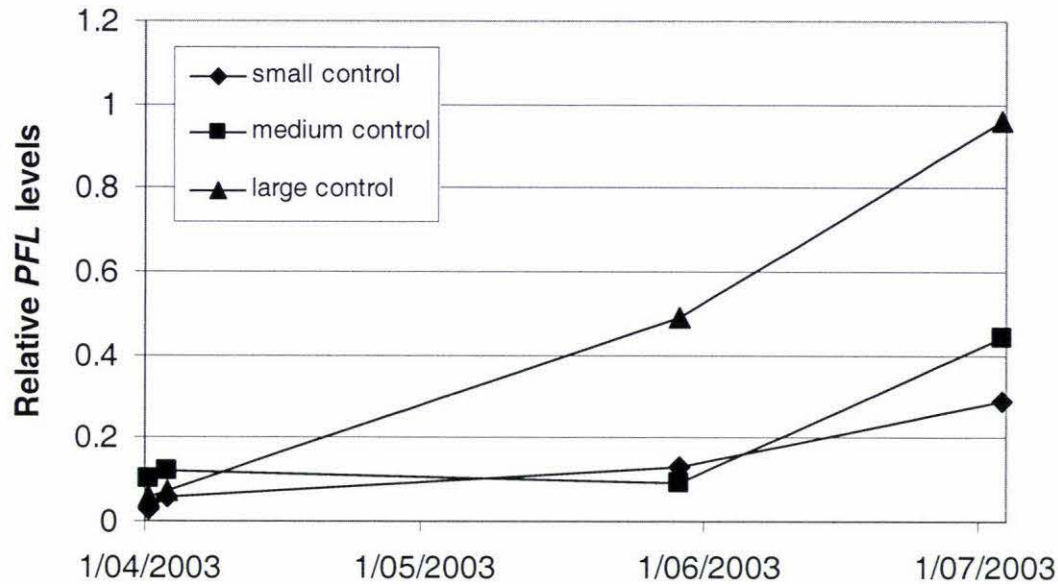


Figure 3.7: Proportion of plants flowering for fans of different sizes treated with EtOH (A) or GA_3 / EtOH (B). Refer to materials and methods for size definitions.

(A)



(B)

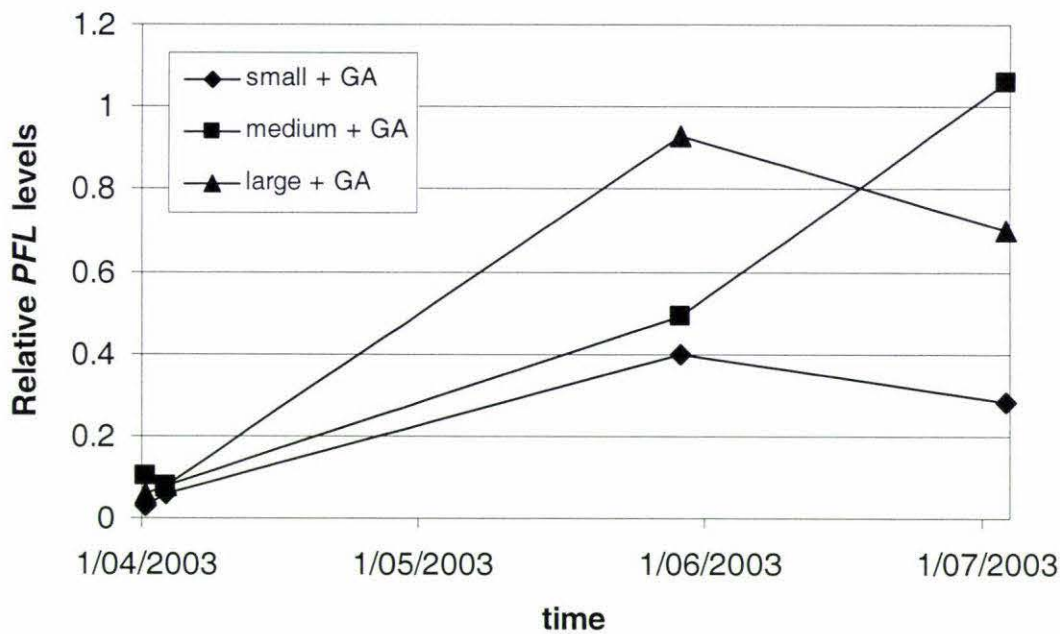


Figure 3.8: **Relative expression of *PFL* in different sized fans treated with EtOH control (A) or GA_3/EtOH (B). See section 3.1.2 *PFL* expression during floral induction and organogenesis upon GA_3 application for treatment details.**

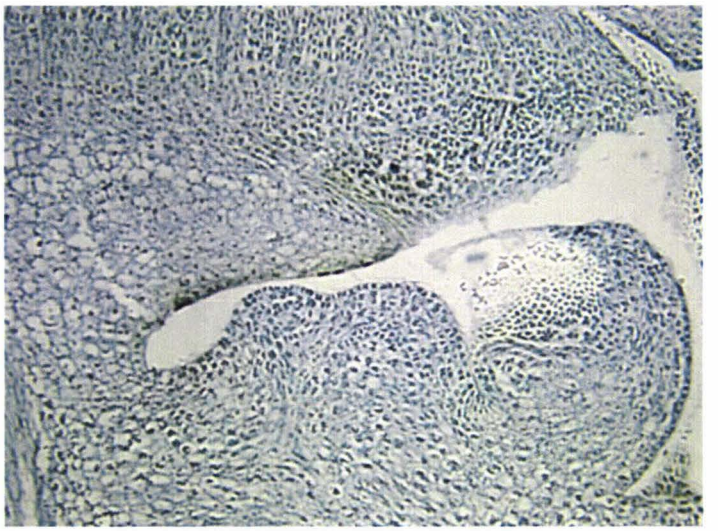


Figure 3.9: **A representative vegetative meristem of large untreated-fans at Time 0 and Time 1.**

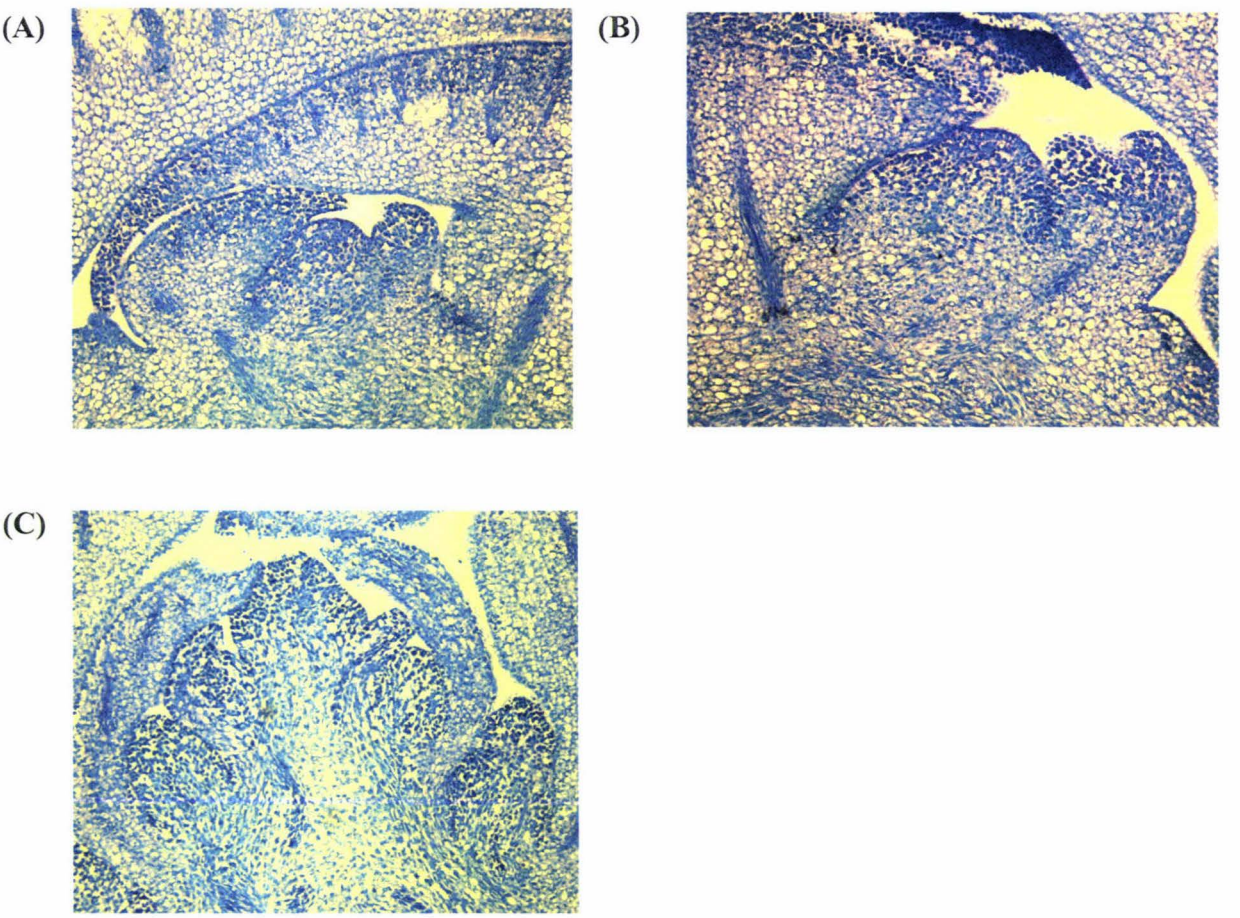


Figure 3.10: **Sections of meristematic samples taken at Time 2 from large untreated-fans displaying varying degrees of floral development.**

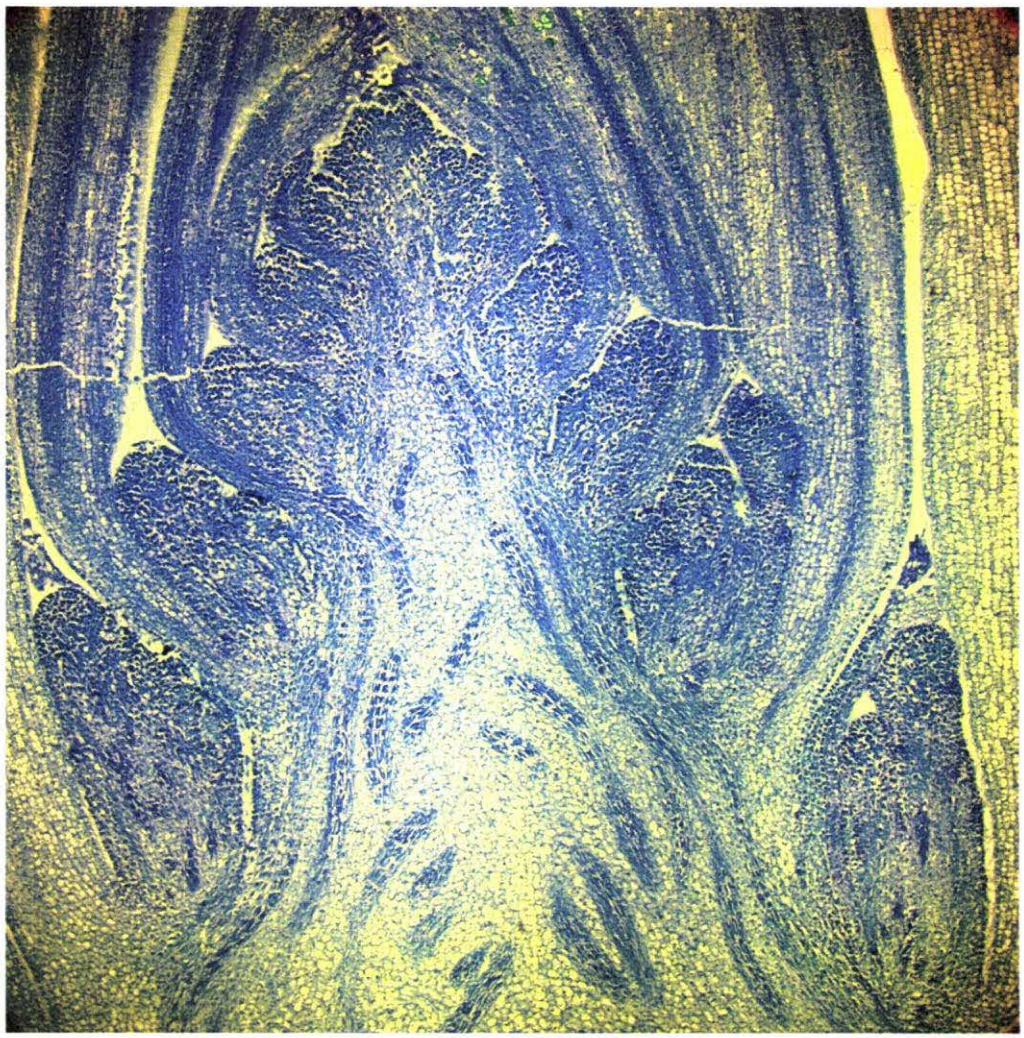


Figure 3.11: Section of meristematic sample of a large untreated-fan, taken at Time 3, displaying advanced inflorescence development.

3.3 Discussion

Evidence provided here suggested *PFL* was the *P. cookianum* homologue of *FLORICAULA / LEAFY*. A high degree of similarity was seen between the cDNA molecule generated from *PFL* mRNA and regions of known *FLO / LFY* homologues. Eighty six percent identity was seen within regions that were 103 and 85 nucleotides long of *Oryza sativa,* and *Lolium temulentum FLO / LFY* homologues respectively (Kyozuka et al. 1998, Gocal et al. 2001). Also, upregulation of *PFL* expression at the meristem coincided with the timing of floral developmental events, as seen in other studies of spatial and temporal expression patterns of the floral meristem identity gene in monocots and dicots. (Weigel et al. 1992, Kyozuka et al. 1998, Molineros-Rosales et al. 1999, Gocal et al. 2001)

PFL mRNA expression increased over the time floral organogenesis occurred. In control plants, the extent to which *PFL* levels increased over time was associated with the size of the fan. The large control fans expressed *PFL* more highly than medium fans which in turn expressed *PFL* more highly than small fans. In the GA_3-treated fans, however, the size related increase was only seen until Time 2. By Time 3 there was a drop in *PFL* mRNA levels in the large and small fans while expression in the medium fans continued to climb. In *O. sativa*, *RFL* levels in the panicle decline after panicle branches form (Kyozuka 1998), so large and small, Time 3, GA_3-treated fans were possibly at a developmental stage where *PFL* expression was decreasing.

