A Molecular Analysis of Flowering in *Metrosideros*

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Abstract

A calendar of floral and vegetative developmental events has been previously determined for the woody perennial *Metrosideros excelsa*. *M. excelsa* flowers just once a year in summer and bud development has been related to seasonality and gene expression. *M. collina* cv. Tahiti is closely related to *M. excelsa* but appeared to flower sporadically through out the year. Using histology, the bud development of *M. collina* has been analysed and a developmental sequence determined. Periodicity of bud development has also been observed by obtaining bud measurements. While it is difficult to relate bud size to developmental phase or determine whether the bud is vegetative or floral, it appears that *M. collina* goes through two periods of bud development in one year and that flowering may be related to warm temperature.

The genetic interactions between pathways controlling floral meristem development have been analysed in the herbaceous annual model plant *Arabidopsis thaliana*. In *Arabidopsis*, expression of the floral meristem identity gene, *LEAFY* (*LFY*), is regulated directly by the florally promotive gibberellin and photoperiodic pathways. *LFY* expression in *Arabidopsis* was upregulated in response to gibberellin application, which hastened the transition from vegetative to floral meristem. Another floral meristem identity gene, *APETALA1* (*AP1*) is upregulated by *LFY* to promote formation of the floral meristem. However, exogenous application of gibberellic acid (GA_3) to woody perennial trees, including *M. collina*, inhibits flowering. Due to the differences between *Arabidopsis* and woody perennial species, it is important to determine if the *Arabidopsis* model is a useful tool to analyse flowering in *Metrosideros* species. *LFY* and *AP1* homologues were isolated previously from *M. excelsa*, and from *M. collina* in this project. The responsiveness of *MEL* (*M. excelsa LFY*-like) and *MESAP1* (*M. excelsa AP1*-like) and *MCL* (*M. collina LFY*-like) and *MTAP1* (*M. collina AP1*-like) to GA_3 and other growth regulators and hormones was examined in juvenile and adult *Metrosideros*. *MEL* or *MCL* expression was not detected in juvenile or adult GA_3-treated *Metrosideros*, which supports GA_3 inhibition of flowering in perennials. It appears that the gibberellin component of the *Arabidopsis* model does not represent appropriately, the response of perennial woody species to gibberellins in terms of flowering.

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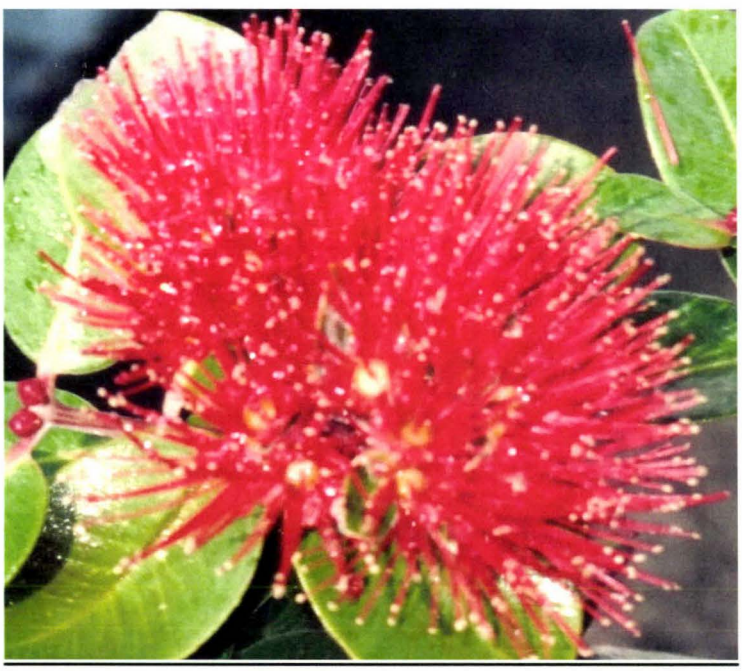

M. collina cv. Tahiti

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CHAPTER ONE

Introduction

1.1 Overview

This project seeks to analyse the phenomenon of flowering in *Metrosideros* by investigating genes involved in three of the pathways implicated in the regulation of floral induction and development in *Arabidopsis* – the vernalisation, the gibberellin (GA) and the photoperiodic pathways.

This research was originally initiated in *Metrosideros excelsa* as it has potential as a native ornamental in the floriculture industry. However, flowering in *Metrosideros* species is not consistent (Clemens *et al.,* 1995). Although *M. excelsa* and *M. collina* are closely related, *M. collina* produces flowers irregularly and appears not to have a specific requirement for a floral inductive signal [J. Clemens, pers. comm.], while *M. excelsa* requires winter chilling of floral initials, which are formed in the autumn (Sreekantan *et al.*, 2004).

The key genes investigated were the flowering time gene, *FLOWERING LOCUS C* (*FLC*), and two floral meristem identity genes, *LEAFY* (*LFY*) and *APETALA1* (*AP1*). In *Arabidopsis* the expression of *FLC* is upregulated by *FRIGIDA (FRI)* to repress flowering and downregulated by vernalisation to induce floral initiation. *FLC* is also important because it appears to exert transcriptional control onto downstream floral integrator genes *SUPPRESSOR OF CONSTANS 1* (*SOC1*) and *FT*. These genes appear to promote flowering with *LFY* at the points where multiple pathways integrate (Araki, 2001). *LFY* and *AP1* are important for floral meristem development and *LFY* also has involvement in the photoperiodic and gibberellin promotive

pathways. LFY upregulates *AP1* expression in *Arabidopsis* to indicate the beginning of floral initiation as the plant is deemed competent to flower (Hempel *et al.*, 1997). It is possible that floral promotion is able to operate via two cross-linked routes, one through the flowering time genes *SOC1* and *FT*, and the other through *LFY*.

This project utilised the model of *Arabidopsis* floral initiation and development as the basis to further understand the genetic and environmental interactions controlling floral development in *Metrosideros* species.

1.2 Pathways that regulate flowering

Arabidopsis thaliana is an herbaceous, annual plant that serves as a model organism for the transition to flowering. *Arabidopsis* is a facultative long day plant that grows as a rosette until long-day inductive conditions occur. As with other annual plants, floral initiation is followed immediately by floral development and flower emergence (Battey, 2000). Important stimuli include photoperiod and temperature, as well as endogenous signals such as hormones. There are many ecotypes of *Arabidopsis* and these respond differently to environmental signals during development. Some *Arabidopsis* ecotypes adopt a winter annual strategy. This allows the plant to establish itself in the autumn and flower in more favourable (warm temperature and long photoperiod) conditions (Battey, 2000). Inductive conditions signal to the plant to flower via promotive and repressive factors in signalling pathways. Some of the loci in these pathways show epistatic interactions, while others have additive effects. This indicates the action of multiple pathways operating during the transition to flowering (Koornneef *et al.*, 1998a; Koornneef *et al.*, 1998b).

The genetic interactions between pathways controlling floral meristem development have been analysed in *Arabidopsis*. Mutant analyses have identified about 80 loci involved in a complex genetic network (Koornneef *et al.*, 1998a; Koornneef *et al.*,

1998b; Levy and Dean, 1998) that monitors plant development and environmental conditions.

Genes have been isolated from multiple input pathways that regulate the transition from the vegetative to the reproductive phase (**Figure 1.1**). The input genes are flowering time genes that regulate flowering by promoting or repressing the activity of floral pathway integrators such as *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*). These floral pathway integrators cause upregulation of the floral meristem identity genes, for example *APETALA1 (AP1)*, *CAULIFLOWER* (*CAL*) and *LFY*. Floral meristem identity genes promote the formation of the floral meristem (Simpson and Dean, 2002). The genes in the input pathways are unlikely to act independently as their signal appears to be integrated by one of the floral pathway integrator genes. The flowering time genes and floral meristem identity genes have been placed into specific pathways that lead to floral induction. These pathways are under specific regulation from photoperiod, temperature, gibberellins, nutrients, and internal developmental cues. Different parts of the plant perceive the environmental stimuli. For example, the shoot apex perceives vernalisation whereas mature leaves perceive changes in photoperiod (Bernier *et al.*, 1993). Floral meristem identity genes promote formation of the floral meristem. Floral organ identity genes, for example, *AP1*, *APETALA2* (*AP2*), *APETALA3* (*AP3*), *PISTILLATA* (*PI*) and *SEPALLATA1-3* (*SEP1-3*), direct the formation of the organs in the four floral whorls (Mandel and Yanofsky, 1995).

The amount of evidence towards alternative signalling pathways to promote and repress flowering has grown exponentially in recent years.

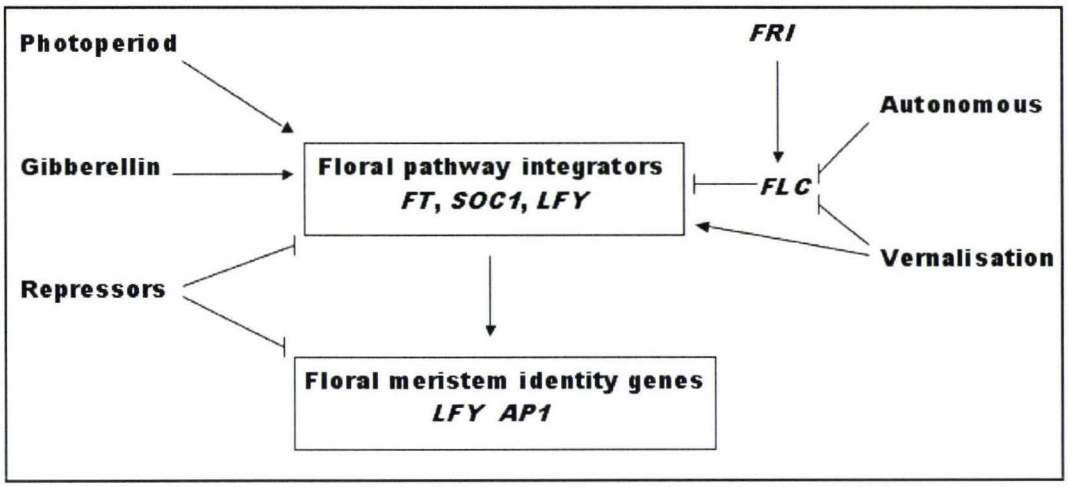


Figure 1.1 The input pathways that regulate the transition to flowering in Arabidopsis. Modified from simpson and Dean, (2003).