Not all of the Time 4, GA_3-treated fans flowered. As only 3-4 meristems were used for each RNA extraction it was possible that less induced meristems were selected for particular samples where *PFL* levels dropped unexpectedly (Time 3 large and small fans). When samples were taken, more tissue not expressing *PFL* could have been left on a floral sample, diluting relative *PFL* levels. The calibrator cDNA used to compare all *PFL* levels was of Time 3, large, GA_3-treated fans and represented a relative *PFL* amount of 1.0. As the compared Time 3, large, GA_3-treated RNA sample has relatively less *PFL* than the calibrator, it was likely that *PFL* was diluted in that sample.

Only 15% of the large Time 4 control plants flowered despite having a level of *PFL* expression comparable with GA_3-treated large fans. It may have been, that more flowering meristems were chosen than was expected due to chance. To reduce this possible variation a larger preparation of RNA could be prepared using more meristems.

Temporal expression of *PFL* in *P. cookianum* correlated well with floral development and was consistent with reports in the literature that increased expression of *FLO/LFY* is an event intricately associated with flowering in both monocots and dicots (Weigel et al. 1992, Kyozuka et al. 1998, Molineros-Rosales et al. 1999, Gocal et al. 2001). Elucidation of spatial expression, however, was not achieved in this study. mRNA *in situ* hybridisation was attempted to gain insight into the spatial expression pattern of *PFL,* but no signal was observed. As *P. cookianum* is a New Zealand native plant, standard probe synthesis techniques could not be used due to the restrictions placed on cloning. Instead attempts were made to generate a probe using PCR products as a template for T7 RNA Polymerase. To further investigate spatial expression by *in situ* methods, permission to clone would be beneficial and make success more likely.

In light of these results the role of *PFL* in comparison to the dicots, and the grasses was speculated upon. Applied GA_3 increased the number of *P. cookianum* plants that flowered and increased the likelihood that a signal cued flowering. It is therefore likely that floral development would be closely synchronised within GA_3-treated plants, resembling the most advanced floral meristems of untreated plants. At Times 0 and 1, there was minimal *PFL* expression and meristems were observed to be vegetative, suggesting *PFL* was expressed to a low level in vegetative tissue. Expression of *FLO / LFY* has been observed in leaf primordia of *Arabidopsis* and in *Z. mays* expression was witnessed in young shoot apices (Weigel et al. 1992, Bomblies et al. 2003). Two months after treatment (Time 2), *PFL* expression increased dramatically, and in samples taken in parallel, clear inflorescence structures were apparent with putative axillary branch primordia developing either side of the inflorescence in an opposite phylotaxy.

There was very little evidence of *FLO / LFY* expression in inflorescence meristems or secondary inflorescence meristems in the dicots, where expression is upregulated in meristems upon floral commitment and in floral primordia and organs. In the grasses, however, the expression patterns appear to have diverged from the conserved patterns

seen amongst the dicots. It was observed that *ZFL* (*Zea mays FLO/LFY*) expression and function remained similar to that of the dicots as *ZFL* was expressed vegetatively and was upregulated in zones on the inflorescence (male and female) that were to become spikelet pair meristems. Expression was maintained right through to floret development and was seen in floret organs. Spatial expression of *FLO / LFY* in *Lolium temulentum* and *Oryza sativa* differed from that of *Z. mays* and the dicots. *LtLFY* expression was first witnessed after exposure to 12 inductive LDs and was not closely linked with the transition to floral meristem as seen in the dicots. In *O. sativa*, *RFL* was expressed in the main panicle but was absent from regions that would differentiate into primary branches. Once primary branches had developed, expression in the main panicle ceased but persisted throughout the branches except for the apex which would terminate in a floret. Down regulation of *RFL* was seen in secondary branches and was entirely absent from florets, an alternate expression pattern to that of the dicots where expression was seen throughout floral tissues and was absent from the tissues of the inflorescence stem.

If *PFL* expression was to follow the patterns of *FLO / LFY* seen in the dicots and *Z. mays*, then expression was likely to occur in meristems destined to become flowers and then in floral organs. If were the case, *PFL* levels at Time 2 suggested the presence of floral meristems. By Time 3 discrete bodies of cells are emerging in the bract axils resembling large floral meristems on elongating branches. The relative levels of *PFL* appear to have dropped in the "small" and "large" shoot apices of GA_3-treated plants by Time 3, which is unlikely if *PFL* expression followed the pattern of *FLO/LFY* homologues in dicots and *Z. mays*. If *PFL* levels have dropped at this stage it was possible that *PFL* played a role in branching rather than floral meristem identity and levels are actually declining as in *RFL* (Kyozuka, et al. 1998). The grasses are a highly diverged group so it is more likely that *PFL* played a role in flowering similar to that of *ZFL* and *FLO/LFY* of the dicots, rather than *LtLFY* and *RFL* of *L. temulentum* and *O. sativa*.

It is possible the decrease of *PFL* at Time 3 in GA_3-treated, "large" and "small" meristems is due to dilution of *PFL* and that levels should have been higher like the "medium" meristems. In that case then, *PFL* expression would be maintained after branching had occurred and floral meristems and organs were developing, and would fulfil the role of a floral meristem identity gene as in the dicot flowering model.

In conclusion, *P. cookianum* had a *FLO/LFY* homologue, the expression of which was upregulated during floral development in agreement with the hypothesis. However, contrary to the hypothesis, the effect of GA_3 on the proportion of flowering plants was not reflected by *PFL* levels.

Chapter 4

General discussion

The aim of the work undertaken here was to expand upon knowledge of floral induction and development in *Myosotidum hortensia* and *Phormium cookianum*, to improve sales potential. Attempts were made to determine the environmental factors that acted as stimuli for floral induction and the ability of the plant hormone GA_3 to substitute for inductive stimuli in *M. hortensia* and *P. cookianum*. The effects of plant size on floral induction, and the expression pattern of *FLORICAULA / LEAFY* were investigated in *P. cookianum*.

Flowering in *P. cookianum* occurred in spring / early summer in 2002 and 2003. Vegetative meristems were seen in plants taken for sectioning on the 1-3 March of 2003 and floral apices were seen by 20 May 2002 and 29 May 2003 suggesting floral induction in *P. cookianum* took place sometime over March, April and May. The proposed time frame for floral induction took place over Autumn (1 March - 30 May) when ambient temperatures were cooling and the daylength shortening. These environmental parameters suggest *P. cookianum* may require SDs or cold temperatures to induce flowering. The CEL treatments of 2002, exposed plants to both these stimuli separately or in combination with no significant effect on floral induction. Floral development was enhanced and accelerated by Cold temperatures and further enhanced by subsequent Warm temperatures, which may have also accelerated development. The lack of response to daylength, the effect of cold temperatures, and the effect of GA_3 on flowering, was consistent with the hypothesis that *P. cookianum* would show no floral response due to different daylengths, but would flower in response to chilling, and that application of GA_3 would enhance this floral response.. Inhibition of flowering under potentially inductive conditions was associated with a lesser node count in *P. cookianum*. These results suggested *P. cookianum* had requirements for cold temperatures and had to be of a large enough size before flowering could take place.

GA_3 promoted flowering when applied on Feb 5, 8, and 11 2002 and March 25 and 31 2003 and even though the GA promoted flowering appeared much more effective in 2003, flowering was still inhibited with node counts of lower size. Improved effectiveness of GA_3 in promoting flowering with different times of application was also noted in *Lolium temulentum* by King and Evans (2003). A single "large", GA_3-treated fan had not flowered in the second trial from 2003, suggesting the inability of GA_3 to induce flowering may come down to inhibition exhibited to different degrees by the individual genome. It could be that node count was not stringently correlated with floral inhibition, and that there was another factor, linked to node number, acting as a marker of size. If increased flowering in larger plants is related to metabolic output, total determinate leaf growth could be a determining factor which is again dependent on the genome of the subject (de Jong et al. 1998).

This study agrees with the literature that *FLO / LFY* expression is upregulated at the meristem upon floral development in the dicots and the monocots. That more fans flowered the larger they got, and that GA_3 promoted flowering in *P. cookianum* explained well the levels of *PFL* expression seen in "small", "medium", and "large" fans. These results agreed with the hypothesis of Chapter 3 as the highest *PFL* levels compared within sizes were seen in GA_3-treated fans, but not in that the highest levels would be seen in "large" fans. A drop in *PFL* expression occurred in the latest meristem samples of "small" and "large" GA_3-treated fans that may be explained by investigation of the spatial expression pattern of *PFL*.

That GA_3 application was still cued in *P. cookianum* suggested a second factor acted to allow GA_3 to promote flowering. It was demonstrated by Sakomoto et al. (2001) that a ring of 2-oxidase activity seen in rice apices may determine the timing of GA access to the apex, and King and Evans (2003) suggested this expression may explain the differences in response to different GAs at different times in *Lolium temulentum*. If this was the case in *P. cookianum*, once 2-oxidase activity was reduced at the apex by a flowering cue, GA_3 could promote flowering. This may explain the similarities seen between the time to flower of GA_3-treated plants in 2002 and 2003. That would also be dependent on the longevity of the presence of GA_3 once applied to the apex.

Further research would be needed to explore the action of GA_3 on flowering in *P. cookianum*. Downstream of GA signalling, the transcriptional activator, *AtMyb33*, was identified in *Arabidopsis* and its binding to a GAMYB domain in the *LFY* promoter has been demonstrated . Investigation of the *PFL* promoter and a GAMYB binding domain could help reveal the action of applied GA_3 on flowering in *P. cookianum*. The presence of 2-oxidase activity and investigation of its expression at the apex, could help explain the timing of flowering seen in GA_3-treated flowering plants in 2002 and 2003. An effect of node number on 2-oxidase expression could also be investigated to determine if the inability of GA_3 to induce flowering in smaller plants lied there.

Flowering did not occur in *M. hortensia* after any treatment. To investigate flowering in this species older / larger plants may be required before application of any treatments would be effective. Application of other GAs, as in *M. alpestris* (Zeevaart 1983), could also be investigated to see if the vegetative effect of GA_3 was GA-dependent.

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Appendix A: Statistical tests of significance for vegetative and floral growth in *Myosotidium hortensia* and *Phormium cookianum*

Table A.1 : Main plot or 'between subjects' ANOVA

Tests of Hypotheses Using the Type I MS for treatment*table as an Error Term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treatment	3	178874.5563	59624.8521	9.69	0.0007
treatment*table	16	98433.0500	6152.0656	.	

Table A.2 : Split plot or 'within subjects' ANOVA

Tests of Hypotheses Using the Type I MS for table*day(treatment) as an Error Term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
day	5	665476.1354	133095.2271	155.29	<.0001
treatment*day	15	71080.8063	4738.7204	5.53	<.0001
table*day(treatment)	80	68566.3500	857.0794	.	

Table A.3 : Linear contrasts

Tests of Hypotheses Using the Type I MS for table*day(treatment) as an Error Term

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
ColdLD V ColdSD	1	1966.5375	1966.5375	2.29	0.1338
ColdLD V WarmLD	1	81918.1500	81918.1500	95.58	<.0001
ColdLD V WarmSD	1	120243.2667	120243.2667	140.29	<.0001
ColdSD V WarmLD	1	58500.0375	58500.0375	68.26	<.0001
ColdSD V WarmSD	1	91455.1042	91455.1042	106.71	<.0001
WarmLD V WarmSD	1	3666.0167	3666.0167	4.28	0.0419
Cold V Warm	1	173242.0021	173242.0021	202.13	<.0001
LD V SD	1	5501.3021	5501.3021	6.42	0.0132

Table A.4 : Observed and expected frequencies of induction on treatment, Cold temperatures versus Warm

treatment induction

Frequency Expected	F	NF	Total
Cold	10 10	10 10	20
Warm	10 10	10 10	20
Total	20	20	40

Table A.5 : Statistics for Table of treatment by induction, Cold temperatures versus Warm

Statistic	DF	Value	Prob
Chi-Square	1	0.0000	1.0000
Likelihood Ratio Chi-Square	1	0.0000	1.0000
Continuity Adj. Chi-Square	1	0.0000	1.0000
Mantel-Haenszel Chi-Square	1	0.0000	1.0000
Phi Coefficient		0.0000	
Contingency Coefficient		0.0000	
Cramer's V		0.0000	

Table A.6 : Observed and expected frequencies of induction on treatment, Short day versus Long day

treatment induction

Frequency / Expected	F	NF	Total
Long	11 10	9 10	20
Short	9 10	11 10	20
Total	20	20	40

Table A.7 : Statistics for Table of treatment by induction, Short day versus Long day

Statistic	DF	Value	Prob
Chi-Square	1	0.4000	0.5271
Likelihood Ratio Chi-Square	1	0.4007	0.5267
Continuity Adj. Chi-Square	1	0.1000	0.7518
Mantel-Haenszel Chi-Square	1	0.3900	0.5323
Phi Coefficient		0.1000	
Contingency Coefficient		0.0995	
Cramer's V		0.1000	

Table A.8 : Observed and expected frequencies of anthesis on temperature

treatment anthesis

Frequency / Expected	A	NA	Total
Cold	10 6	0 4	10
Warm	2 6	8 4	10
Total	12	8	20

Table A.9 : Statistics for table of treatment by anthesis

Statistic	DF	Value	Prob
Chi-Square	1	13.3333	0.0003
Likelihood Ratio Chi-Square	1	16.9124	<.0001
Continuity Adj. Chi-Square	1	10.2083	0.0014
Mantel-Haenszel Chi-Square	1	12.6667	0.0004
Phi Coefficient		0.8165	
Contingency Coefficient		0.6325	
Cramer's V		0.8165	

Fisher's Exact Test	
Cell (1,1) Frequency (F)	10
Left-sided Pr <= F	1.0000
Right-sided Pr >= F	3.572E-04
Table Probability (P)	3.572E-04
Two-sided Pr <= P	7.145E-04

Sample Size = 20

Table A.10 : T-test procedure for the number of flowers per plant under Cold and Warm temperatures

T-Tests					
Variable	Method	Variances	DF	t Value	Pr > |t|
flowers	Pooled	Equal	18	0.70	0.4904
flowers	Satterthwaite	Unequal	16	0.70	0.4916

Equality of Variances					
Variable	Method	Num DF	Den DF	F Value	Pr > F
flowers	Folded F	9	9	2.11	0.2821

Table A.11 : T-test procedure for the node with the first floral branch under Cold and Warm temperatures

T-Tests					
Variable	Method	Variances	DF	t Value	Pr > |t|
first	Pooled	Equal	18	-8.16	<.0001
first	Satterthwaite	Unequal	17.1	-8.16	<.0001

Equality of Variances					
Variable	Method	Num DF	Den DF	F Value	Pr > F
first	Folded F	9	9	1.59	0.5032

Table A.12 : T-test procedure for the number of floral axes per plant under Cold and Warm temperatures

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
branches	Pooled	Equal	18	-1.60	0.1272
branches	Satterthwaite	Unequal	13.6	-1.60	0.1328

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
branches	Folded F	9	9	3.63	0.0683

Table A.13: T-test procedure for the height of inflorescence bolt under Cold and Warm temperatures

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
inflorescence	Pooled	Equal	18	2.14	0.0462
inflorescence	Satterthwaite	Unequal	18	2.14	0.0462

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
inflorescence	Folded F	9	9	1.00	0.9960

Table A.14 : Main plot or 'between subjects' ANOVA

Tests of Hypotheses Using the Type I MS for treatflow*table as an Error Term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treatflow	7	327408.560	46772.651	7.06	0.0001
treatflow*table	25	165626.531	6625.061	.	

Table A.15 : Split plot or 'within subjects' ANOVA

Tests of Hypotheses Using the Type I MS for table*day(treatflow) as an Error Term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
day	5	1031707.208	206341.442	265.48	<.0001
treatflow*day	35	181468.585	5184.817	6.67	<.0001
table*day(treatflow)	125	97156.581	777.253		

Table A.16 : Linear contrasts

Tests of Hypotheses Using the Type I MS for table*day(treatflow) as an Error Term

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
ColdLDF V ColdLDNF	1	9130.08333	9130.08333	11.75	0.0008
ColdSDF V ColdSDNF	1	18960.75000	18960.75000	24.39	<.0001
WarmLDF V WarmLDNF	1	3771.88021	3771.88021	4.85	0.0294
WarmSDF V WarmSDNF	1	23009.46759	23009.46759	29.60	<.0001

Table A.17 : T-test procedure for the number of flowers for transferred and Cold grown plants

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
flowers	Pooled	Equal	18	-0.68	0.5069
flowers	Satterthwaite	Unequal	13.5	-0.68	0.5097

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
flowers	Folded F	9	9	3.75	0.0621

Table A.18 : T-test procedure for the node with the first floral branch for transferred and Cold grown plants

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
first	Pooled	Equal	18	3.79	0.0013
first	Satterthwaite	Unequal	13.9	3.79	0.0020

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
first	Folded F	9	9	3.41	0.0816

Table A.19 : T-test procedure for the number of floral branches for transferred and Cold grown plants

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
branches	Pooled	Equal	18	-2.50	0.0223
branches	Satterthwaite	Unequal	12.8	-2.50	0.0269

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
branches	Folded F	9	9	4.57	0.0336

Table A.20 : T-test procedure for the height of inflorescence for transferred and Cold grown plants

T-Tests

Variable	Method	Variances	DF	t Value	Pr > |t|
inflorescence	Pooled	Equal	18	-0.69	0.4999
inflorescence	Satterthwaite	Unequal	18	-0.69	0.4999

Equality of Variances

Variable	Method	Num DF	Den DF	F Value	Pr > F
inflorescence	Folded F	9	9	1.09	0.9010

Table A.21 : Observed and expected frequencies of flowering in *P. cookianum* on GA_3 concentration

[GA]	induction		
Frequency Expected	F	NF	Total
0	2 6	8 4	10
25	5 6	5 4	10
250	8 6	2 4	10
2500	9 6	1 4	10
Total	24	16	40

Table A.22 : Statistics for table of GA_3 concentration by induction

Statistic	DF	Value	Prob
Chi-Square	3	12.5000	0.0059
Likelihood Ratio Chi-Square	3	13.4602	0.0037
Mantel-Haenszel Chi-Square	1	11.7000	0.0006
Phi Coefficient		0.5590	
Contingency Coefficient		0.4880	
Cramer's V		0.5590	

Fisher's Exact Test	
Table Probability (P)	8.119E-05
Pr <= P	0.0060

Sample Size = 40

Table A.23 : Completely randomised design ANOVA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GAconc	3	700.722403	233.574134	1.93	0.1603
Error	18	2174.232143	120.790675		
Corrected Total	21	2874.954545			

Table A.24 : Main plot or 'between subjects' ANOVA

Tests of Hypotheses Using the Type I MS for treatment*table as an Error Term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treatment	3	94208.9000	31402.9667	10.57	0.0004
treatment*table	16	47530.5600	2970.6600	.	

Table A.25 : Split plot or 'within subjects' ANOVA

Tests of Hypotheses Using the Type I MS for table*day(treatment) as an Error Term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
day	4	125915.0600	31478.7650	128.93	<.0001
treatment*day	12	29998.1000	2499.8417	10.24	<.0001
table*day(treatment)	64	15625.7400	244.1522	.	

Table A.26 : Linear contrasts

Tests of Hypotheses Using the Type I MS for table*day(treatment) as an Error Term

Contrast	DF	Contrast SS	Mean Square	F Value	Pr > F
ColdLD V ColdSD	1	873.62000	873.62000	3.58	0.0631
ColdLD V WarmLD	1	70650.40500	70650.40500	289.37	<.0001
ColdLD V WarmSD	1	31425.24500	31425.24500	128.71	<.0001
ColdSD V WarmLD	1	55811.40500	55811.40500	228.59	<.0001
ColdSD V WarmSD	1	21819.60500	21819.60500	89.37	<.0001
WarmLD V WarmSD	1	7837.52000	7837.52000	32.10	<.0001
Cold V Warm	1	85497.76000	85497.76000	350.18	<.0001
LD V SD	1	1738.89000	1738.89000	7.12	0.0096

Appendix B: Statistical tests for homogeneity and normality of the residuals from vegetative growth data (Chapter 2).

As the same leaves and plants were measured repeatedly to observe their response, the following general linear model was constructed to explain the repeated measures observations:

$y_{ijk}=\mu+U_i+S_{j(i)}+T_k+(UT)_{ik}+\varepsilon_{ijk}$

where,

y_{ijk} is the response of the j^{th} plant at k^{th} time for i^{th} level of factor U,

μ is the overall mean,

U_i is the main effect due to the i^{th} level of factor U,

$S_{j(i)}$ is the (random) effect due to the j^{th} plant in the i^{th} level of U, the between subjects error structure,

T_k is the main effect due to the k^{th} level of repeated measures factor T,

$(UT)_{ik}$ is the interaction effect between the i^{th} level of U and the j^{th} level of T,

ε_{ijk} is the within-subject error structure (Ganesh 2002).

When making use of an ANOVA, certain assumptions about the data need to be verified. These assumptions are that, the distribution of the population from which the samples are drawn is normally distributed; populations may have different means but the variance is equal or common; samples are randomly drawn from the population and are independent; and the fitted model is additive. Tests for the homogeneity of variances assumption were performed and are presented here along with a visual interpretation.

It is required by the homogeneity of variances assumption that the treatments do not change the variability of the response (the spread of the residuals) but change the magnitude or mean of the response. In terms of environmental treatments and plants, as growth increased the mean inevitably increased, but the differences between the plants in each room at each time should have remained the same. To visualise differences between residuals they were standardised and plotted against the fitted or

expected values derived from the model in SAS v8.2. Residuals were standardised by division by the estimated population variance or Mean Square Error.

It was assumed, that like the distributions of the populations of observations, the variances were normally distributed and displayed symmetry about a single peak. The variances when normally distributed should have mean 0 and variance. The distribution was visualised by plotting the residuals on a normal probability scale using SAS v8.2. Residuals were ordered by magnitude, and the i^{th} ordered value was plotted against the $((i - ½)/n)^{th}$ quantile of the normal distribution (n is the total number of observations).

Analysis of the *Phormium cookianum* vegetative growth data residuals (Chapter 2, section 2.2.1.1.1 Vegetative growth) revealed ANOVA assumptions were not validated. The standardised residuals were not spread evenly about the mean and there was an outlier at coordinates outside of the inner fence (-2.69) (Fig. B.1), which after further investigation was considered a valid measurement. Levene's test for homogenous variance also suggested heterogeneity (p=0.0003, Table B.2). Analysis of the normality of the variance distribution (Fig. B.2) suggested the assumptions could be void. The data display a distinct cyclic pattern about the expected trendline and the Kolmogorov-Smirnov test was negative for normality (p=0.0100, Table B.1). When the residuals were calculated without blocking effects (table), the variance occurring between the growth of the leaves suggested an increase as average growth increased (Fig. B.3). The wedge shaped pattern was indicative of a functional relationship between the variance of the residuals and the mean of the responses. Several outliers were present in the residuals when analysed without blocking effects.

Variances in growth between rooms may have occurred as treatments affected growth rates of subjects differently (Section 2.2.1.1.1 Vegetative growth). The differences in growth under each separate treatment, however, were dependent on the capabilities of each of the individual subjects of that room. There were a number of factors that may have influenced the growth of an individual in response to exposure to different growth conditions. Growth may have varied due to the leaf chosen for measuring (differences between nodes) or the individual's morphogenetic potential (Atwell et al. 1999).

As time proceeded differences between the individual growth rates became magnified. The amplification opposed the additivity of the model, which was required for validity of an ANOVA. The amplification of variance was initially magnified in Warm rooms, compared with Cold, due to enhanced vegetative growth. It was possible that variance was associated with growth by a constant factor, such as log or an exponential, which could be used to transform the data. Once the data was transformed additivity could be restored to the model as

$Log(y_{ijk})=Log(\mu)+Log(U_i)+Log(S_{j(i)})+Log(T_k)+Log((UT)_{ik})+Log(\varepsilon_{ijk})$,

is additive.

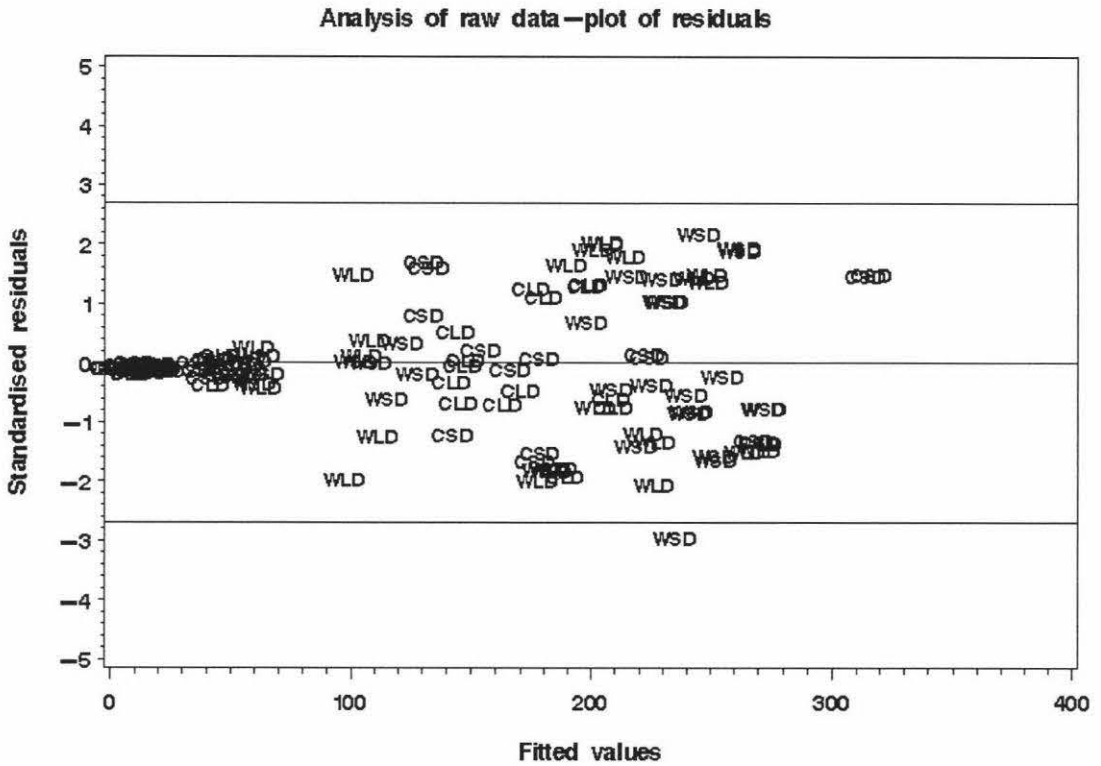


Figure B.1: Residual plot of *P. cookianum* vegetative growth data, with the General Linear Model used to account for the observations taking blocking effects into consideration.

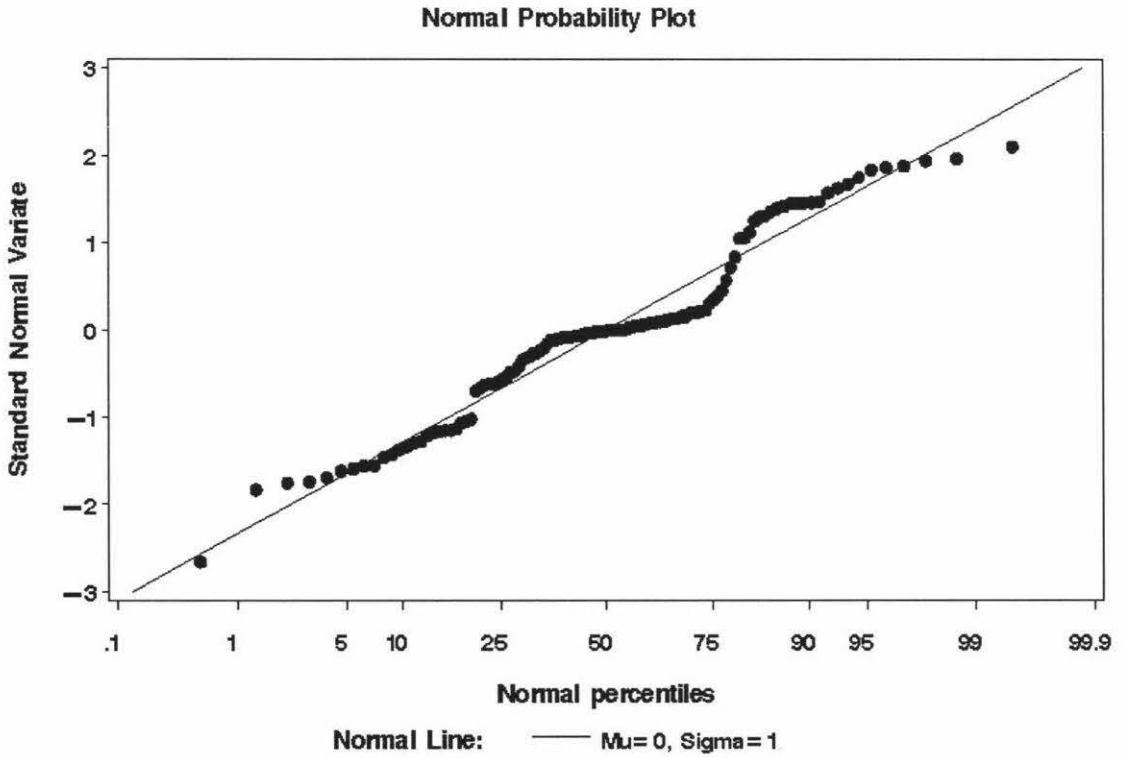


Figure B.2: Normal probability plot of *P. cookianum* vegetative growth data, with the General Linear Model used to account for the observations taking blocking effects into consideration.