1.2.1 Regulation of flowering by photoperiod

The photoperiodic pathway mediates the response of the plant to day length initiated through genes that encode photoreceptors (**Figure 1.2**). Depending upon its wavelength, light is received by phytochrome and cryptochrome proteins (Beveridge *et al.,* 2003). The phytochrome proteins, PHYTOCHROME B (PHYB) and PHYTOCHROME A (PHYA), mediate the reception of red and far red light signals during flowering, respectively. The cryptochrome proteins CRYPTOCHROME 1 (CRY1) and CRYPTOCHROME 2 (CRY2) receive blue light. PHYA, CRY1 and CRY2 regulate the protein components of the circadian clock in *Arabidopsis*, ELF3, TOC1, and LHY. ZTL, FKF, and LPK2, putative photoreceptors, are floral promoters, while LHY, CCA1, TOC1, ELF3, and ELF4 are involved in floral repression (Beveridge *et al.,* 2003). The expression of these genes regulates the oscillations of *GIGANTEA* (*GI*), *CONSTANS* (*CO*), *TERMINAL FLOWER 1* (*TFL1*) and *FT*, genes specific for the transition from vegetative to floral development. *CO* appears to be the link between the circadian oscillator and flowering time (Simpson and Dean, 2002). Yanovsky and Kay (2002) demonstrated that CO coordinates integration of temporal information and signals from photoreceptors, as light perceived through *cry2* or *phyA* is required for CO activation of *FT*. *TFL1* also displays a photoperiod dependent regulation in *Arabidopsis*, but not in *Antirrhinum* (Bradley *et al.*, 1996).

The transcriptional activation of *LFY* was observed in *Arabidopsis* through the expression of *CO* (Araki, 2001). While *LFY* is involved in mediating the long day promotive pathway (Blazquez *et al.,* 1997), the short day pathway also appears to involve *LFY* regulation and interacts with the gibberellin (GA) pathway. *LFY* upregulation is abolished in the *ga1-3* (gibberellin requiring) mutant under treatment with short days. Endogenous GA levels are severely reduced in these mutant plants but exogenous GA application can rescue this phenotype (Blazquez *et al.,* 1998; Wilson *et al.,* 1992), and allow flowering under short days.

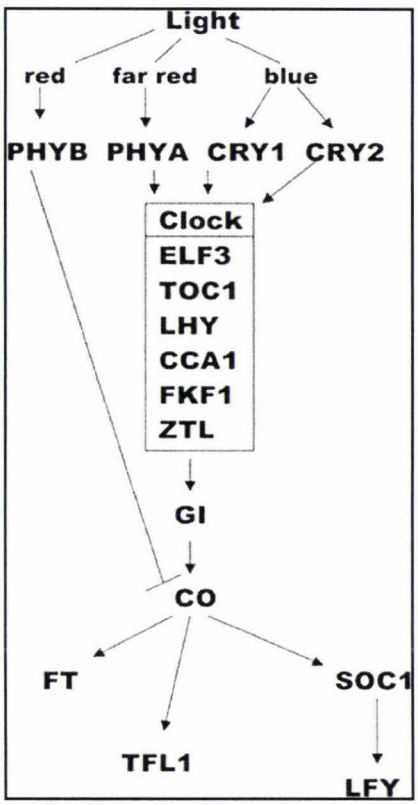


Figure 1.2 The interactions of proteins involved in regulating the photoperiodic pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

Further photoperiodic experiments with the GA-deficient mutant, *ga1-3*, have revealed that failure to flower under short days correlated with an absence of *LFY* promoter activity (Blazquez *et al.*, 1998). *LFY* promoter deletion analyses in *Arabidopsis* have found that the photoperiodic response and the GA response are mediated by different *cis* elements in the *LFY* promoter (Blazquez and Weigel, 2000).

The PHYTOCHROME B (PHYB) protein in *Arabidopsis* functions as part of the shade-avoidance mechanism of a plant, and regulates genes responsive to red light. Under both long and short photoperiods, the null *phyb* mutation causes earlier flowering and hypocotyl elongation (Reed *et al.*, 1993). Phytochromes regulate seed germination, cell expansion and flowering, which are also regulated by GAs. It was found that GAs and PHYB have opposing roles in floral development. GAs promote flowering in *Arabidopsis* especially under short days, while *PHYB* delays flowering (Blazquez and Weigel, 1999). It is thought that *PHYB* can regulate flowering via a GA-independent mechanism.

It appears that differences in floral promotion have evolved between short day- and long day-responsive species with respect to involvement by gibberellins and daylength.

1.2.2 Regulation of flowering by gibberellins

While in *Arabidopsis* there appears to be a link between gibberellins and the *LFY* promoter (Blazquez and Weigel, 2000), for gibberellins to have a role in floral evocation, King *et al.* (2001) suggest that they must conform to the following criteria. In the shoot apex, long day exposure should increase levels of florally active gibberellins. Inhibitors of gibberellin biosynthesis should block flowering, and there should be molecular *and* biochemical links between floral induction and gibberellins at the apex.

King *et al.* (2003) found that florigenic GAs, GA_5 and GA_6, were strong contenders

as floral stimuli under long day inductive conditions in the long day grass *Lolium temulentum*. These florigenic GAs were found to reach a concentration in the excised shoot apex in vitro that was considered to be florally inductive.

There have been several studies in perennial species assessing the effect of GAs on delaying flowering in horticultural crops until favourable conditions come about (Looney *et al.*, 1985). In the past, grape plants were sprayed with GA_3 and a GA_{4+7} mixture to repress floral initiation as it was observed that GA application inhibited flowering of fruit trees (Bradley and Crane, 1960). But this generalisation had to be re-examined once it became clear that some GAs had a promotive effect on floral transition (Looney *et al.*, 1985).

Exogenous applications of GA_3 to the perennial *M. collina* resulted in delayed flowering and inhibition of bud break (Clemens *et al.*, 1995). This is indicative of GA having an inhibitory effect on flowering in woody perennial species. In *M. excelsa*, exogenous application of GA_3 to juvenile plants was sufficient to upregulate *MEL* expression but not to provoke flowering (Sreekantan *et al.*, 2004). However, *MESAP1* expression was not detected in juvenile *M. excelsa* plants upon GA_3 application (Sreekantan *et al.*, 2004). This indicates that the transition to floral development may be different in herbaceous annual plants (*Arabidopsis*) and woody perennial trees (*Metrosideros*).

Growth retardants have been used to promote flowering in *Eucalyptus*. A single application of the gibberellin biosynthesis inhibitor, paclobutrazol, decreased the concentrations of endogenous GA in *Eucalyptus nitens* before flowering (Moncur *et al.*, 1994). It appears that cold temperatures are also required for floral promotion in *Eucalyptus* as paclobutrazol-treated trees, kept outside in winter in Canberra, Australia, produced flower buds. Paclobutrazol-treated trees kept in a warm glasshouse did not produce flower buds. It appears to be a combination of lower

temperatures and decreased endogenous GA levels that enhance reproductive development. If GA was applied to trees that had been treated with paclobutrazol, the GA reduced the reproductive enhancement produced by the paclobutrazol (Moncur and Hasan, 1994).

Boss and Thomas (2002) presented genetic evidence that flowering in grapevine is inhibited by GAs. The *vvgai1* dwarf mutant is derived from the L1 cell layer of the Pinot Meunier cultivar. The *VvGAI1* (*Vitis vinifera GA INSENSITIVE 1*) gene is a homologue of the *Arabidopsis GAI* (*GA Insensitive*) gene that is normally involved in GA signalling. The tendrils of the mutant plants are converted to inflorescence, which suggests that in WT plants GA inhibits floral development, by converting inflorescence to tendrils. VvGAI1 proteins in WT plants are thought to function as growth repressors, whose action is repressed by GA action (Boss *et al.*, 2003).

It has been suggested that GAs do not totally inhibit flowering, and that *VvGAI1* action to convert floral meristems to tendrils follows the production of uncommitted primordia (Boss and Thomas, 2002).

It has been observed that GA insensitive dwarf mutants such as *gai* in *Arabidopsis* accumulate GA_1 and GA_4, non-florigenic GAs (Talon *et al.*, (1990). In the *vvgai* dwarf mutant, the amount of GA_1 is 4-fold higher than in control plants, and GA_4 is 12-fold higher than in control plants (Boss and Thomas, 2002). King and Evans (2003) suggest in *L. temulentum*, that although GA_1 and GA_4 are responsible for stem elongation, they may be degraded before reaching the shoot apex and as a consequence be inactive for floral promotion.

The grass, *Lolium temulentum*, flowers in response to a single long day inductive signal. Unlike *Arabidopsis*, grasses have a limited lengthening of the stem before flowering. It is the florigenic GA_5 and GA_6, which are less effective for stem

elongation, that are found in the shoot apex (King and Evans, 2003). King *et al.* (2001) found that the concentration of GA_5 in the shoot apex doubled the day after inductive conditions.

Arabidopsis plants that are responsive to short days require gibberellins to flower. This is unlike *Metrosideros* (Clemens *et al.*, 1995) and *Eucalyptus* (Moncur and Hasan, 1994), another member of the Myrtaceae family, where GAs appear to inhibit flowering.

There is little known about the early events of GA signaling in floral promotion, but LFY has been determined as one of the factors that integrate the signal (**Figure 1.3**). Research into gene regulation by gibberellins has shown that *HvGAMYB* encodes an activator of transcription that specifically binds to a GA response element in the α-amylase promoter in barley aleurone cells (Gubler *et al.*, 1995). Research into GA signalling in *Arabidopsis* discovered three *GAMYB*-like genes whose expression occurred in the floral stage. During transcriptional regulation of the floral transition, a potential target for the GAMYB proteins is the *LFY* promoter, which has a putative 8 bp MYB-binding domain (Gocal *et al.*, 2001). There are separate elements that respond to either photoperiod or GA-response regions on the *LFY* promoter in *Arabidopsis* (Blazquez and Weigel, 2000). The AtMYB33 protein, in gel shift assays, could bind the *LFY* promoter fragment that carries this putative MYB-binding domain. This indicates that *AtMYB33* may influence the GA-mediated flowering response (Gocal *et al.*, 2001).

Blazquez *et al.* (1998) established that an absence of *LFY* promoter induction correlated to failure of *ga1-3* mutants to flower in short days. A constitutively expressed *LFY* transgene could rescue flowering in these gibberellin-deficient mutants in short days.