Table B.1: **Tests for Normality performed on *P. cookianum* vegetative growth data residuals.**

Test		--Statistic---		-----p Value------
Kolmogorov-Smirnov	D	0.150052	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.47016	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	2.377082	Pr > A-Sq	<0.0050

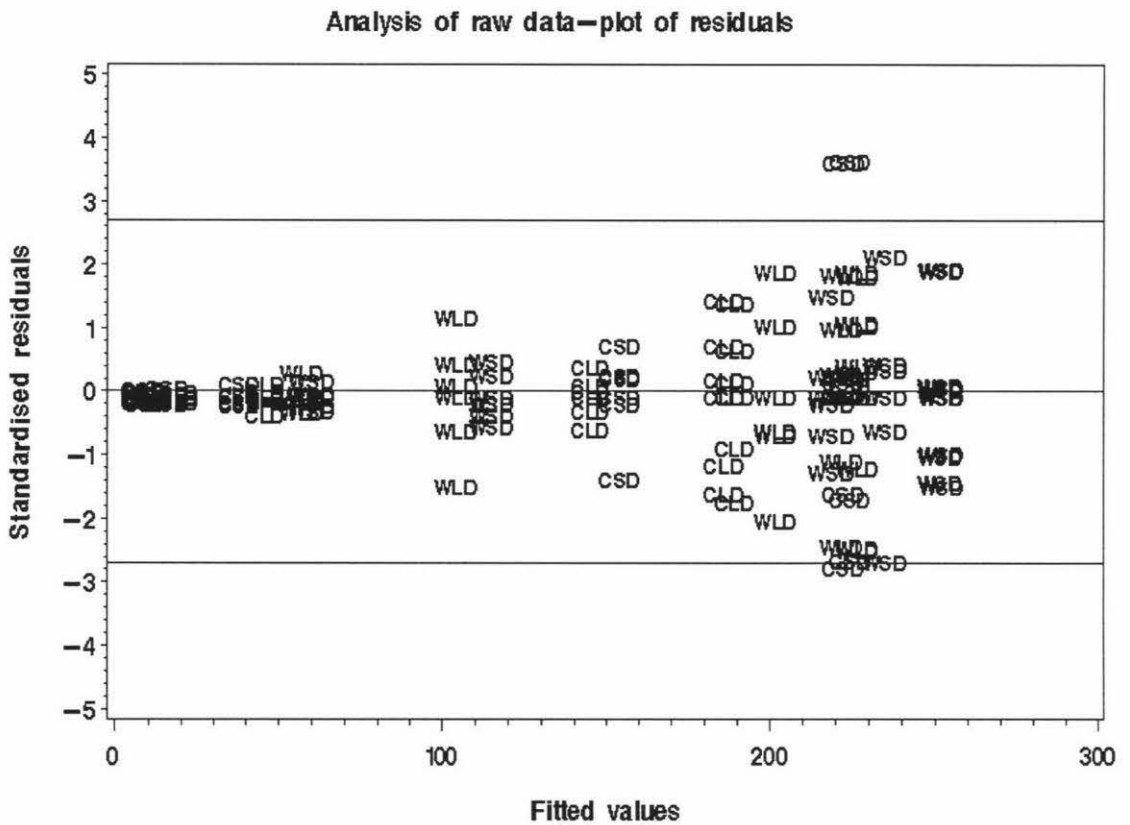


Figure B.3: **Residual plot of *P. cookianum* vegetative growth data, with the General Linear Model used to account for the observations not taking into account any effects blocking within the rooms may have had on growth.**

Table B.2: **Levene's Test for homogeneity of *P. cookianum* vegetative growth data variance, with the General Linear Model used to account for the observations not taking into account any effects blocking within the rooms may have had on growth.**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment*day	23	35973.7	1564.1	2.68	0.0003
Error	120	69923.9	582.7		

To find whether a functional relationship between the mean and variance of the *P. cookianum* growth data was proportional, or was proportional to the square root of the mean, logarithm and square root transformations were performed.

The homogeneity and normality of the residuals were visualised by methods similar to those above. The model used to explain the transformed data still omitted the blocking effects for easy comparison.

Standardised residuals of the logarithm transformed data had a much more homogenous spread about the variance mean (0) (Fig. B.4). A significance test still tested marginally significant (p=0.1459) indicating slight heterogeneity of the spread (Table B.3). A normal probability plot (Fig. B.5) showed improvement of the log transformed residuals in adhering to a normal distribution scale, but a cyclic pattern about the trend line was evident. Non-normality of the distribution was confirmed by a Kolmogorov-Smirnov test, testing critically significant (p=0.010, Table B.4).

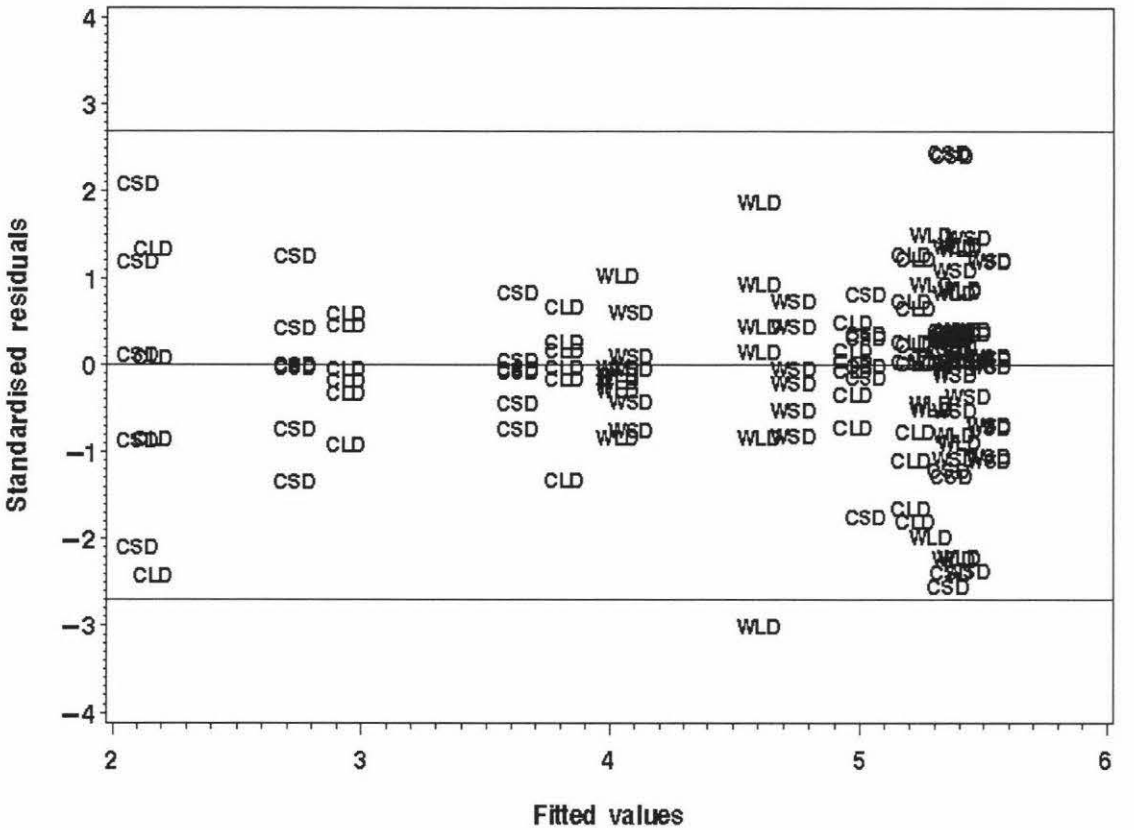


Figure B.4: **Residual plot of logarithm transformed *P. cookianum* vegetative growth data, with the General Linear Model used to account for the observations not taking into account any effects blocking within the rooms may have had on growth.**

Table B.3: **Levene's Test for Homogeneity of logarithm transformed *P. cookianum* vegetative growth data variance, with the General Linear Model used to account for the observations not taking into account any effects blocking within the rooms may have had on growth.**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment*day	23	0.5211	0.0227	1.36	0.1459
Error	120	2.0004	0.0167		

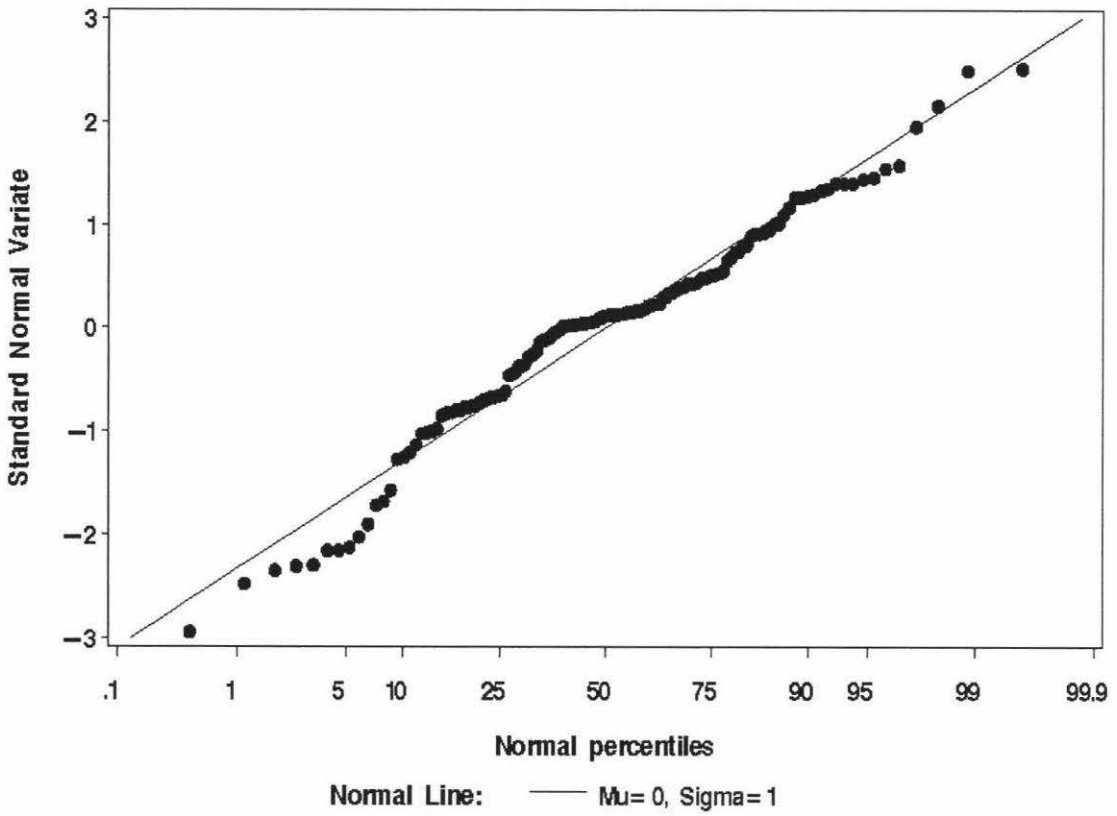


Figure B.5: **Normal probability plot of Logarithm transformed *P. cookianum* vegetative growth data, with the General Linear Model used to account for the observations not taking into account any effects blocking within the rooms may have had on growth.**

Table B.4: **Tests for Normality performed on Logarithm transformed *P. cookianum* vegetative growth data residuals.**

Test	--Statistic---		-----p Value-----	
Kolmogorov-Smirnov	D	0.116749	Pr > D	<0.010
Cramer-von Mises	W-Sq	0.330729	Pr > W-Sq	<0.005
Anderson-Darling	A-Sq	1.759265	Pr > A-Sq	<0.005

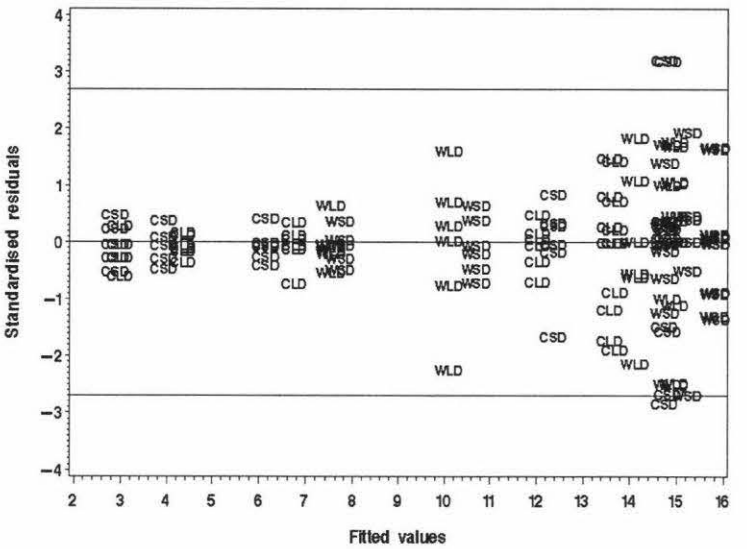
A square root transformation was performed after the log transformation proved invalid. Residuals of the square root transformed data still increased as mean growth increased, suggesting the two were not functionally related by the square root of the mean, as the variances were not stabilised (Fig. B.5 A). Levene's test for homogeneity of variances further confirmed differences were occurring between variances of different measurements (Fig. B.5 C). The normal probability plot had a cyclic pattern about the trend (Fig. B.5 B), and a Kolmogorov-Smirnov test confirmed the non-normal distribution of the residuals (Fig. B.5 D).

A further attempt to find a transformation to stabilise variance involved an empirical method, where by $\log_e$(standard deviation) was plotted against $\log_e$(mean) (Fig. B.6), and the slope of the fitted line is subtracted from 1 to find the power by which to transform data (in that case, 1- 1.0504, Fig. B.6). Once the data was transformed by -0.054 the tests for homogeneity and normality of the distribution of the variances were performed using SAS v8.2. The spread of the variances, about the variance mean of 0, was heterogeneous with increased spread at the polar ends (Fig. B.7 A) and a very weakly significant homogeneity test ($p=0.1107$, Fig. B.7 C). The variance followed the normal probability distribution trend line weakly and displayed a cyclic pattern (Fig. B.7 B). The tests for normality of the distribution tested significant, implying the data had not displayed a normal distribution (Kolmogorov-Smirnov test, $p<0.010$).

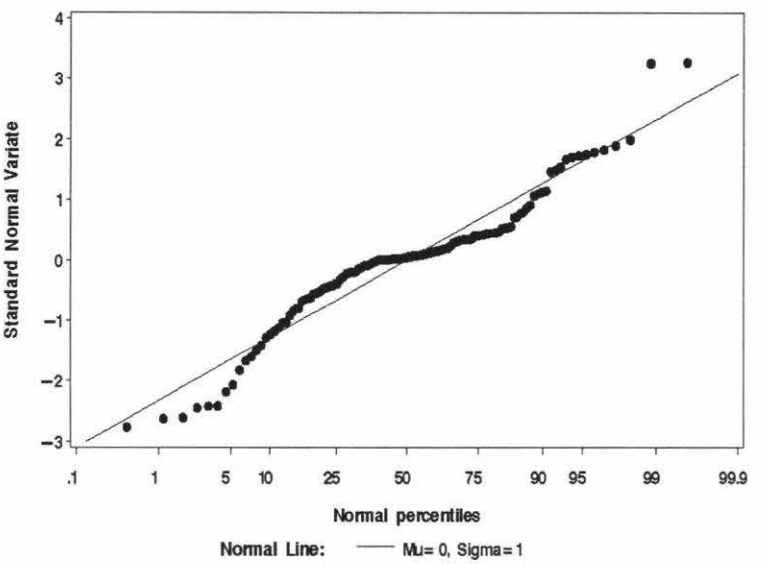
Plants were divided further within the four treatment groups into flowering and non-flowering and tests on differences in growth were performed. The data were the same as those used to analyse the variances of the vegetative growth, and results were similar. No variance stabilising transformation could be found.

Though the power transformation improved the homogeneity of the variance generated by the General Linear Model, adherence to a normal distribution could not be made. Ten plants per treatment were used, two at each of five tables. Even though the duplicates at each table were averaged to reduce variance, heterogeneity was still found. Growing more plants under each treatment may have reduced the differences in variance seen between time points and a transformation may have been found to stabilise the variance-mean relationship.

A)



B)



C)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment*day	23	34.5001	1.5000	2.25	0.0025
Error	120	80.0168	0.6668		

D)

Tests for Normality				
Test	--Statistic---		-----p Value-----	
Kolmogorov-Smirnov	D	0.133420	Pr > D	<0.010
Cramer-von Mises	W-Sq	0.803800	Pr > W-Sq	<0.005
Anderson-Darling	A-Sq	4.084970	Pr > A-Sq	<0.005

Figure B.6: Analysis of Square Root transformed *P. cookianum* vegetative growth data. A) Residual plot. B) Normal Probability plot. C) Levene's test of variance homogeneity. D) Tests of Normality.

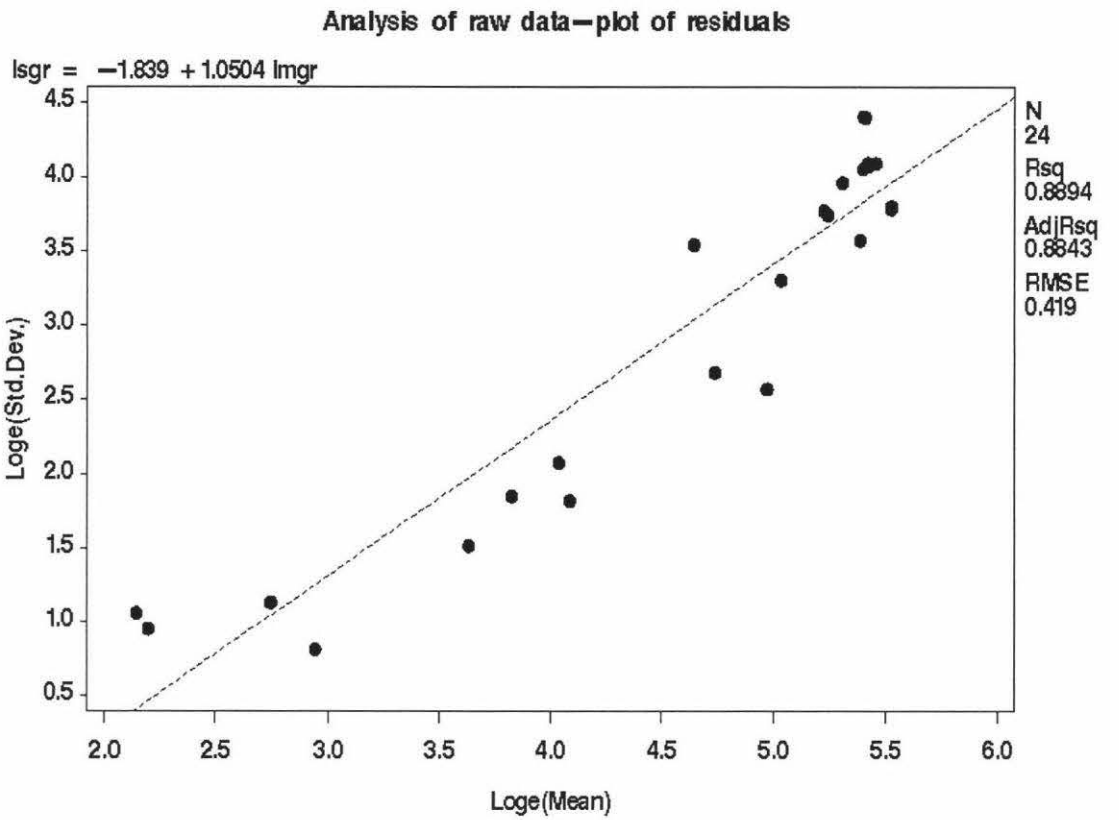
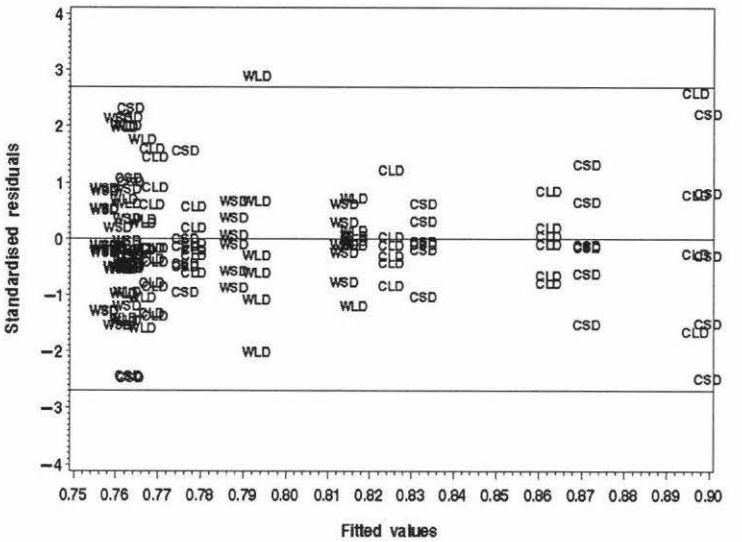
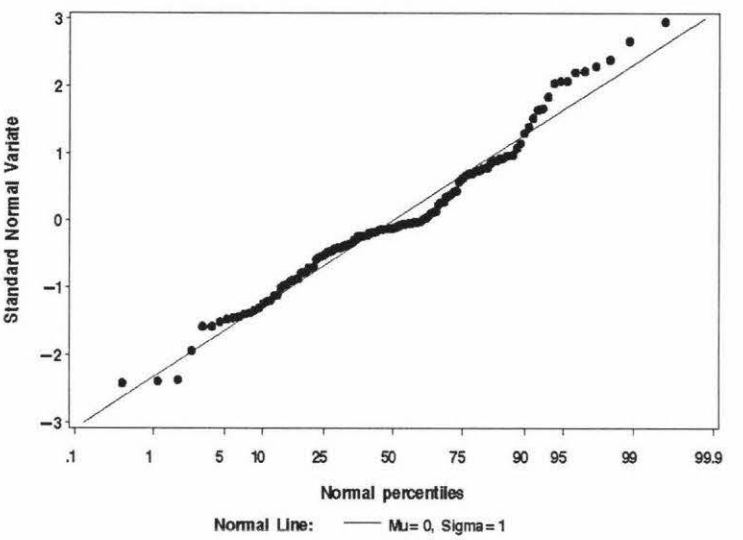


Figure B.7: **Linear regression model of logarithm transformed *P. cookianum* vegetative growth data.**

A)



B)



C)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment*day	23	0.000877	0.000038	1.43	0.1107
Error	120	0.00320	0.000027		

D)

Tests for Normality				
Test	--Statistic---		-----p Value-----	
Kolmogorov-Smirnov	D	0.116893	Pr > D	<0.010
Cramer-von Mises	W-Sq	0.331182	Pr > W-Sq	<0.005
Anderson-Darling	A-Sq	1.728276	Pr > A-Sq	<0.005

Figure B.8: Analysis of Power transformed *P. cookianum* vegetative growth data. A) Residual plot. B) Normal Probability plot. C) Levene's test of variance homogeneity. D) Tests of Normality.

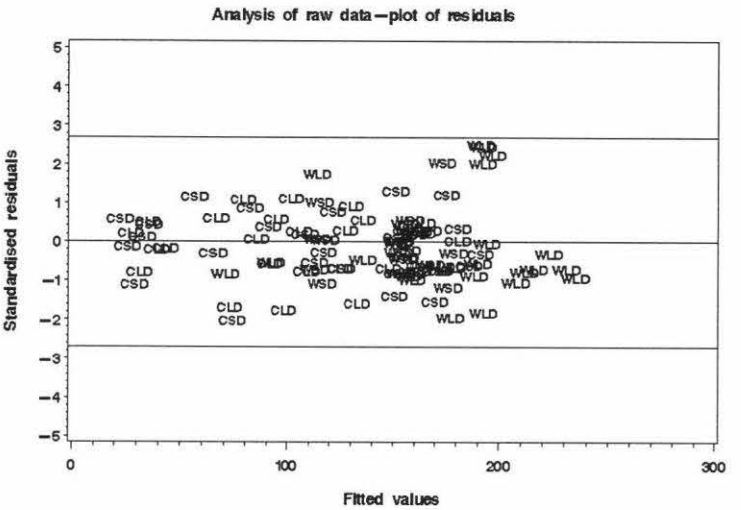
Myosotidium hortensia residuals

The residuals were analysed for *Myosotidium hortensia* vegetative growth data (section 2.3.2.1.1 Vegetative growth), as they were for *P. cookianum*, to validate the ANOVA.

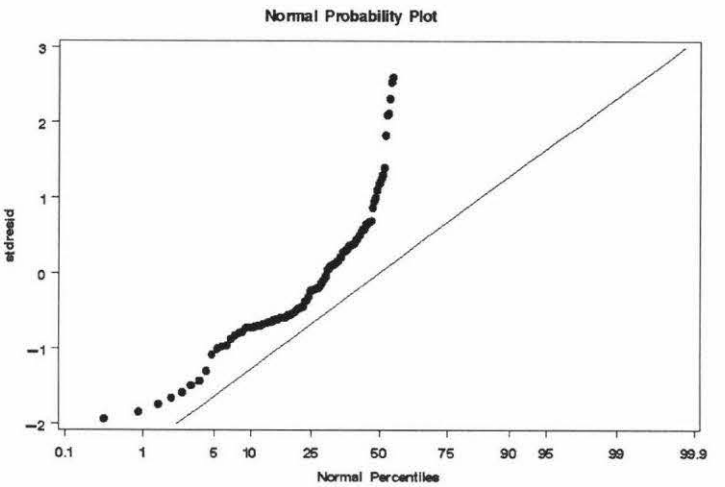
The standardised residuals that existed when the observations were modelled on the effects of treatment, day, and table had a fairly uniform distribution about the mean, with the suggestion of a slight fan shape (Fig. B.8 A). Plotting the residuals on a normal probability plot (Fig. B.8 B) revealed drift from the expected trendline. The normality tests confirmed the non-normal distribution of the residuals, the Kolmogorov-Smirnov test found the distribution to be very significant (p=0.0896, Fig. B.8 C)

There may still be a wedge shape emerging in the standardised residuals, though measurements were not repeated for as long as the *P. cookianum* data, so they were analysed without the effects of the tables to better see any size-variance relationship (Fig. B.9). A wedge shape was apparent, implying the variance was a function of mean growth. Levene's test for homogeneity suggested there was a weak difference between the residuals (p=0.1275, Table. B.5).