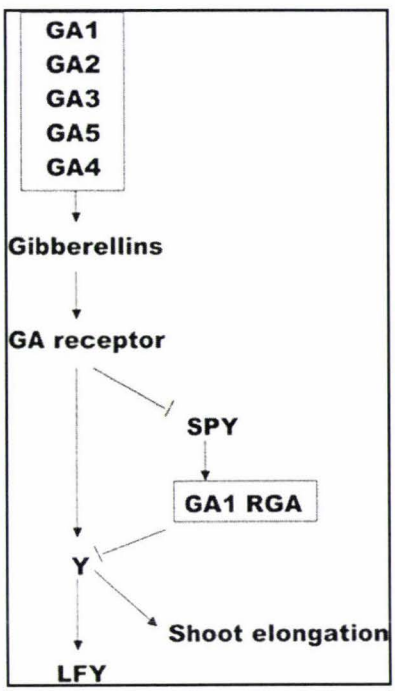


Figure 1.3 The interactions of proteins involved in regulating the gibberellin pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

1.2.3 Regulation of flowering by temperature

The vernalisation pathway involves the regulation of certain genes in response to a period of cold treatment. It acts redundantly with the autonomous pathway to accelerate floral initiation indirectly. Vernalisation prepares winter ecotypes of *Arabidopsis* to respond to other developmental and environmental signals to promote flowering in more favourable conditions in the spring (Michaels and Amasino, 2000). Vernalisation was defined by Chouard (1960) as "the acquisition or acceleration of the ability to flower by a chilling treatment". Winter annual ecotypes will exhibit delayed flowering until they have been exposed to winter conditions. They grow vegetatively throughout the winter season and flower in the spring. Winter annual ecotypes have dominant alleles at the *FRI* and *FLC* loci. In comparison, summer annual ecotypes of *Arabidopsis* do not require vernalisation and have null alleles of *FRI* and *FLC* (Michaels and Amasino, 2000). The floral initials are not developed before chilling occurs.

Winter chilling differs from vernalisation. Chilling, a specific period of time where dormant buds are exposed to temperatures around 2-4°C, is responsible for relieving bud dormancy so deciduous plants flower in spring. For many woody perennials, especially those in temperate climates, a delay between floral initiation and flowering often coincides with bud dormancy (Battey, 2000). The onset of bud dormancy is subsequent to floral initiation and is promoted by decreasing photoperiod (Wareing, 1956; Vaartaja, 1959). Temperature can also interact with photoperiod to influence the onset of dormancy (Heide, 1974). Chilling is the 'breaker of bud dormancy' (Cannell, 1989) but has the parallel function to control flowering, which for temperate trees, is in the spring (Battey *et al.*, 2000). If the chilling requirement is not met, long photoperiods can, to a degree, compensate (Cannell and Smith, 1983; Nizinski and Saugier, 1988). The length of photoperiod is not a critical breaker of dormancy unless chilling is not satisfied (Wareing, 1956). Chilling has a cumulative

effect. It increases up to a specific threshold that releases buds from winter dormancy (Samish, 1954).

It has previously been suggested that *M. excelsa* and *M. collina* have different chilling and daylength requirements for floral initiation and organogenesis, but neither undergo vernalisation or exhibit bud dormancy. *M. excelsa* floral initials develop in the autumn and undergo a period of stalling until spring when organogenesis occurs (Sreekantan *et al.*, 2001). When the floral initials did not receive the required period of chilling during winter the flowers did not develop (Henriod *et al.*, 2000).

The perennial *Fragraria vesca* (strawberry) exhibits seasonal flowering that is conferred by the dominant allele of a particular gene. The alpine type *F.v. semperflorens* is ever flowering and has the recessive allele of this gene (Brown and Waring, 1965; Battey *et al.*, 1998). Guttridge (1985) and Battey *et al.* (1998) suggest that a lifting of floral repression in *F. vesca* (the end of winter chilling) allows floral initiation to begin (Metzger, 1996).

Vernalisation shows epigenetic features and is thought to involve chromatin remodelling. The stimulus (cold) and the response (flowering) are separated temporally and the transient stimulus is remembered as a seasonal memory. Since vernalisation does not involve permanent genetic changes it therefore requires resetting through mitosis each generation (Goodrich and Tweedie, 2002).

Genes that mediate vernalisation have been isolated from *Arabidopsis*. These are *VERNALISATION1* (*VRN1*) and *VERNALISATION2* (*VRN2*). *VRN1* encodes a non-specific DNA binding protein that may function to stabilise *FLC* repression during vernalisation to promote flowering (Levy *et al.*, 2002). *VRN2* is also required for this stable repression of *FLC* (Levy *et al.*, 2002), and the vernalisation response is reduced in plants containing mutations of these genes (Chandler *et al.*, 1996). Sung and Amasino (2004a) identified a gene, *VERNALISATION INSENSITIVE 3* (*VIN3*),

which has a role in establishment of the vernalised state by measuring the length of time the plant has been exposed to cold temperatures. VIN3 is only expressed after a long period of cold. (Sung and Amasino, 2004a). *VIN3* is expressed in *vrn1* and *vrn2* mutants, and *FLC* is silenced by VIN3 in response to cold exposure in these mutants. However, *FLC* repression is blocked during cold exposure in *vin3* mutants, and no histone modification is detected, in contrast to WT plants. Therefore, it is suggested that *VIN3* is further upstream than the *VRN* genes (Sung and Amasino, 2004b). **Figure 1.4** shows how vernalisation appears to regulate flowering in *Arabidopsis*.

Levels of FLC protein appear to be regulated by vernalisation and by the FRIGIDA (FRI) protein (Michaels and Amasino, 2001). Vernalisation acts to promote flowering by suppressing *FLC* activity or expression so downstream integrator and floral meristem genes are expressed.

1.2.4 Regulation of flowering by sucrose

Photosynthesis is responsible for the assimilation of carbon intermediates that are required for sugar production in plants. Growth and development is governed by internal and environmental cues that are regulated by sugar status (Koch, 1996; Sheen *et al.*, 1999; Smeekens, 2000).

Sucrose has been exposed as a signalling molecule. It has been shown to regulate a variety of genes (Koch, 1996) including *LFY*, indirectly, as shown in **Figure 1.5** (Blazquez, 2000). Lejeune *et al.* (1991, 1993) reported that sucrose was the major sugar in apical and leaf exudates of *Arabidopsis*. When photosynthesis is limited at floral induction, starch mobilisation is required to produce the sucrose signal (Corbesier *et al.*, 1998). It is thought that the extra sucrose required for signalling is produced from starch reserves (Bodson *et al.*, (1977). Upon floral initiation by photoperiod in both long day and short day plants, levels of soluble sugars in the apical bud and/or sucrose in the phloem sap increase (Bodson and Outlaw, 1985;

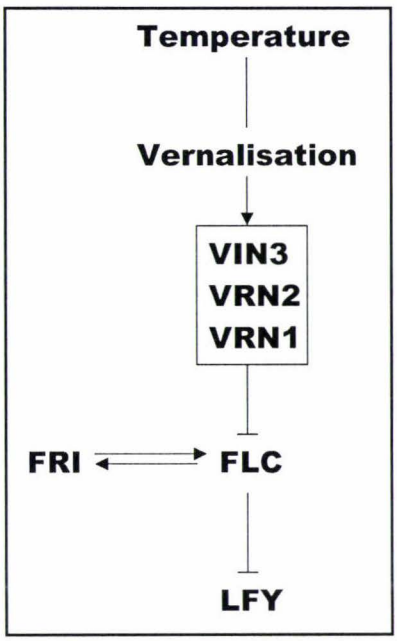


Figure 1.4 The interactions of proteins involved in regulating the vernalisation pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

Lejeune *et al.*, 1991, 1993). ADP glucose pyrophosphorylase small subunit 1 (ADG1) is involved in starch biosynthesis from glucose (Lin *et al.*, 1988). Phosphoglucomutase (PGM) is involved in controlling photosynthetic carbon flow. The starch in excess (SEX1) protein is required for starch catabolism to make sucrose (Corbesier *et al.*, 1998). These three genes are important for sucrose production and, therefore, signalling to LFY, as seen in **Figure 1.5**.

There is evidence from research in *Arabidopsis* that sugar can promote and inhibit flowering depending on the concentration applied to the growth media, the time the sugar was applied, and the genetic background of the plants (Ohto *et al.*, 2001). An increase in cell division observed in the shoot apical meristem during floral evocation is preceded by a rapid and transient increase of sucrose translocation from the phloem to the apical meristem in *S. alba* in response to a promotive stimulus of a single LD or displaced short day (DSD) (Bernier *et al.*, 1993). A similar rapid and transient increase in sucrose export from the leaves following floral induction by one LD or DSD is also observed in WT *Arabidopsis* plants (Corbesier *et al.*, 1998).

The partial rescue of late flowering mutants of *co*, *gi*, *fca*, *fpa*, and *fve* mutants when grown on medium containing 1% (w/v) sucrose was reported by Roldan *et al.* (1999). These results support the promotive effect of sucrose on the floral transition of *Arabidopsis*. It was also revealed that high levels of glucose in growth media delayed flowering significantly in *Arabidopsis* (Zhou *et al.*, 1998). A concentration of 1% (w/v) sucrose promoted flowering in late flowering mutants such as *co*, *fca*, *fha*, *gi*, and *ld*, but inhibited flowering of WT plants. In contrast, a concentration of 5% (w/v) sucrose delayed flowering in all *Arabidopsis* plants examined, including late flowering mutants (Ohto *et al.*, 2001).

Corbesier *et al.* (1998) suggested that carbohydrates from the phloem have an essential role in the transition to floral development. They noted that when only a low percentage of induced plants resulted from an inductive treatment, carbohydrate

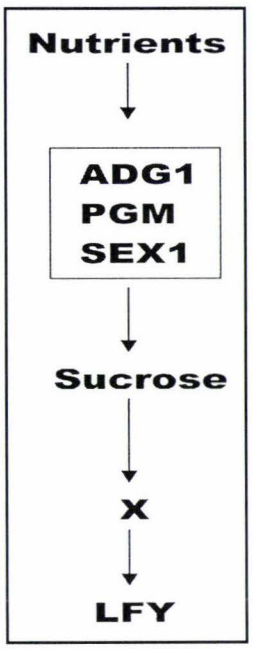


Figure 1.5 The interactions of proteins involved in regulating the nutrients and sucrose pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

export from the leaf was not increased. In contrast, a large, early and transient increase in carbohydrate export correlated with a large percentage of induced plants resulting from an inductive treatment. The capacity of the leaf phloem loading system was increased by floral induction.

No research into sucrose signalling has yet been documented in perennials.

1.2.5 Autonomous floral development

In *Arabidopsis*, the autonomous pathway monitors internal developmental cues and will enable flowering to eventually occur in the absence of external stimuli such as correct photoperiod in long day responsive plants, or vernalisation in winter annual ecotypes. The autonomous pathway is utilised by annuals with short life cycles (12-16 weeks) that must flower and set seed before they die to ensure the next generation.