A)



B)



C)

Test	--Statistic---		-----p Value------	
Kolmogorov-Smirnov	D	0.082854	Pr > D	0.0896
Cramer-von Mises	W-Sq	0.150842	Pr > W-Sq	0.0232
Anderson-Darling	A-Sq	0.994026	Pr > A-Sq	0.0131

Figure B.9: **Analysis of *M. hortensia* vegetative growth data. A) Residual plot. B) Normal Probability plot. C) Tests of Normality.**

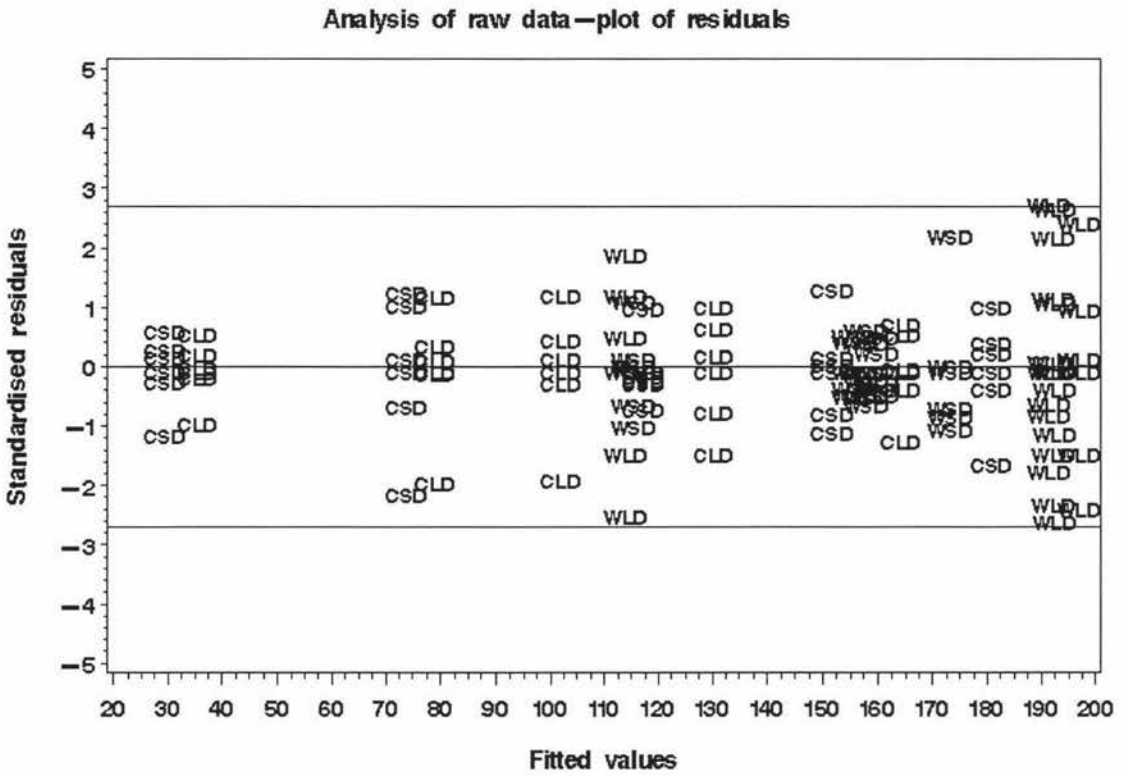


Figure B.10: **Residual plot of *M. hortensia* vegetative growth data, with the General Linear Model used to account for the observations not taking into account any effects blocking within the rooms may have had on growth.**

Table B.5: **Levene's Test for homogeneity of *M. hortensia* vegetative growth data variance, with the General Linear Model used to account for the observations not taking into account any effects blocking within the rooms may have had on growth.**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment*day	19	6884.6	362.3	1.44	0.1275
Error	100	25237.3	252.4		

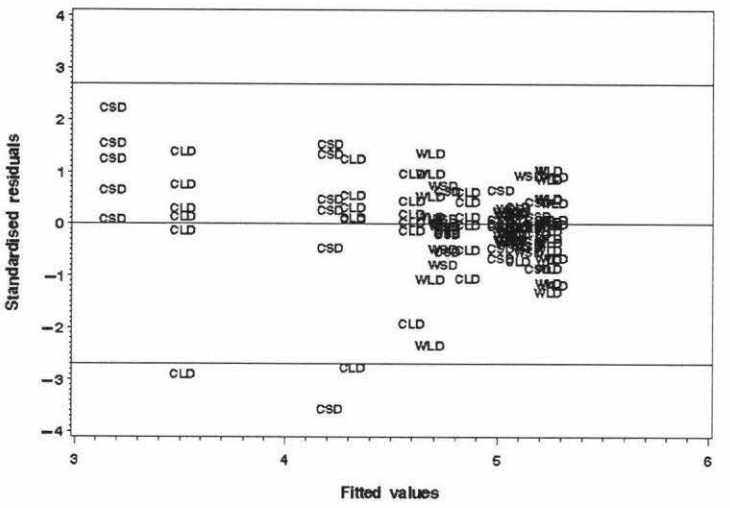
To stabilise the variance, data were transformed by logarithm, the square root of the mean, and the power 0.7061 (1- 0.2939). The logarithm transformation increased the spread of the standardised residuals about the mean (Fig. B.10 A), but a Levene's test found the differences to be significantly different (p=0.0020, Fig. B.10 C). The transformation stabilised the normality of the distribution but points below the 50^{th} quantile were above the trendline, and vice versa (Fig 2.10 B). The Kolmogorov-Smirnov test for normality found the variances followed a non-normal distribution (Fig. B.10 D).

The square root transformation stabilised the variance (p=0.2077, Fig. B.11 C) and the spread about the mean appeared more homogenous (Fig. B.11 A). The distribution was still non-normal, a Kolmogorov-Smirnov test suggesting a non-normal distribution was followed (p<0.010, Fig 2.11 D). The plot follows the trend line better than for untransformed data, but the trend seen in log transformed data, where there is an inflection about the 50^{th} percentile, has still occurred (Fig. B.11 B).

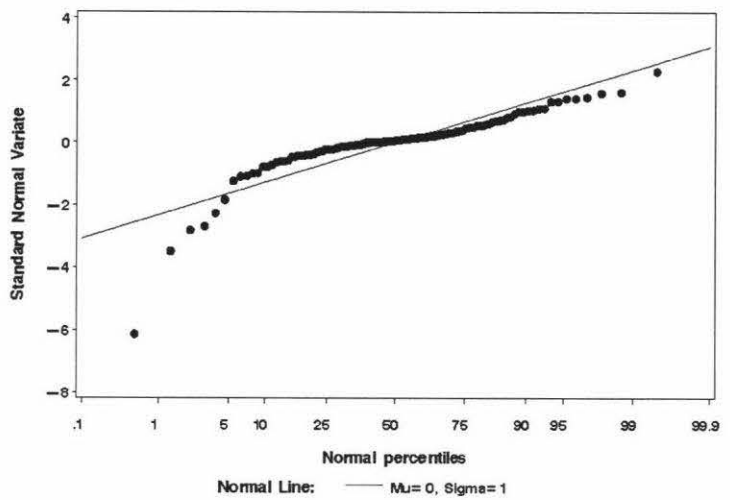
Like the logarithm and square root transformation, the power transformation stabilised the spread of variance but not the distribution. The residuals showed a more homogenous spread about the mean (Fig. B.12 A) than the other transformations and tested homogenous by Levene's test (p=0.2405, Fig. B.12 C). The residuals plotted on a normal probability plot showed more of a cyclic pattern than previous transformations, but again there was an inflection about the 50^{th} quantile (Fig. B.12 B). The distribution of the residuals tested non-normal by a Kolmogorov-Smirnov test (Fig. B.12 D).

The rate of growth of *M. hortensia* has generated differences between plants that could not be accounted for by the model. Using more than ten plants per treatment would reduce the variation and possibly enable a transformation to account for the functionality of the variance-mean relationship.

A)



B)



C)

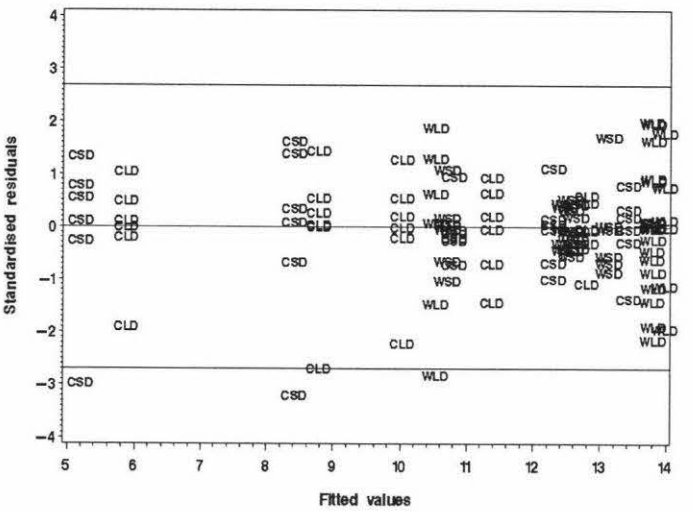
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment*day	19	1.8386	0.0968	2.47	0.0020
Error	100	3.9187	0.0392		

D)

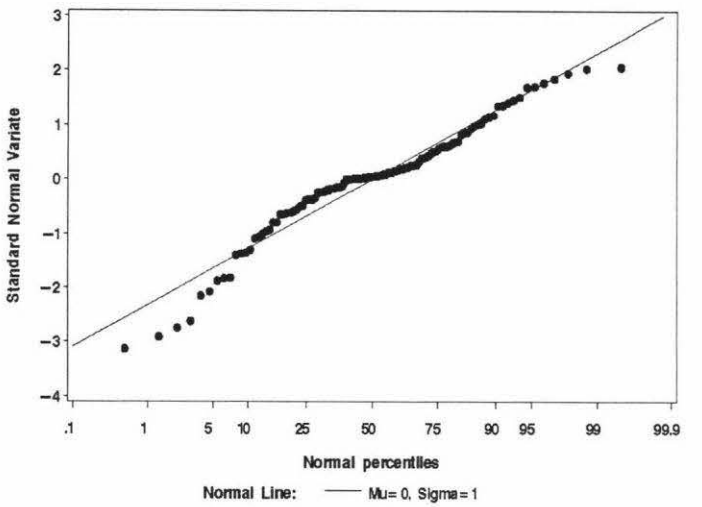
Test	--Statistic---		-----p Value-----	
Kolmogorov-Smirnov	D	0.169914	Pr > D	<0.010
Cramer-von Mises	W-Sq	1.030962	Pr > W-Sq	<0.005
Anderson-Darling	A-Sq	5.655423	Pr > A-Sq	<0.005

Figure B.11: Analysis of Logarithm transformed *M. hortensia* vegetative growth data. A) Residual plot. B) Normal Probability plot. C) Levene's test of variance homogeneity. D) Tests of Normality.

A)



B)



C)

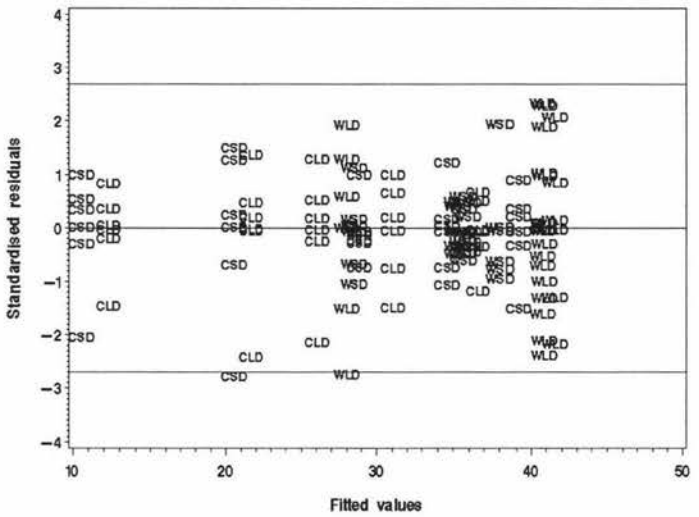
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment*day	19	13.2316	0.6964	1.29	0.2077
Error	100	54.0239	0.5402		

D)

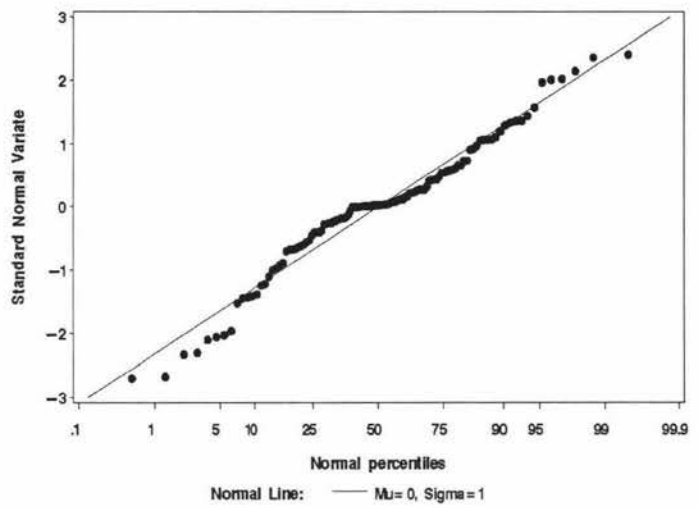
Test	--Statistic---		-----p Value-----	
Kolmogorov-Smirnov	D	0.120274	Pr > D	<0.010
Cramer-von Mises	W-Sq	0.367087	Pr > W-Sq	<0.005
Anderson-Darling	A-Sq	1.945809	Pr > A-Sq	<0.005

Figure B.12: Analysis of Square Root transformed *M. hortensia* vegetative growth data. A) Residual plot. B) Normal Probability plot. C) Levene's test of variance homogeneity. D) Tests of Normality.

A)



B)



C)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment*day	19	170.3	8.9645	1.24	0.2405
Error	100	721.7	7.2172		

D)

Test	--Statistic---		-----p Value-----	
Kolmogorov-Smirnov	D	0.111211	Pr > D	<0.010
Cramer-von Mises	W-Sq	0.324808	Pr > W-Sq	<0.005
Anderson-Darling	A-Sq	1.660420	Pr > A-Sq	<0.005

Figure B.13: Analysis of Power transformed *M. hortensia* vegetative growth data. A) Residual plot. B) Normal Probability plot. C) Levene's test of variance homogeneity. D) Tests of Normality.

Appendix C: Alignment of *Phormium cookianum* sequences with homologues

Figure C.1: Comparison of partial Mitochondrial 26S rRNA homologues

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
T.aestivum       GCCATGAACA TGCGATAGCC AGTAACTCCT TATGAATCCG AATCTCCCAC
P.cookianu       GCCGCT--CG TGCGATAGCC AGTAACTCCT TATGAATCCG AATCTCCCAC
O.sativa         GCCATGAACA TGCGATAGCC AGTAACTCCT TATGAATCCG AATCTCCCAC
Z.mays           GCCATGAACA TGCGATAGCC AGTAACTCCT TATGAATCCG AATCTCCCAC
O.berteria       GCCATGAACA TGCGATAGCC AGTAACTCCT TATGAATCCG AATCTCCCAC
B.vulgaris       GCCATGAACA TGCGATAGCC AGTAACTCCT TATGAATCCG AATCTCCCAC
                 ***      *  ********** ********** ********** **********

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
T.aestivum       CAGGGGGAAA GAGCGCATTT TCGCTAGTAG TAAGCTTGTG CGGCGCAAAA
P.cookianu       CAGGGGGAAA GAGCGCATTT TCGCTAGTAG TAAGCTTGTG CGGCGCAAAA
O.sativa         CAGGGGGAAA GAGCGCATTT TCGCTAGTAG TAAGCTTGTG CGGCGCAAAA
Z.mays           CAGGGGGAAA GAGCGCATTT TCGCTAGTAG TAAGCTTGTG CGGCGCAAAA
O.berteria       CAGGGGGAAA GAGCGCATTT TCGCTAGTCT TAAGCTTGTG CGGCGCAAAA
B.vulgaris       CAGGGGGAAA GAGCGCATTT TCGCTAGTCT TAAGCTTGTG CGGCGCAAAA
                 ********** ********** ********   ********** **********

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150
T.aestivum       TGACCCTTCC TAGCTTGGTA CCCTTGCTTA CTTGTCCCGA TAGTGGAAGA
P.cookianu       TGACCCTTCC TAGCTTGGTA CCCTTGCTTA AGTGTCCCGA TAGTGGAAGA
O.sativa         TGACCCTTCC TAGCTTGGTA CCCTTGCTTA CTTGTCCCGA TAGTGGAAGA
Z.mays           TGACCCTTCC TAGCTTGGTA CCCTTGCTTA CTTGTCCCGA TAGTGGAAGA
O.berteria       TGACCCTTCC TAGCTTGGTA CCCTTGCTTA GATGTCCCGA TAGTGGAAGA
B.vulgaris       TGACCCTTCC TAGCTTGGTA CCCTTGCTTA GATGTCCCGA TAGTGGAAGA
                 ********** ********** **********   ******** **********
```

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
T.aestivum      AACCGGCCTA GAAGGTTAGA AAAATCTTTT GGTCAAGTGA CGCG--CGGG
P.cookianu      AACCGGTCTA GAAGGTTGGA AAAGTCTTTT GGTCAAGTGA CGCG--CGGG
O.sativa        AACCGGCCTA GAAGGTTAGA AAAATCTTTT GGTCAAGTGA CGCG--CGGG
Z.mays          AACCGGCCTA GAAGGTTAGA AAAATCTTTT GGTCAAGTGA CGCG--CGGG
O.berteria      AACCGGTCTA GAAGGTTGGA AAAGTGTTAA TGTCAAGTGA CGAAAGCGAG
B.vulgaris      AACCGGTCTA GAAGGTTGGA AAAGTGTTAA TGTCAAGGGA CGCG---GGA
                ****** *** ******* ** *** * **    ****** ** **     *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
T.aestivum      GTG-GGGCAG TAGTCAGGAC ACAACGGATA CGAACGCGGG GTTTTGCCAA
P.cookianu      GTG-GGGCAT T---CATGAC ACAACGGATG CGAACGCGGG GTTTTGCCAA
O.sativa        GTG-GGGCAG TAGTCAGGAC ACAACGGATA CGAACGCGGG GTTTTGCCAA
Z.mays          GTG-GGGCAG -------GAC ACAACGGATA CGAACGCGGG GTT------A
O.berteria      GAGAGGGCAA CCAGCTCACC GTACAACGAG GAAATGGAAA TCCTTTCTCG
B.vulgaris      ATGCGGATAA AGAATCCGC- ---------- ---------- ----------
                  * **  *

                ....|....| ....|....| ....|....| ....|....| ..
                       260        270        280        290
T.aestivum      CGACTGAAAC ATGTTGAGGC ATCCCGCGCG ATGCCGTTGA AA
P.cookianu      CGACTGAAAC ATGTTCATTA GGCCCGCTCG ATGTAGTTGT TC
O.sativa        CGACTGAAAC ATGTTGAGGC ATCCCGCGTG ATGCCGTTGT --
Z.mays          CGACTGAAAC ATGTTGAGGC ATCC-GCGCG ATGCC----- --
O.berteria      CCGCCGAACG ---------- ---------- ---------- --
B.vulgaris      ---------- ---------- ---------- ---------- --
```

Figure C.2: Partial cDNA comparison of *FLO / LFY* homologues

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
OrcLFY           GAGCCGGGCG AGGTGGCACG AGCTAAGAAG AACGGGCTCG ATTACCTCTT
AtLFY            GAGCCTGGGG AAGTGGCACG TGGCAAAAAG AACGGCTTAG ATTATCTGTT
PFL              GAGCCTGGGG AGGTGGCACG TGCGAAAAAG AACGGCTTGG ATTATTTRTT
OSL              GAGCCCGGCG AGGTGGCGAG GGCCAAGAAG AACGGGCTGG ACTACCTGTT
RFL              GAGCCCGGCG AGGTGGCGAG GGCCAAGAAG AACGGGCTGG ACTACCTGTT
LtLFY            GAGCCCGGGG AGGTGGCGAG GGCCAAGAAG AACGGGCTGG ACTACCTGTT
ZFL1             GAGCCCGGCG AGGTGGCGAG GGCCAAGAAG AACGGGCTCG ACTACCTCTT
ZFL2             GAGCCCGGCG AGGTGGCCAG GGCCAAGAAG AACGGGCTTG ACTACCTCTT
HLY              GAGTCGGGGG AGGTGGCGCG CGCGAGGAAG AACGGGCTCG ACTACCTCTT
                 *** * ** * * *****  *  *  *  *** *****  * * * **  * **

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
OrcLFY           CAATCTTTAC GAGCAATGCC ATGAATTTCT GCTCCAAGTC CAGTCCGTGG
AtLFY            CCACTTGTAC GAACAATGCC GTGAGTTCCT TCTTCAGGTC CAGACAATTG
PFL              CCATTTGTAC GAGCAGTGCG GGGAGTTRTT GGCACARGTG CAGTCGCTAG
OSL              CCATCTGTAC GAGCAGTGCC GCCTCTTCCT GCTGCAGGTG CAATCCATGG
RFL              CCATCTGTAC GAGCAGTGCC GCCTCTTCCT GCTGCAGGTG CAATCCATGG
LtLFY            CCATCTCTAC GAGCAGTGCC GCCTCTTCCT GCTCCAGGTG CAGTCCATGG
ZFL1             CCACCTGTAC GAGCAGTGCC GCGTCTTCCT GCTCCAGGTG CAGTCCATCG
ZFL2             CCATCTGTAC GAGCAGTGCC GCGTCTTCCT GCTGCAGGTG CAGTCCCTTG
HLY              TCACCTCTAC GAGGAGTGCC GCGGGTTCTT GGCCCAGGTC CAGGCGCTCG
                   *  * *** **  * ***       **  *     ** **  **  *  * *

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
OrcLFY           CCAAGGAGCG CGGGGACAAG TGCCCAACTA AGGTGACGAA CCTGGTATTC
AtLFY            CTAAAGACCG TGGCGAAAAA TGCCCCACCA AGGTGACGAA CCAAGTATTC
PFL              CCAAGGAGCG AGGGGACAAG TGTCCGACCA AGGTTACAAA CCAAGTGTTC
OSL              CTAAGCTGCA TGGACACAAG TCCCCAACCA AGGTGACGAA CCAGGTGTTC
RFL              CTAAGCTGCA TGGACACAAG TCCCCAACCA AGGTGACGAA CCAGGTGTTC
LtLFY            CCAAGCTGCA TGGCCACAAG TCTCCAACCA AGGTGACGAA CCAGGTGTTC
ZFL1             CTAAGCTGGG CGGCCACAAA TCCCCTACCA AGGTGACCAA CCAGGTGTTC
ZFL2             CTAAGCTGGG CGGCCACAAG TCCCCTACAA AGGTGACCAA CCAGGTGTTC
HLY              CCAAGGAGCG CGGCGACAAG TGCCCGACCA AGGTAACGAA TCAAGTGTTC
                 * **        **  * **  *  ** ** * **** ** **  *  ** ***
```

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
OrcLFY           CGATATGCGA AGAAGAAAGT GGGAGCAAGC TACATCAATA AGCCGAAGAT
AtLFY            AGGTACGCGA AGAA---ATC AGGAGCGAGT TACATAAACA AGCCTAAAAT
PFL              CGATACGCGA AGAA---AGT CGGTGCRCAC TACATCAACA AGCCGAAGAT
OSL              CGGTACGCGA AGAA---GGT CGGGGCGAGC TACATCAACA AGCCCAAGAT
RFL              CGGTACGCGA AGAA---GGT CGGGGCGAGC TACATCAACA AGCCCAAGAT
LtLFY            AGGTACGCGA GCAA---GGT GGGGGCGAGC TACATCAACA AGCCCAAGAT
ZFL1             CGGTACGCGA ACAA---GTG CGGGGCGAGC TACATCAACA AGCCCAAGAT
ZFL2             CGGTACGCCA AGAA---GTG CGGCGCGAGC TACATCAACA AGCCCAAGAT
HLY              CGATACGCGA AGAA---GGT GAGGGCGTCG TACATAAACA AGCCCAAGAT
                  * ** ** *   **         * **     ***** ** * **** ** **

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
OrcLFY           GAGGCACTAC GTGCACTGCT ACGCGCTCCA CGTGCTGGAC GAAGATGCGT
AtLFY            GCGACACTAC GTTCACTGTT ACGCTCTCCA CTGCCTAGAC GAAGAAGCTT
PFL              GCGSCACTAC GTCCACTGCT ATGCGYTGCA CTGCCTCGAC GAGGCWGCCT
OSL              GCGGCACTAC GTGCACTGCT ACGCGCTGCA CTGCCTGGAC GAGGAGGCGT
RFL              GCGGCACTAC GTGCACTGCT ACGCGCTGCA CTGCCTGGAC GAGGAGGCGT
LtLFY            GCGCCACTAC GTGCACTGCT ACGCGCTGCA CTGCCTCGAC CAGGAGGCCT
ZFL1             GCGGCACTAC GTGCACTGCT ACGCGCTGCA CTGCCTGGAC GAGGAGGCCT
ZFL2             GCGGCACTAC GTGCACTGCT ACGCGCTGCA CTGCCTGGAC GAGGATGCCT
HLY              GAGGCACTAC GTCCACTGCT ACGCCCTCCA CTGCCTCGAC CAGCAGGCCT
                 * * ****** ** ***** * * **  * ** *   ** ***  *    ** *

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
OrcLFY           CCAACTCCCT CCGGCGG-GT GTTCAAGGAA CGCGGGGAGA ACGTCGGCGC
AtLFY            CAAATGCTCT CAGAAGA-GC GTTTAAAGAA CGCGGTGAGA ACGTTGGCTC
PFL              CTGACTCGCT GCGCAGTTGA GTTCAAGCAG CGGGGGGAGA ACGTTGGGTC
OSL              CGGACGCGCT GCGGCGC-GC CTACAAGGCC CGCGGCGAGA ACGTGGGGGC
RFL              CGGACGCGCT GCGGCGC-GC CTACAAGGCC CGCGGCGAGA ACGTGGGGGC
LtLFY            CCGACGCGCT GCGCCGC-GC GTACAAGGCC CGCGGCGAGA ACGTCGGCGC
ZFL1             CCAACGCGCT GCGCCGG-GC GTACAAGTCC CGCGGCGAGA ACGTGGGCGC
ZFL2             CCAACGCGCT GCGCCGG-GC GTACAAGGCC CGTGGCGAGA ACGTCGGTGC
HLY              CCGACGCGCT CCGGCGT-GA GTACAAGGCG CGCGGCGAGA ACGTCGGCGC
                 *  *  * **   *  *  *   *  **     ** ** **** **** **  *
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
OrcLFY          CTGGCGGCTC GCCTGCTACA AGCCTCTGGT GGCCATCTCC GCCTCCCACA
AtLFY           ATGGCGTCAG GCTTGTTACA AGCCACTTGT GAACATCGCT TGTCGTCATG
PFL             GTGGCTCCAG GCCTGCTACC AGCCCCTCGT GGAYATTWCG GCYCGCCACG
OSL             GTGGAGGCAG GCCTGCTACG CGCCGCTCGT CGACATCTCC GCGCGCCACG
RFL             GTGGAGGCAG GCCTGCTACG CGCCGCTCGT CGACATCTCC GCGCGCCACG
LtLFY           CTGGAGGCAG GCATGCTACG CGCCGCTCGT CGACATCGCC GCCGGCCACG
ZFL1            CTGGAGGCAG GCCTGCTACG CGCCGCTCGT CGAGATCGCC GCGCGCCACG
ZFL2            CTGGAGGCAG GCCTGCTACG CGCCGCTCGT CGAGATCGCC GCGCGCCACG
HLY             GTGGCGGCAG GCTTGCTACC AGCCTCTGGT GGCCATCTCG GCGCGGCACG
                 ***   *   ** ** ***   *** ** **     **  *        **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
OrcLFY          GCTTCGACAT CGACGCCGTT TTCAACGCGC ATCCCCGCCT CTCCATCTGG
AtLFY           GCTGGGATAT AGACGCCGTC TTTAACGCTC ATCCTCGTCT CTCTATTTGG
PFL             GCTGGGACAT CGATGCTGTC TTCAGCGCCC ACCCTCGGCT CTCCATCTGG
OSL             GATTCGACAT CGACGCCGTC TTCGCCGCGC ACCCGCGCCT CGCCATCTGG
RFL             GATTCGACAT CGACGCCGTC TTCGCCGCGC ACCCGCGCCT CGCCATCTGG
LtLFY           GCTTCGACGT CGACGCCGTC TTCGCCGCGC ACCCGCGACT CGCCATCTGG
ZFL1            GCTTCGACAT TGACGCCGTC TTCGCCGCGC ACCCGCGCCT CGCCGTCTGG
ZFL2            GCTTCGACAT CGACGCCGTC TTCGCCGCGC ACCCGCGCCT CACCATCTGG
HLY             CCTGGGACAT CGACGCCGTC TTCAACGCCC ACCCCCGCCT ATCCGTCTGG
                  *  **  *  ** ** **  **   *** * * ** ** **   *  * ***

                ....|....| .
                       410
OrcLFY          TACGTCCCCA C
AtLFY           TATGTTCCAA C
PFL             TATGTCCCCA C
OSL             TACGTGCCCA C
RFL             TACGTGCCCA C
LtLFY           TACGTGCCCA C
ZFL1            TACGTGCCCA C
ZFL2            TACGTGCCCA C
HLY             TACGTCCCCA C
                ** ** ** * *
```