FCA, *FY*, *FPA*, *FVE*, *LD* and *FLD* are genes that have been identified as being involved in the autonomous pathway of floral promotion (Koornneef *et al.*, 1998; Lee *et al.*, 1994; Sanda and Amasino, 1996) (**Figure 1.6**). Mutations in these genes produce late-flowering phenotypes under both short and long days (Koornneef *et al.*, 1998) and this lateness in flowering can be rescued by vernalisation (Lee and Amasino, 1995). These genes have been placed in the autonomous pathway because they have been shown to promote flowering independently of the photoperiod pathway (Koornneef *et al.*, 1998). Michaels and Amasino, (1999) and Sheldon *et al.* (1999) have shown that mutants of the autonomous pathway have increased levels of *FLC* RNA. This suggests that the genes in the autonomous pathway have a role in *FLC* repression to promote flowering, along with the vernalisation pathway.

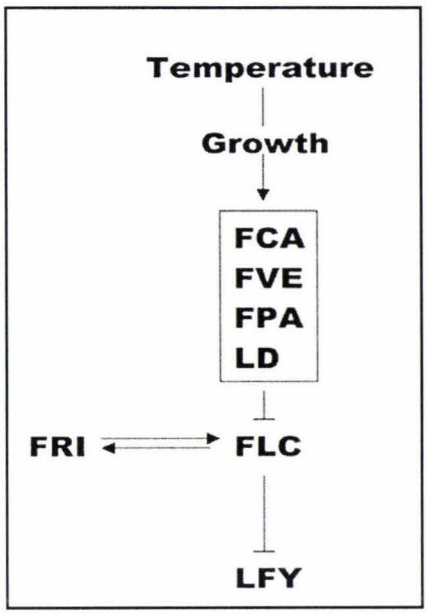


Figure 1.6 The interactions of proteins involved in regulating the autonomous pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

Recently, it has been discovered that the FLD protein, which is related to a protein found in mammalian histone deacetylase complexes, is involved in regulating *FLC* expression. *fld* mutants display hyperacetylation of *FLC* (He *et al.*, 2003).

Simpson *et al.* (2003) reported that FCA and FY, two flowering time regulatory proteins, form a complex by interacting through FCA's WW-domain and promotes production of an *FCA* transcript that encodes a non-functional protein. Since this complex is able to target *FCA* pre-mRNA, it is suggested that the FCA-FY complex could target *FLC* pre-mRNA also. FCA has an RNA-binding domain and a WW domain. FY is similar to Psf2p, the yeast polyadenylation factor. Ausin *et al.* (2004) recently cloned a gene involved in the autonomous pathway, *FVE*, which encoded *AtMS14*, a putative retinoblastoma-associated protein. *FLC* chromatin from *fve* mutants contained increased amounts of acetylated histones. This indicates that FVE may be part of a protein complex involved in histone deacetylation, a mechanism through which the complex represses *FLC* transcription and promotes flowering in *Arabidopsis*.

1.3 Flowering time genes

Flowering time genes are identified as such because their mutants affect the time to flower in *Arabidopsis*. Flowering time genes that promote flowering have mutants with delayed flowering (known as late-flowering mutants), while repressors of flowering are characterised by their early-flowering mutants. The flowering time genes include *SOC1*, *CO*, *GI*, *FLC*, *FT*, *LFY*, and *FRI*. *LFY* is considered to be a flowering time gene, as well as a floral meristem identity gene, because *LFY* mutations can delay or accelerate the change in phase from vegetative to floral development. Many flowering time genes have been described in previous sections of this chapter.

Late-flowering genes, GI and CO (Koornneef *et al.*, 1991), transduce signals to LFY, FT (Samach *et al.*, 2000), and SOC1, which act as pathway integrators or key floral promoters. Downstream floral organ identity genes, AP3, PI and AG, are activated by an increase of LFY in the floral meristem. Floral organ identity genes facilitate the formation of floral organs form the meristem (Wagner *et al.*, 1999; Blazquez and Weigel, 2000; Busch *et al.*, 1999; Krizek and Meyerowitz, 1996).

Flowering time gene products are most likely to repress flowering, and therefore maintain vegetative growth, as chromatin-associated epigenetic silencers. Levels of floral promoters must increase to eventually activate the change in phase to floral development (Sung *et al.*, 2003).

The flowering time gene, *SOC1*, has emerged as an important floral activator gene. Watson and Brill (2004) isolated two functional orthologues of the *Arabidopsis* SOC1, EgrMADS 3 and EgrMADS 4, from *Eucalyptus grandis* Hill ex Maiden. Ectopic expression of either *EgrMADS* 3 or *EgrMADS* 4 in the late flowering *SOC1*-deficient mutant of *Arabidopsis Ler* rescued flowering. A *SOC1* homologue has also been studied in *S. alba* (Menzel *et al.*, 1996, Borner *et al.*, 2000). *S. alba* is florally responsive after induction by one long day photoperiod. Bonhomme *et al.* (2000), discovered that *SaMADSA* upregulation is initiated 24 h after the start of the long day inductive condition, and this activation is imitated by the application of zeatin riboside, a cytokinin, to the apex of the meristem. GA_3 application also induced *SaMADSA* expression, as seen in *Arabidopsis*.

1.3.1 *FLOWERING LOCUS C*

An important flowering time gene is the floral repressor *FLOWERING LOCUS C* (*FLC*). This gene codes for a MADS-box transcription factor that is believed to repress flowering until environmental conditions are suitable (Michaels and Amasino, 1999). The level of *FLC* expression correlates with flowering time in the different

ecotypes of *Arabidopsis*. It has been shown that vernalisation promotes flowering by reducing the level of FLC transcript and protein in late flowering ecotypes and late flowering mutants of *Arabidopsis* (Sheldon *et al.*, 2000). The differences in the alleles of *FRI* and *FLC* can explain the variation in flowering time between the naturally occurring *Arabidopsis* ecotypes (Koornneef *et al.*, 1998a; Koornneef *et al.*, 1998b). The dominant allele of *FRI* appears to affect flowering time by causing upregulation of *FLC* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999) to inhibit flowering. This indicates that *FLC* is a key repressor of flowering in *Arabidopsis*.

FLC expression was thought to be downregulated by a period of chilling before flowering could occur, but recently it has been observed, in vernalisation-responsive late flowering ecotypes and mutants of *Arabidopsis* (Lun and Kr-2), that non-chilled plants do not have diminished FLC protein levels before flowering. The levels of protein were equivalent to the levels of *FLC* transcript. This suggests that floral induction was not mediated at the level of translation by a decline in FLC protein, but at the level of transcription or transcript stability. Rouse *et al.* (2002) suggested that there may be an accumulation of a floral inductive signal that may activate flowering by overcoming the repression from FLC.

Bastow *et al.* (2004) described the relationship between vernalisation and histone methylation and the effect on the *FLC* expression. Vernalisation was found to increase methylation of the *FLC* locus, specifically of lysines 9 and 27 of histone H3. In both *vrn 1* and *vrn 2* mutants, there is no methylation of *FLC* H3 K9, but methylation of H3 K27 is lost in *vrn 2* mutants only. It appears that a 'histone code' mediates the epigenetic memory of winter.

Michaels *et al.* (2004) isolated a gene similar to *FRI* in a winter annual strain of *Arabidopsis*, *FRIGIDA LIKE 1* (*FRL 1*). FRL 1 is required specifically for FRI to upregulate FLC expression to repress flowering in winter.

Sung and Amasino, (2004b) suggest that VIN3 functions as a vernalisation-specific regulator of flowering for a number of reasons. Induction of *VIN3* by cold temperature shows only a transient expression pattern. On return to room temperature, *VIN3* mRNA cannot be detected. *VIN3* is only expressed in response to a long period of cold temperature and *FLC* expression decreases as *VIN3* induction occurs. *VIN3* is only expressed in sites of vernalisation perception and *FLC* expression during vernalisation. Repression of FLC expression after vernalisation is never detected in *vin3* mutants. This indicates that VIN3 may be responsible for initial *FLC* repression during vernalisation to promote flowering after vernalisation.

1.4 Inflorescence meristem identity genes

Inflorescences can be described as either determinate or indeterminate based on the presence of a terminal flower at the shoot apex (Weberling, 1989). The vegetative and inflorescence meristems of *Arabidopsis* are characterised by an indeterminate growth pattern. The vegetative meristem produces a compact rosette of short stems and leaves, while the inflorescence meristem produces an elongated stem with secondary shoot and an indeterminate number of floral meristems. The floral meristem produces four whorls of organs (sepals, petals, stamens and carpels) and has a determinate growth pattern that is terminated after floral organogenesis (Clark *et al.*, 1993).

Inflorescence meristem identity genes are those that have a role in maintaining the meristem at the inflorescence stage, such as *TERMINAL FLOWER 1* (*TFL1*) in *Arabidopsis* or *CENTRORADIALIS* (*CEN*) in *Antirrhinum majus*. These gene products have a repressive effect on the development of the floral meristem.

1.4.1 *TERMINAL FLOWER 1*

In *Arabidopsis*, *TERMINAL FLOWER 1* (*TFL1*) is expressed in the shoot apical

meristem and prevents the expression of *LFY* and *AP1*, thereby maintaining the inflorescence meristem and inhibiting the transition to floral meristem (Weigel *et al.*, 1992; Bowman *et al.*, 1993). *TFL1* in *Arabidopsis* may also have a role in flowering time, as well as in meristem maintenance, as *tfl1* mutants flower earlier than WT *Arabidopsis*. This was not seen in *cen* mutants of *Antirrhinum*, which just produced a terminal flower (Bradley *et al.*, 1996).

Arabidopsis and *Antirrhinum* mutants presenting a determinate inflorescence, instead of indeterminate, are known as *terminal flower 1* and *2* (*tfl1*, *tfl2*) and *centroradialis* (*cen*) respectively (Shannon and Meeks-Wagner, 1991; Bradley *et al.*, 1997; Larsson *et al.*, 1998). The *SELF-PRUNING* gene in tomato is a homologue of *TFL1* and *CEN* and controls the determinate and indeterminate growth of the compound shoot (Carmel-Goren *et al.*, 2003).

TFL1 acts to repress floral meristem production and *tfl1* mutants flower early (Bradley *et al.*, 1997). *TFL1* sequence is very closely related to that of the *FT* gene, which is involved in floral promotion (Kardailsky *et al.*, 1999), but the two proteins have opposite roles in the control of floral transition. TFL1 in *Arabidopsis* acts antagonistically to LFY to maintain inflorescence meristem identity (Liljegren *et al.*, 1999) and therefore repress the transition from vegetative to floral development.

The *TFL1* sequence seems to encode a membrane-associated protein that may function in signal transduction pathways to regulate the floral meristem identity genes (Bradley *et al.*, 1997).

Homologous sequences of *Arabidopsis TFL1* have also been isolated from tobacco (*CET*) (Amaya *et al.*, 1999), *Lolium perenne* (*LpTFL1*) (Jensen *et al.*, 2001), pea (*PsTFl1a*, *PsTFL1b*, and *PsTFL1c*) (Foucher *et al.*, 2003) and *Metrosideros* (*METFL1*) (Sreekantan *et al.*, 2004).