Figure C.3: Partial amino acid sequence comparison of *FLO / LFY* homologues

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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             10         20         30         40         50
AtLFY               EPGEVARGKK NGLDYLFHLY EQCREFLLQV QTIAKDRGEK CPTKVTNQVF
PFL                 EPGEVARAKK NGLDY-FHLY EQCGE-LAQV QSLAKERGDK CPTKVTNQVF
OSL                 EPGEVARAKK NGLDYLFHLY EQCRLFLLQV QSMAKLHGHK SPTKVTNQVF
RFL                 EPGEVARAKK NGLDYLFHLY EQCRLFLLQV QSMAKLHGHK SPTKVTNQVF
LtLFY               EPGEVARAKK NGLDYLFHLY EQCRLFLLQV QSMAKLHGHK SPTKVTNQVF
ZFL1                EPGEVARAKK NGLDYLFHLY EQCRVFLLQV QSIAKLGGHK SPTKVTNQVF
ZFL2                EPGEVARAKK NGLDYLFHLY EQCRVFLLQV QSLAKLGGHK SPTKVTNQVF
OrcLFY              EPGEVARAKK NGLDYLFNLY EQCHEFLLQV QSVAKERGDK CPTKVTNLVF
HLY                 ESGEVARARK NGLDYLFHLY EECRGFLAQV QALAKERGDK CPTKVTNQVF
                    *.*****.:* ***** *:** *:*   * ** *::**  *.* .****** **

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                             60         70         80         90        100
AtLFY               RYAKK-SGAS YINKPKMRHY VHCYALHCLD EEASNALRRA FKERGENVGS
PFL                 RYAKK-VG-H YINKPKM-HY VHCYA-HCLD -EASDSLRS- ----------
OSL                 RYAKK-VGAS YINKPKMRHY VHCYALHCLD EEASDALRRA YKARGENVGA
RFL                 RYAKK-VGAS YINKPKMRHY VHCYALHCLD EEASDALRRA YKARGENVGA
LtLFY               RYASK-VGAS YINKPKMRHY VHCYALHCLD QEASDALRRA YKARGENVGA
ZFL1                RYANK-CGAS YINKPKMRHY VHCYALHCLD EEASNALRRA YKSRGENVGA
ZFL2                RYAKK-CGAS YINKPKMRHY VHCYALHCLD EDASNALRRA YKARGENVGA
OrcLFY              RYAKKKVGAS YINKPKMRHY VHCYALHVLD EDASNSLRRV FKERGENVGA
HLY                 RYAKK-VRAS YINKPKMRHY VHCYALHCLD QQASDALRRE YKARGENVGA
                    ***.*       ******* ** ***** * **  :**::**

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            110        120        130        140        150
AtLFY               WRQACYKPLV NIACRHGWDI DAVFNAHPRL SIWYVPTKLR QLCHLERNNA
PFL                 ---------V QAAGGERWVV APGLLPAPRG -----GPRLG HRCCLQRP--
OSL                 WRQACYAPLV DISARHGFDI DAVFAAHPRL AIWYVPTRLR QLCHQARSSH
RFL                 WRQACYAPLV DISARHGFDI DAVFAAHPRL AIWYVPTRLR QLCHQARSSH
LtLFY               WRQACYAPLV DIAAGHGFDV DAVFAAHPRL AIWYVPTRLR QLCHQARSAH
ZFL1                WRQACYAPLV EIAARHGFDI DAVFAAHPRL AVWYVPTRLR QLCHQARGSH
ZFL2                WRQACYAPLV EIAARHGFDI DAVFAAHPRL TIWYVPTRLR QLCHQARGSH
OrcLFY              WRLACYKPLV AISASHSFDI DAVFNAHPRL SIWYVPTKLR QLCHLARSST
HLY                 WRQACYQPLV AISARHAWDI DAVFNAHPRL SVWYVPTKLR HLCHLARSNA
                            *   :  . : :  . : . **        .:*  : *   *
```

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                 ....|....| ....|....| ....|...
                         160        170
AtLFY            VAAAAALVG- ---------- --GISCTG
PFL              -PSALHLV-- ---------- -----CPH
OSL              AAAAA----- ---------- ----ALPP
RFL              AAAAA----- ---------- ----ALPP
LtLFY            EAAAANANA- ---------- --NGAMPP
ZFL1             AHAAA----- ---------- ----GLPP
ZFL2             AHAAA----- ---------- ----GLPP
OrcLFY           SQLPQSSPPT TTTLSTHFPK AFKTQLQQ
HLY              SAAASHCS-- ---------- ----VLPS
                    .
```

Figure C.4: Partial cDNA comparison of actin homologues

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
S.italica       CTCCTTCACC ACCACTGCCG AGCGGGAAAT TGTAAGAGAC ATCAAGGAGA
Z.mays          CTCCTTCACC ACGACCGCCG AGCGAGAGAT TGTCAGGGAC ATCAAGGAAA
L.perenne       CTCCTTCACC ACCACCGCTG AGCGGGAAAT TGTAAGGGAC ATCAAGGAGA
H.vulgare       CTCCTTCACA ACCTCAGCTG AGCGGGAAAT TGTAAGGGAC ATCAAGGAGA
P.cookianu      ATTCTTCACC ACCACAGCCG AGCGGGAAAT TGTGAGAGAC ATAAAGGAGA
M.denudata      CTCGTTCACC ACCACTGCTG AGCGGGAAAT TGTCCGGGAT ATGAAAGAGA
A.thaliana      CTCATTCACT ACCTCAGCAG AGCGTGAAAT CGTAAGGGAT GTGAAAGAGA
                 *  *****  **  * ** * **** ** **  **  * **   * ** ** *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
S.italica       AGCTCGCCTA TGTGGCACTT GACTATGAGC AGGAGCTTGA GACTGCTAAG
Z.mays          AGCTTGCCTA CGTTGCTCTT GATTATGAAC AGGAGCTGGA GACTGCCAAG
L.perenne       AGCTTGCATA TGTGGCTCTT GACTATGAAC AGGAGCTGGA GAATGCCAAG
H.vulgare       AGCTTGCGTA CGTTGCCCTT GATTATGAAC AGGAGCTGGA GACTGCCAAG
P.cookianu      AGCTTGCATA CW-TGCCCTT GATTAAGARC CRGAGCTCGA GACCGCCAAG
M.denudata      AGCTTGCCTA TGTGGCCCTT GACTATGAAC AAGAGCTTGA GACTGCCAAG
A.thaliana      AACTTGCTTA CATAGCACTT GACTACGAGC AAGAGATGGA AACAGCAAAC
                * ** ** **     ** *** ** ** ** *   *** * **  *  ** **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
S.italica       ACCAGCTCCT CTGTGGAGAA GAGCTATGAG CTGCCTGATG GGCAGGTGAT
Z.mays          AGCAGCTCAT CTGTGGAGAA GAGCTACGAG CTGCCTGATG GCCAGGTGAT
L.perenne       AGCAGCTCCT CTGTGGAGAA GAGCTACGAG CTGCCCGATG GGCAGGTGAT
H.vulgare       AACAGCTCCT CAGTTGAGAA GAGCTACGAG CTTCCTGATG GTCAGGTGAT
P.cookianu      AGCAGCTCCT CTGTGGAGAA GAGCTAYGAR CTGCCTGACG GTCAGGTTAT
M.denudata      AGCAGCTCCT CGATTGAGAA GAGCTATGAG CTGCCTGACG GGCAGGTGAT
A.thaliana      ACAAGCTCAT CCGTTGAGAA GAGCTATGAG TTGCCTGATG GACAGGTGAT
                *  ***** * *  * ***** ****** **   * ** ** * * ***** **
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
S.italica       CACCATCGGG GCAGAGAGGT TCAGATGCCC TGAGGTCCTT TTCCAGCCTT
Z.mays          CACCATTGGG GCAGAGAGGT TCAGATGCCC TGAGGTCCTC TTCCAGCCTT
L.perenne       CACCATTGGG GCTGAGAGGT TCCGTTGCCC TGAGGTCCTT TTCCAGCCAT
H.vulgare       CACGATTGGC GCAGAGAGGT TCAGGTGCCC TGAGGTCCTC TTCCAGCCAT
P.cookianu      CACAATTGGR GCWGAGAGAT TCAGATGTCC TGAGGTTCTY TTCCAACCWT
M.denudata      CACCATTGGA GCTGAGAGGT TCCGTTGCCC AGAAGTGCTC TTCCAGCCTT
A.thaliana      CACCATTGGA GGAGAGAGAT TCCGCTGCCC GGAGGTTCTG TTCCAACCGT
                *** ** **  *  ***** * ** * ** **  ** ** **  ***** ** *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
S.italica       CATTCATTGG TATGGAGTCG CCTGGAATCC ATGAGACCAC CTACAACTCT
Z.mays          CCTTCATTGG TATGGAAGCT CCTGGCATCC ATGAGACCAC CTACAACTCC
L.perenne       CTTTCATTGG CATGGAAGCT GCTGGAATCC ATGAGACCAC CTACAACTCT
H.vulgare       CCATGATCGG CATGGAGTCT TCTGGAATCC ACGAGACGAC CTACAACTCC
P.cookianu      CTTTGATTGG AATGGAAGCT GCTGGKATCC ATGAGACCAC YTACAACTCC
M.denudata      CTCTCATTGG GATGGAAGCT GCTGGTATCC ACGAGACCAC TTACAACTCG
A.thaliana      CTTTGGTGGG AATGGAAGCT GCTGGTATTC ACGAGACCAC TTACAATTCC
                *  *  * **  *****  *   **** ** * * ***** **  ***** **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
S.italica       ATCATGAAGT GTGATGTGGA TATTAGGAAG GACCTCTATG GTAACATTGT
Z.mays          ATCATGAAGT GCGACGTCGA TATCAGGAAG GACTTGTATG GTAACATTGT
L.perenne       ATCATGAAGT GTGATGTGGA CATCAGGAAG GACCTCTACG GTAACATTGT
H.vulgare       ATCATGAAGT GTGACGTGGA TATCAGGAAG GACTTGTATG GAAACATCGT
P.cookianu      ATYATGAAGT GTGAYGTGGA TATCAGGAAG GATTTGTATG GAAAYATTGT
M.denudata      ATCATGAAGT GTGATGTCGA TATCAGGAAG GATTTGTATG GCAACATCGT
A.thaliana      ATCATGAAGT GTGATGTGGA TATCAGGAAG GATCTGTATG GAAACATTGT
                ** ******* * ** ** **  ** ****** **  * ** * * ** ** **
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```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
S.italica       GCTCAGCGGT GGCTCAACCA TGTTCCCTGG ---TATTGCT GACCGCATGA
Z.mays          GCTCAGTGGT GGCACGACCA TGTTCCCTGG ---TATTGCT GACCGTATGA
L.perenne       GCTCAGTGGT GGCTCAACTA TGTTCCCTGG ---TATTGCT GACCGCATGA
H.vulgare       GCTCAGTGGT GGCACAACTA TGTTCCCAGG ---TATCGCT GACCGTATGA
P.cookianu      GCTYAGTGGA GGGTCTACYC TGTTCCCCGG GRAWATTGCT GATCGWATGA
M.denudata      GCTTAGTGGT GGGTCTACCA TGTTCCCGGG ---CATTGCT GATCGTATGA
A.thaliana      GCTCAGTGGT GGAACCACCA TGTTCCCTGG ---AATTGCT GATAGGATGA
                *** ** **  **  * **   ******* **     ** *** **  * ****

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
S.italica       GCAAGGAGAT CACTGCCCTT GCACCAAGCA GTATGAAGAT TAAGGTGGTG
Z.mays          GCAAGGAGAT CACTGCCCTT GCGCCGAGCA GCATGAAAAT CAAGGTGGTG
L.perenne       GCAAGGAGAT CACTGCCCTT GCACCAAGCA GCATGAAGAT CAAGGTTGTT
H.vulgare       GCAAGGAGAT CACCGCCCTT GCTCCGAGCA GCATGAAGAT CAAGGTCGTC
P.cookianu      GCAAAGAGAT CACTGCKCTT GCACCYAGCA GCATGAAGAT TAAGGTGGTT
M.denudata      GCAAGGAGAT CACTGCCCTT GCTCCTAGCA GCATGAACAT CAAGGTGGTG
A.thaliana      GCAAAGAGAT CACTGCTTTG GCTCCAAGCA GCATGAAGAT TAAGGTGGTT
                **** ***** *** **  *  ** ** **** * ***** **  ***** **

                ....|....| ....|....| ....|....| ....|....| .
                       410        420        430        440
S.italica       GCACCACCTG AGAGGAAATA CAGTGTCTGG ATTGGAGGGT C
Z.mays          GCACCGCCTG AGAGGAAATA CAGTGTCTGG ATAGGAGGAT C
L.perenne       GCTCCACCCG AGAGGAAGTA CAGTGTCTGG ATTGGAGGCT C
H.vulgare       GCTCCACCTG AGAGGAAGTA CAGTGTCTGG ATCGGAGGGT C
P.cookianu      GCTCCYCCKG AG-SGAAGTA CAGTGTCTGG GATTCGGGTT C
M.denudata      GCTCCACCAG AGAGGAAGTA CAGTGTCTGG ATTGGAGGGT C
A.thaliana      GCTCCACCAG AGAGGAAGTA CAGTGTCTGG ATTGGAGGCT C
                ** ** ** * **  *** ** **********       ** * *
```

Appendix D: Primer sequences used in PCR and Real Time PCR reactions (Chapter 3)

JHLFYF1: 5'CTTCACGGCGAGCACGCT 3'

JHLFYR: 5'GGGCTTGTTGATGTAGCTCGC 3'

LFYF1B: 5'CAAGGCTGCHRTHMGRGC 3'

LFYF2: 5'CAGAGCCWGGDGARSYKGC 3'

LFYR1: 5'GGTARGTNCCDACKAAGC 3'

LFYR2: 5'GKGYSACTARGTHCARTGCTATG 3'

LFYLF: 5'GCGATTCCAAGCCGAAGATGAGGCACATAC 3'

LFYLR: 5'CATCGACGCTATCTTCAATGC 3'

Figure D.1: Sequences of primers used to isolate *PFL* cDNA.

Actin forward: 5' GCGAATTCTTCACCACYACHGCYGARCG 3'

Actin reverse: 5' GCGGATCCCCRATCCARACACTGTAYTTC 3'

Figure D.2: Sequences of primers used to isolate *P. cookianum* actin cDNA.

PFL forward: 5' AAACCAAGTGTTCCGATACGC 3'

PFL reverse: 5' TACCAGATGGAGAGAGCCGAGG 3'

Actin forward: 5' CTTCACCACCACAGCCGAG 3'

Actin reverse: 5' GAACCCGAATCCCAGACACT 3'

Figure D.3: Sequences of *PFL* and actin specific primers used in Real Time reverse transcriptase-PCR