In *M. excelsa*, *METFL1* was expressed in vegetative buds and during floral development. Spatially, the expression pattern for *METFL1* is similar to that of *TFL1* in *Arabidopsis* (Liljegren *et al.*, 1999). Therefore, it is possible that METFL1 could function to delay inflorescence development in *M. excelsa* (Sreekantan *et al.*, 2004).

1.5 Floral meristem identity genes

The expression patterns of *LFY* and *AP1* have come under scrutiny in multiple species, as their upregulation correlates to the development of floral meristems. A critical threshold level of *LFY*, and the proteins it interacts with, appears to be required before flowering can proceed, whereas *AP1* expression indicates actual floral initiation has occurred (Hempel *et al.*, 1997). *LFY* expression increases in the *Arabidopsis* meristem as the transition from vegetative to floral meristem progresses. The relative level of *LFY* affects the timing of the transition to flowering (Blazquez *et al.*, 1997). The number of copies of *LFY* and *AP1* genes appears to vary in different families of plants.

Floral meristem identity genes such as *LFY* and *AP1* are regulated by flowering time genes. LFY and AP1 mediate the decision to form floral meristems from the flanks of the inflorescence meristem (Clark *et al.*, 1993). Lateral meristems that develop into either shoots or flowers are produced from the apical meristem (Mandel and Yanofsky, 1995). In *Arabidopsis*, *LFY* and *AP1* are not transcribed in the shoot apical meristem, but in the floral meristem (Sharma and Fletcher, 2002).

1.5.1 *LEAFY*

The transcription factor *LFY* has two roles in floral development. *LFY* has been shown to act both as a floral meristem identity gene and as a flowering time gene to promote floral transition. It is also expressed in the vegetative phase in some species. (Bowman *et al.*, 1993).

The *lfy* mutation causes the formation of flowers with vegetative shoots or leaves in the place of petals (Bowman *et al.,* 1993). Loss-of-function mutations in the *lfy* gene result in *Arabidopsis* plants with delayed flowering. They continue to produce leaves while *WT* plants start to produce flowers under inductive conditions. (Bowman *et al.,* 1993; Rottmann *et al.,* 2000)

The overexpression of *LFY* hastens the transition to flowering in many species (Leandro *et al.,* 2001; Mouradov *et al.,* 1998; Pena *et al.,* 2001; Rottmann *et al.,* 2000; Southerton *et al.,* 1998). In *Arabidopsis*, *LFY* appears to upregulate *AP1* (Liljegren *et al.,* 1999) to promote floral organogenesis.

Wagner and Meyerowitz (2002) identified SPLAYED (SYD), a protein that, along with LFY, regulates apical meristem identity, and plays a role in temporal regulation for the change from vegetative to reproductive phase. The *SYD* sequence was similar to the yeast *Snf2p* ATPase involved in the remodelling of chromatin during transcriptional regulation. It is thought that SYD may alter the activity of LFY, and functions as a LFY-dependent repressor of the transition from vegetative to floral meristem identity.

The homologous genes *LFY* and *FLO* regulate the formation of determinate meristems in *Arabidopsis* and *Antirrhinum*, respectively. However, in *Eucalyptus globulus*, another member of the Myrtaceae family and close relative to *Metrosideros*, there are two genes that have sequence homology to *Arabidopsis LFY* and *Antirrhinum FLO*. These are *Eucalyptus Leafy 1* and *2* (*ELF1* and *ELF2*). These genes were found to be functional equivalents of LFY as the *ELF1*::GUS fusion protein expressed in *Arabidopsis* had similar temporal and tissue specific expression patterns as *LFY*::GUS. Further, *35S::ELF1* could cause premature flowering in *Arabidopsis*, just like *35S::LFY*. However, *ELF2* appeared to be a pseudo gene (Southerton *et al.,* 1998).

A *LFY*-like gene, *NFL1* (*Nicotiana FLO/FLY*), has been isolated in *Nicotiana tabacum* along with the *NFL2* gene. These two homologous genes share 97% identity and 73% amino acid sequence identity with *LFY* from *Arabidopsis*. The results from *N. tabacum* plants that constitutively express *NFL1* cDNA indicate that *NFL1* has a critical role in distribution of cells during development of the vegetative and floral meristems. Ectopic overexpression of *LFY* in *N. tabacum* plants caused severe early flowering which was not seen for *35S::NFL1 N. tabacum* plants (Ahearn *et al.*, 2001). This indicates that *LFY* is capable of regulating the floral homeotic genes that are positioned downstream of *NFL1*. Transcripts of *NFL1* and *NFL2* have been found in both the floral meristems and in indeterminate vegetative meristems. This may indicate that expression of this gene is not sufficient for formation of the floral meristem in *N. tabacum*. It has been suggested that *NFL* gene products may function in the progenitor cells for flowers and leaves to specify determinacy (Kelly *et al.*, 1995).

Another homologue of *LFY/FLO* has been isolated in the woody, perennial species *Populus trichocarpa* (*PTLF*). *In situ* hybridisation studies have shown that *PTLF* is expressed in the floral meristems and developing male and female flowers (Rottmann *et al.*, 2000). It was also found that overexpression of *PTLF* cDNA in *Populus* caused early flowering, albeit, infrequently. Functional studies showed that the *PTLF* gene was functional in *Arabidopsis* as *PTLF* expressed in *Arabidopsis* caused flowering to occur five days earlier than control plants (Rottmann *et al.*, 2000).

Perennial trees have a longer generation time than annual species as they have a lengthy juvenile phase. The juvenile phase of citrus ranges from 6 – 20 years depending on the species which has been a limiting factor in their genetic improvement (Pena *et al.*, 2001). When *LFY* or *AP1* from *Arabidopsis* are overexpressed in transgenic citrange plants, the juvenile phase is shortened and early flowering is observed (Pena *et al.*, 2001).

AFL1 and *AFL2*, two orthologues of *LFY* with high homology to each other, were isolated from apple (*Malus* x *domestica* Borkh) floral tissue where they were expressed during floral bud differentiation. When ectopically expressed in *Arabidopsis*, *AFL2* accelerated floral transition more strongly than *AFL1*. Alignments of AFL1 and AFL2 show a difference of 4 amino acids, which may be responsible for the differences in functionality between AFL1 and AFL2 when expressed in *Arabidopsis* (Wada *et al.*, 2002). It is believed that cultivated apples are complex polyploids (Korban and Chen, 1992) and that apple may have additional *LFY* homologues than other plant species because of their polyploid origin (Wada *et al.*, 2002).

Expression of *VFL*, an orthologue of *LFY* from grape (*Vitis vinifera*), was detected in meristematic tissue. Both floral and vegetative meristems developed from this over the two seasons of inflorescence development (Carmona *et al.*, 2002).

In *M. excelsa* there is only a single copy of a *LFY* orthologue, (McKenzie *et al.*, 1997; Sreekantan, 2002). The *MEL* fragment from *M. excelsa* has over 80% to homology to other *LFY*-like sequences in the GenBank database (Sreekantan, 2002). Further details are provided in **Section 1.7**.

A functional orthologue of the *LFY* (*Arabidopsis*) and *FLO* (*Antirrhinum*) (*LFY/FLO*-like genes) was identified in the gymnosperm *Pinus radiata*. *NEEDLY* (*NLY*) is expressed during the vegetative stage, which is at least five years before the transition to reproductive development (Mouradov *et al.*, 1998). The NLY protein, although it lacks the putative LFY transcriptional activation domains, can function as a floral meristem identity gene in *Arabidopsis* when fused to the *LFY* promoter (Mouradov *et al.*, 1998). Orthologues of *LFY* have been studied in other gymnosperms, for example, *Gnetum* (Winter *et al.*, 1999; Shindo *et al.*, 1999). All *LFY* orthologues so far detected in gymnosperms are only expressed in reproductive organs but not in vegetative tissue (Himi *et al*, 2001), unlike *LFY* expression in angiosperms.

A gene related to *LFY* has been isolated from the monocot *Lolium temulentum* (*LtLFY*) that has 84% amino with the rice orthologue, *RFL*, and 54% amino acid identity to *LFY* from *Arabidopsis*. *LtLFY* was expressed in spikelet meristems, glumes, and lemma primordia (Gocal *et al*, 2001).

The discovery of the *LFY* gene in so many flowering plants, and across the evolutionary boundary between gymnosperms, pteridophytes (Himi *et al*, 2001) and angiosperms, shows that *LFY* has an important role in the transition to flowering that cannot be fulfilled by other genes. It has been suggested that there has been divergence in the functional roles of *LFY*-like genes as there are variations in the amino acid sequence between species (Ahearn *et al.*, 2001). The different response of LFY to GA and photoperiod seen in annuals and perennials also indicates this.

1.5.2 *APETALA1*

Time to flowering in *Arabidopsis* is reduced in plants ectopically expressing AP1 compared with WT *Arabidopsis* plants, and AP1 alone is capable of converting inflorescence meristems to floral meristems (Mandel and Yanofsky, 1995). Since *AP1* is only detected in *Arabidopsis* meristems after floral induction, it is useful as a marker of floral determination (Hempel *et al.*, 1997)

The *ap1* mutation causes the partial transformation of flowers into inflorescences (Bowman *et al.*, 1993). *AP1* appears to function once the plant is deemed competent to flower, and interacts with *APETALA2* (*AP2*) to control the development of the outer two whorls of floral organs (Bowman *et al.*, 1993; Rottmann *et al.*, 2000).

The transcriptional activation of *AP1* was also shown in the sterile *lfy-6*-null *Arabidopsis* mutant constitutively expressing a fusion construct of *LFY* and the rat glucocorticoid receptor hormone binding domain [*35S::LFY-GR*]. The development of *lfy-6 35S::LFY-GR* flowers was rescued by dexamethasone treatment to induce *LFY* expression, indicating that *LFY* is important in floral meristem formation. *AP1*

expression was monitored to test whether *LFY* acted as a transcriptional activator *in vivo*. After hormone induction, *AP1* was detected in the early floral primordia compared with no *AP1* detection in *lfy-6* inflorescences (Wagner *et al.*, 1999).

In *Arabidopsis* there is only one copy of the *AP1* gene. In *Antirrhinum*, *SQUAMOSA* (*SQUA*) is the *AP1* homologue. *PEAM4* is the functional homologue of *AP1* in pea (*Pisum sativum*). PEAM4 shares 76% amino acid identity with AP1 from *Arabidopsis*, and is able to hasten flowering when overexpressed in *Arabidopsis* (Berbel *et al*, 2001). A mutation in pea that disrupted floral meristem identity and delayed floral development, *pim* (*proliferating inflorescence meristem*), was shown to be a defect in the *PEAM4* gene (Taylor *et al*, 2002).

Nicotiana tabacum also has an *AP1*-like gene, *NtMADS11*. This cDNA was isolated from tobacco floral bud libraries and was found to be expressed in both vegetative and floral tissue. In a yeast two-hybrid screen, NtMADS11 interacted with NtMADS4, in the AGL2-like family, via the K domain. NtMADS11 may act during floral initiation (Jang *et al.*, 2002).

Brassica oleracea (cauliflower) has two copies of the *AP1* gene, *BoAP1-A* and *BoAP1-B*. The *BoAP1-B* allele is present in most *B. oleracea* subspecies and encodes a truncated BoAP1 protein that produces defects in the function of the floral meristem (Lowmann and Purugganan, 1999).

E. globulus also has functional equivalents of the *AP1* gene, *EAP1* and *EAP2*. They are mainly expressed in floral structures and *EAP2* undergoes alternative splicing to produce two proteins, EAP2 L and EAP2 S. Overexpression of any of these three *Eucalyptus* genes hastens the transition to flowering in *Arabidopsis* (Kyozuka *et al.*, 1997).

A homologous fragment of *AP1* was also isolated in apple (*Malus sylvestris* var.

domestica) and named *MdAP1*. When analyzing the expression of the *LFY* equivalent, *AFL*, and *MdAP1* genes over the growing season, it was observed that *AFL* exhibited transcriptional upregulation two months before any upregulation of *MdAP1* (Kotoda *et al.*, 2000). MdAP1 was renamed MdMADS5. Introduction and expression of this gene in *Arabidopsis* caused early flowering compared with WT *Arabidopsis*. Therefore, MdMADS5 may have similar function to *Arabidopsis* AP1 (Kotoda *et al.*, 2002).

Kiwifruit (*Actinidia deliciosa*) is a perennial that has a two-year cycle of axillary bud, flower and fruit development. The *LFY* and *AP1* orthologues in kiwifruit are *ALF* and *AAP1*, respectively. The patterns of expression of *AFL* and *AAP1* are consistent with the growth and development of first and second order meristems. During the first growing season, *ALF* is expressed throughout first order bud development, and *AAP1* is expressed in second order axillary meristems. *ALF* and *AAP1* transcripts accumulate in developing flowers during the spring of the second growing season. This indicates that floral commitment may be observed during the first growing season when *AAP1* is detectable during initiation of the second order meristems (Walton *et al.*, 2001).

In *M. excelsa* there is also only a single homologue of *AP1*, *MESAP1* (McKenzie *et al.*, 1997; Sreekantan, 2002). *MESAP1* had over 70% homology to other *AP1* sequences (Sreekantan *et al.*, 2004).

Elo *et al.* (2001) cloned and characterised from *Betula pendula* (silver birch), *BpMADS3*, a MADS-box gene similar to *AP1* from *Arabidopsis*. *BpMADS3* expression was observed in the early stages of the transition to flowering, and in inflorescence development. Constitutive expression of *BpMADS3* in tobacco resulted in extreme hastening of floral development.

The AP1 gene and protein are critical in flowering. Expression of *AP1* gives an

indication in most species that the phenomenon of flowering is going to happen and that a floral meristem is developing. Although, some species of plants have more than one copy of the AP1 gene, the second copy of the gene appears to be non-functional or a pseudogene.

1.6 The genetic interactions of floral genes in *Arabidopsis*

Flowering time genes are responsible for detecting changes in the environment or the developmental phase of the plant and regulating the expression of inflorescence meristem identity genes and floral meristem identity genes. The expression of floral meristem identity genes regulates the expression of floral organ identity genes to produce floral organs.

It has been proposed that the effect of environmental stimuli on plant development may be attained by chromatin remodelling, since SYD may alter the effect of transcription factors such as LFY (Wagner and Meyerowitz, 2002). Evidence for chromatin remodelling as part of flowering time regulation has also been obtained from the autonomous and vernalisation pathways. Many of the factors in these pathways also interact with factors in other pathways.

The complex interactions that promote and repress flowering in *Arabidopsis* are shown in **Figure 1.7**. To regulate the transition from vegetative to reproductive meristem, environmental and developmental signals are integrated by flowering time proteins, inflorescence meristem identity proteins, and floral meristem identity proteins.

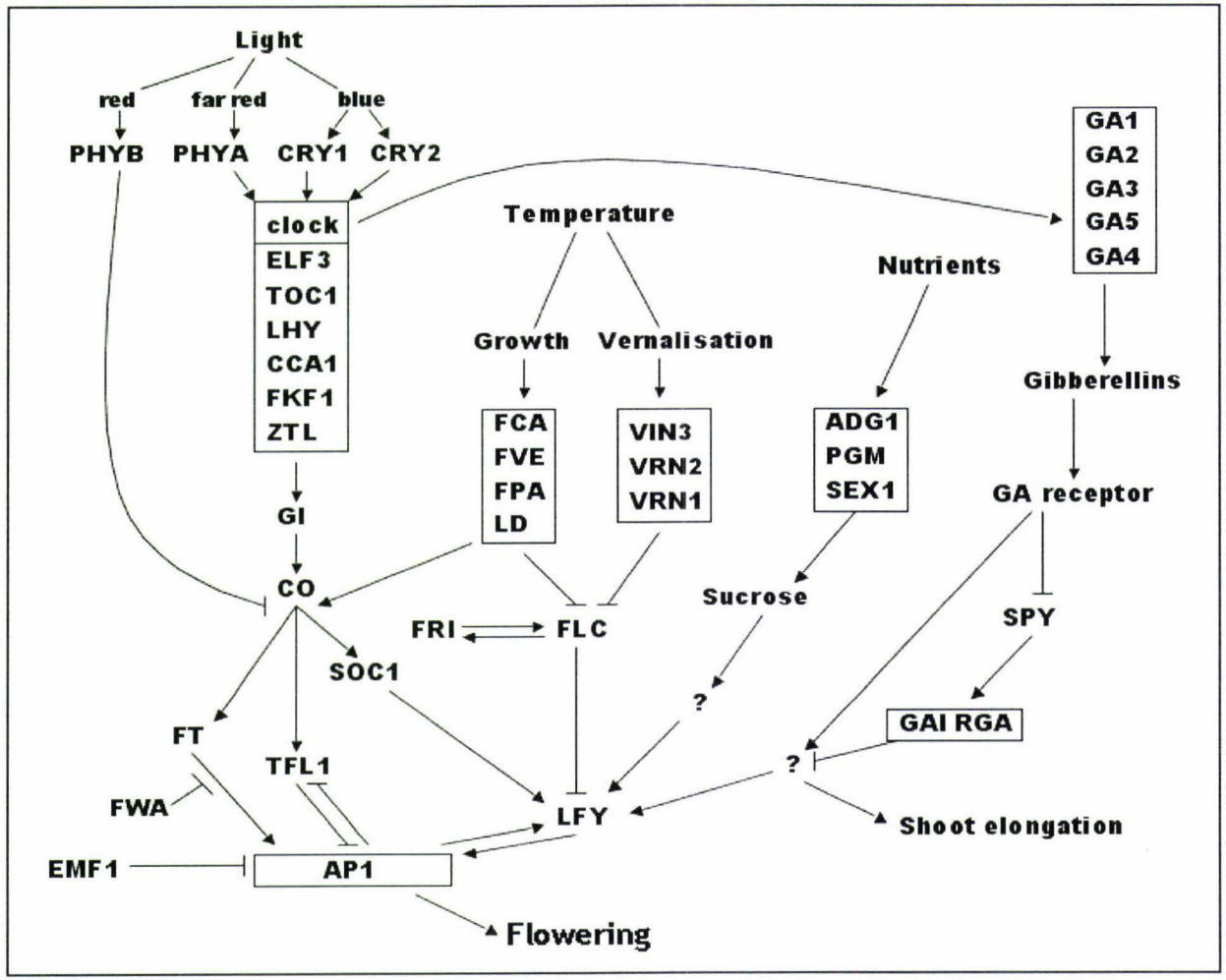


Figure 1.7 The interactions between flowering time gene products, inflorescence meristem identity genes, and floral meristem identity genes in *Arabidopsis* to promote and repress flowering. Modified from Blazquez (2000).

1.7 Flowering in *Metrosideros*

Although it is possible to use the *Arabidopsis* model for flowering as a basis for studying flowering in other species, there are major differences between *Metrosideros* and *Arabidopsis* that must be considered. *Arabidopsis* is a long day responsive plant. It is an herbaceous annual with a rapid life cycle and, as a consequence of this, has no significant juvenile period. GA_3 application has been found to upregulate *LFY* in *Arabidopsis*, and also enhance stem elongation and hasten flowering (Bagnall, 1992). *M. excelsa* is a facultative short day woody perennial plant (Henriod *et al.*, 2000). Many woody perennials have a significant juvenile period of up to five years (Pena *et al.*, 2001) or more. GA_3 application has been found to upregulate *LFY* expression in juvenile *M. excelsa*, but not hasten floral development (Sreekantan *et al.*, 2004). Observations of flowering in *M. collina* would suggest that *M. collina* and *M. excelsa*, although closely related Myrtaceae family members, have different chilling and/or daylength requirements for floral initiation and organogenesis [Clemens, J. pers. comm.].

M. excelsa requires lowered temperatures for completion of floral development, but only after floral development has been initiated (Sreekantan *et al*, 2001). This appears to be a period of stalling of development after floral bud initiation (Sreekantan *et al.*, 2004) and contrasts with winter annual ecotypes of *Arabidopsis*, which require vernalisation to promote floral development. *M. collina* appears to flower spasmodically with no winter chilling requirements although this has not been experimentally determined [Clemens, J. pers. comm.].

Previous research by Henriod *et al.* (2000) determined that *M. excelsa* plants developed a greater proportion of flowers if they were treated with cold temperatures and short days. This indicated that *M. excelsa* is a facultative short day plant and that inductive conditions for flowering in *M. excelsa* were a combination of short days and cold temperatures. When buds from field grown *M. excelsa* were examined, it

was determined that floral initiation occurred during the shortest autumn days (Sreekantan *et al.*, 2001). This correlates to a developmental time frame documented in *M. excelsa*, which establishes the development of floral structures from autumn (April) to spring (September) with flowering in summer (December). It appears that floral initiation is promoted by short days in autumn with the development of cymule primordia. It is these structures that require a period of winter chilling so that they can complete their development in spring (Sreekantan *et al.*, 2001).

M. excelsa exhibits a bimodal pattern to flowering. Development of floral initials in bud axils takes place in late autumn. Development stalls at this point over winter (short days and colder temperatures) until further differentiation and organogenesis occurs during spring when warmer temperatures and longer days return (Sreekantan, 2002).

Temporally, *MEL* and *MESAP1* were expressed in a bimodal pattern throughout the year. In early autumn (March), *MEL* was expressed at low levels. Expression of *MEL* and *MESAP1* was upregulated at floral initiation (May to early June) and detectable in the early inflorescence apex. In winter (May – June), *MEL* expression was downregulated during this latent period of bud development and expression shifted to the early cymule primordia. Expression was upregulated once more during floral organogenesis/development in August, and increased in the following months of spring. At this stage, *MEL* expression was localised in cymule primordia. Subsequently, expression was observed in the sepals and petals, and later in the stamens and gynoecium during continuing floral development in late spring. *MESAP1* expression was similar to *MEL*: detected in the cymule primordia in August and moved into the sepals, petals and perianth during further flower development (Sreekantan *et al.*, 2004).

A bimodal pattern of expression of *LFY* homologues before floral development has also been documented in kiwifruit (Walton et al., 2001), apple (Kotoda *et al.*, 2000),

and has also been found to precede floral development in grape (Carmona *et al.*, 2002).

MEL and *MESAP1* expression, and endogenous GA content in *M. excelsa* have been compared during development of *M. excelsa* over the growing season. *MEL* and *MESAP1* were downregulated during the particular stage of flower development that coincided with an absence of GA_1 from floral buds and a stalling of floral development over winter, after floral initiation but before floral organogenesis. GA_1 was detected at its highest levels during spring when major vegetative growth occurred in *M. excelsa*, just prior to the continuance of floral organogenesis (Sreekantan *et al.*, 2004).

The effect of exogenous application of GA_3 on *MEL* and *MESAP1* expression was also investigated in juvenile *M. excelsa* plants. GA_3 upregulated *MEL* expression compared with control plants but flowering did not occur. A change to *MESAP1* expression was not detected (Sreekantan, 2002).

1.8 Rationale and aims of this research

The general aim of this project was to investigate the involvement of genes required for floral induction in two closely related *Metrosideros* species, *M. excelsa* and *M. collina*. This research utilises the model of *Arabidopsis* floral initiation and development as the basis for comparison of flowering in *Metrosideros,* and focuses predominantly on *LFY*- and *AP1*-like genes, which are key genes involved in floral meristem identity in *Arabidopsis*. The main floral pathway studied was the GA pathway. This pathway promotes flowering in *Arabidopsis* by upregulating *LFY*, which in turn upregulates *AP1* expression. As *M. excelsa* and *M. collina* have different requirements for floral induction signals, specifically differing in chilling

requirement for floral development, the flowering time gene, *FLC*, was also examined. Comparisons between *Metrosideros* (short day perennial) and *Arabidopsis* (long day annual), and comparisons between *M. excelsa* and *M. collina* are made.

1.8.1 Specific objectives

Specific objectives of this work were:

To examine the growth of *Metrosideros* plants in response to a variety of plant growth regulators and plant hormones.

To determine whether *M. collina* has a regular pattern to flowering that is determined by seasonal or developmental stimuli.

To use histological methods to build up a developmental sequence for *M. collina* vegetative and floral bud growth that will be used to determine if there is a correlation between bud developmental stage and the decision of the bud to be floral.

To determine a framework that can be used to predict floral status from bud size in order to explore the effect of seasonality on *M. collina* floral development.

To optimise the methods for DNA and RNA extraction for *M. collina* in order to isolate, sequence and analyse *LFY*-like, *AP1*-like and *FLC*-like genes from *M. collina*.

To examine the expression of *LFY* and *AP1* in both juvenile and adult forms of *M. excelsa* and *M. collina* plants in order to determine the response of these genes to the application of growth regulators, including gibberellic acid (GA_3) and determine whether the response is GA-specific.

To develop a method for detecting Southern blots with non-radioactive probes in order to determine the number of *LFY*-, *AP1*- and *TFL1*-like genes present in *M.*

collina.

To isolate the flowering time gene *FLC* and examine its role in floral initiation in *M. excelsa*, which requires a chilling period, and in *M. collina*, which does not appear to require chilling for flowering.

1.8.2 Presentation of results

The results are presented in three chapters. Chapter Three presents the results from morphological data of juvenile and adult *Metrosideros* shoots collected over 50 days in response to the exogenous application of plant hormones and growth regulators. *M. collina* bud measurement data taken to determine floral status is also described. These measurements allowed the description of a method to differentiate between *M. collina* vegetative and floral buds. It was also predicted to be a useful way to observe if *M. collina* is responsive to external signals, as *M. excelsa* is, to promote/repress flowering throughout the year, and to produce a calendar of developmental events for vegetative and floral bud growth in *M. collina*.

The isolation and identification, sequence analysis and evolutionary relationships for *LFY*- and *AP1*-like genes in *Metrosideros* species are described in Chapter Four. Also presented in this chapter is the expression of these genes in response to the exogenous application of plant growth regulator and hormones and the relationship this may have to flowering. Chapter Five contains a description of the brief venture into the isolation of *FLC*-like genes in *Metrosideros*.

CHAPTER TWO

Materials and Methods

2.1 Plant material and measurements of shoot growth

Micropropagated plants of *M. excelsa* 'Scarlet Pimpernel' were obtained from Lyndale Nurseries, Auckland, October 2001. Ninety glasshouse-grown micropropagated *M. excelsa* plants were divided into six treatment groups. On December 4th, 2001, each plant was randomly assigned a number from 1 to 90. Two shoots on each plant were labelled 'A' or 'B' by labelling a fully expanded leaf that was growing from below the apex. The node that subtended these leaves then acted as a marker from which to monitor the growth of shoots in response to growth regulator or hormone application. Measurements (mm) were subsequently taken from the labelled node to the most apical node of the shoot (**Figure 2.1**).

The distance between the node belonging to leaves 'A' or 'B' and the most apical node was monitored every two days as a measure of shoot growth. Measurements were taken from December 4th, 2001, until January 21st, 2002, when the growth of the plants treated with paclobutrazol (Section 2.2) had slowed significantly when compared to the growth of the five groups of untreated plants.

2.2 Plant growth regulator and plant hormone applications

Adult *M. excelsa* adult plants were obtained from Duncan & Davies Nursery, New Plymouth. Adult and juvenile *M. collina* cv. Tahiti plants were obtained from Lyndale Nurseries, Auckland. Adult and juvenile *M. excelsa* and *M. collina* plants

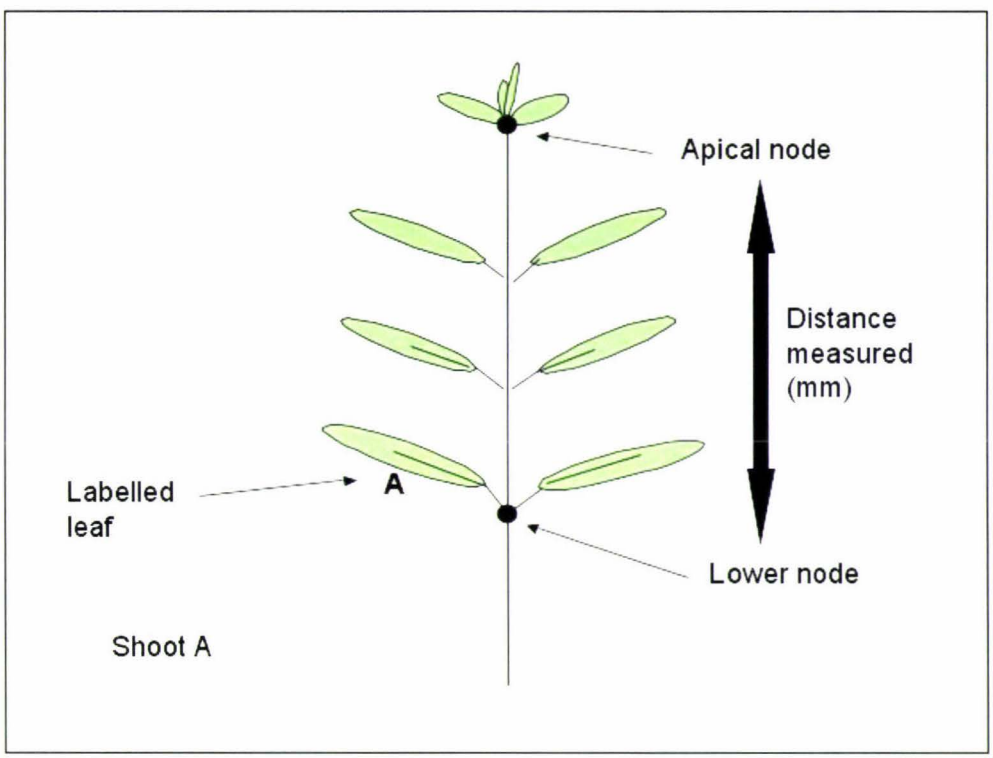


Figure 2.1 Measurement of shoot growth.
The distance between the labelled leaf and shoot apex was measured on micropropagated *M. excelsa* plants treated with water or paclobutrazol to facilitate quantification of significant growth differences between treated and untreated plants.

were treated with water, GA_3, paclobutrazol, 6-BAP, or ABA. The numbers of plants in each treatment groups are shown in **Table 2.1**. Initially, on December 6th, 2001, the soil of the plants in treatment group 2 was treated by drench application with 'Cultar' (ICI Crop Care). 'Cultar' contains the active ingredient paclobutrazol (250 g/L^{-1}) in the form of suspension granules. It was used as a soil drench application at a concentration of 10 mg a.i. L^{-1}. On December 7th, 2001, a follow-up foliar application of 'Cultar' at 100 mg a.i. L^{-1} was applied. A second drench application was applied on January 3rd, 2002.

On January 22nd, 2002, when the paclobutrazol-treated plants showed significant growth retardation, each group of untreated plants underwent foliar applications of the other growth regulators and hormones as shown in **Table 2.1**. A follow-up foliar application was repeated on January 24th, 2002, with the exception of the paclobutrazol-treated plants in Group 2. Adult *M. excelsa* and adult and micropropagated *M. collina* were also treated with 0.05 mg mL^{-1} GA_3 or water at the same time. Samples were collected early in the morning on January 25th, 2002, by excising the vegetative apices, immediately placing them into liquid nitrogen and storing them at -80°C until they were required for RNA extraction.

Each foliar application contained the surfactant Tween-20 as a 0.05% solution to assist in the absorption of growth regulators and hormones into the foliage.

2.3 Measurements of bud growth across the season

Two groups of 15 shoots were selected and the two leaves below the two buds at the shoot apex were labelled 'A' or 'B'. Shoots 1 - 15 were chosen because their buds appeared to be newly developed (2mm or less) and their growth and development could be monitored throughout their development to establish whether they would

Table 2.1 Details of the first foliar applications of the growth regulators and hormones applied to *Metrosideros* plants on January 22nd, 2002. These were applied to the plants when group 2, previously treated with paclobutrazol, showed significant growth inhibition when compared to the control plants.

Group	Description	Treatment	Num. of plants	ml L^{-1} per plant	mg mL^{-1} regulator/hormone
1	*M. excelsa* micropropagated	water	15	5	
2	*M. excelsa* micropropagated	pac	15	5	100 mg a.i L^{-1}
3	*M. excelsa* micropropagated	GA_3	15	5	0.5
4	*M. excelsa* micropropagated	GA_3	15	5	0.05
5	*M. excelsa* micropropagated	6-BAP	15	5	0.05
6	*M. excelsa* micropropagated	ABA	15	5	0.05
7	*M. excelsa* seedling	water	50	5	
8	*M. excelsa* seedling	GA_3	50	5	0.5
9	*M. collina* micropropagated	water	70	2	
10	*M. collina* micropropagated	GA_3	70	2	0.5
11	*M. excelsa* adult	water	5	10	
12	*M. excelsa* adult	GA_3	5	10	0.5
13	*M. collina* adult	water	5	10	
14	*M. collina* adult	GA_3	5	10	0.5

become vegetative or floral. Shoots 16 – 30 were chosen because the buds appeared to be either floral or vegetative. Potential floral buds were chosen based on the following criteria: 1) The buds measured more than 5 mm at their widest part, and 2) the buds appeared to have floral characteristics as they were rounder than the vegetative buds and cymules could be felt through the bud scales. Vegetative buds of the same size were also chosen for measurement. Potential vegetative buds were determined to be vegetative as they were less hard to the touch since they contained only leaves, not many floral organs like the potential floral buds. These buds were labelled as 'Set 1' buds. The growth of these buds was monitored until they finished developing as either floral buds or vegetative buds. If they were floral buds then no new growth was initiated after flowering. However, vegetative buds produced a new set of buds which were either floral or vegetative. The development of the subsequent sets, Set 2 and Set 3, respectively, was also monitored.

All buds were measured across their widest point (mm) (**Figure 2.2**) at weekly intervals from October 26th, 2002 until July 4th, 2003.

2.4 Methods for extracting genomic DNA

Solutions that are specific to each protocol are listed in this chapter. General lab solutions are found in **Appendix I**.

Several methods were trialed to extract DNA from vegetative tissue of *M. excelsa*, *M. collina* and *Arabidopsis*.

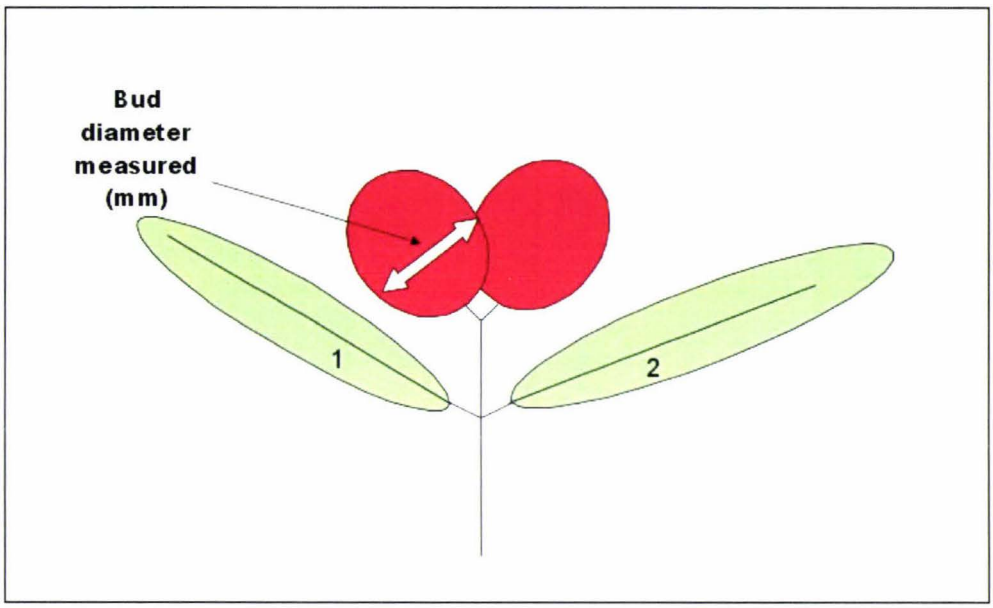


Figure 2.2 *M. collina* floral and vegetative bud measurements. All buds were measured across their widest point (mm) at weekly intervals from October 26th, 2002 until July 4th, 2003.

2.4.1 CTAB maxi preparation

The procedure of Reichardt and Rogers (1994) was followed as described below. To the required volume of cetyltrimethylammoniumbromide (CTAB), extraction solution (0.5 mL per gram of leaf tissue) 2-mercaptoethanol was added to a final amount of 2% (w/v). This solution was heated to 65°C. At the same time, the required amount of CTAB/NaCl solution (1/10 volume) was heated to 65°C.

An autoclaved mortar and pestle was chilled to -196°C with liquid nitrogen and used with liquid nitrogen to grind the leaf tissue into a fine powder. The liquid nitrogen was allowed to evaporate and using a chilled metal spatula, the ground tissue was transferred to an Oak Ridge tube (Nalgene) and the required amount of warm 2-mercaptoethanol/CTAB extraction solution was added and mixed. The homogenate was then transferred to Oak Ridge tubes, 1% (w/v) polyvinylpyrrolidone-40 was added, and the mixture incubated at 65°C for 45-60 min with occasional mixing. The homogenate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1 v/v), centrifuged for 5 min at 8,000 rpm in the Sorvall RC5C centrifuge (Du Pont Instruments) using the Sorvall SS34 rotor at 4°C.

The top aqueous layer was transferred to a clean Oak Ridge tube and one tenth of the volume of warm CTAB/NaCl solution was added. The samples were then mixed by inversion and extracted as before using chloroform/isoamyl (24:1 v/v) alcohol. To the recovered aqueous phase, one volume of CTAB precipitation solution was added and mixed. The DNA precipitated during this step. The DNA was collected by centrifugation for 5 min at 2000 rpm, 4°C. The supernatant was removed and the pellet was resuspended in high-salt TE buffer, pH 8 (0.5-1 mL per gram of starting material). The DNA was precipitated by adding 0.6 vol cold isopropanol and mixing. The samples were then centrifuged for 15 min at 8,000 rpm, 4°C. The pellet was washed with 80% ethanol and resuspended in a minimal volume of TE buffer, pH 8.

CTAB extraction buffer:

2% (w/v) CTAB (cetyltrimethylammonium bromide)

0.1 M Tris-HCl, pH 8

20 mM EDTA, pH 8

1.4 M NaCl

Add 2% (v/v) 2-Mercaptoethanol to the required volume of CTAB extraction buffer just before use.

CTAB/NaCl solution:

10% CTAB (w/v)

0.7 M NaCl

CTAB precipitation solution:

1% (w/v) CTAB

50 mM Tris-HCl, pH 8

10 mM EDTA, pH 8

High-salt TE buffer:

10 mM Tris-HCl, pH 8

0.1 mM EDTA, pH 8

1 M NaCl

2.4.2 Cesium chloride density centrifugation

The procedure of Richards (1994) was followed as described below. Two 10 g amounts of new vegetative tissue were collected from adult *M. collina* and adult *M. excelsa* plants and immediately frozen in liquid nitrogen. The tissue was ground to a fine powder with a mortar and pestle using liquid nitrogen and transferred to two 250 mL centrifuge bottles. To each centrifuge bottle, 100 mL of extraction buffer was added and the ground tissue gently dispersed. 10mL of a 10% N-lauroylsarcosine solution was added to the solution and the samples were incubated for 1-2 h in a 55°C water bath. The samples were centrifuged for 10 min at 6,000 rpm, 4°C (Sorvall GSA rotor in the Sorvall RC5C centrifuge). The supernatants were recovered and centrifuged again to remove any debris.

The DNA was precipitated by adding 0.6 vol of isopropanol to each bottle and mixed gently. The DNA was collected by centrifugation for 15 min at 8,000 rpm, 4°C and the supernatant discarded. The pellet was resuspended in 9 mL of TE buffer, pH8, and incubated at 55°C to aid resuspension. Solid cesium chloride (9.7 g) was added to each tube, mixed gently until dissolved and incubated on ice for 30 min. Both samples were then centrifuged for 10 min at 8,000 rpm, 4°C and the supernatant transferred into Oak Ridge tubes. Ethidium bromide (0.5 mL of 10 mg/mL stock solution) was added to each tube and the tubes were incubated on ice overnight. The samples were centrifuged for 10 min at 8,000 rpm, 4°C and the supernatant transferred to 4 x 6 mL quick seal ultracentrifuge tubes. The tubes were balanced and sealed. Samples were centrifuged overnight at 56,000 rpm using the Sorvall TV-865 rotor in the Beckman L5-75 ultracentrifuge at 20°C.

The next day the tubes were removed from the centrifuge and viewed under UV light to look for the DNA bands. To recover the DNA, a 15 G hypodermic needle was used to punch one hole in the top of the tube and another hole in the side of the tube directly below the DNA band. The needle was attached to a 1 mL syringe and the DNA drawn out of the tube and placed into Oak Ridge tubes. The ethidium bromide was removed from the DNA by repeatedly extracting the collected DNA with water-saturated n-butanol until the pink colour was removed. Two volumes of water and six volumes of absolute ethanol were added to each DNA preparation, mixed gently and incubated for one hour at -20°C. The DNA was collected by centrifugation in a Sorvall SS34 rotor for 10 minutes at 8,000 rpm, 4°C. The pellet was resuspended in TE buffer, pH8, and the DNA precipitated again using 1/10 vol of 3 M sodium acetate and two volumes of ethanol. The pellet was collected by centrifugation and briefly air dried before being resuspended in 0.5 mL – 2 mL TE buffer, pH8.

Extraction buffer:

100 mM Tris-HCl, pH 8

100 mM EDTA, pH 8

250 mM NaCl

Add 100 μg mL^{-1} of Proteinase K added just before use.

2.4.3 Modified CTAB mini preparation

A mortar and pestle and liquid nitrogen were used to grind 200 mg of tissue into a fine powder. Using a chilled metal spatula, the ground tissue was divided into two portions and each portion placed into a 1.5 mL microtube containing 700 μL of CTAB extraction buffer. The tubes were shaken vigorously for 15 sec and incubated in a water bath at 60°C for 45 min (*M. excelsa*) or 30 min (*M. collina*), with occasional mixing. The tubes were centrifuged at 4,000 rpm for 5 min until the pellet had separated from supernatant in the Eppendorf Benchtop 5417R Centrifuge.

The supernatant was transferred to a clean 1.5 mL tube and 700 μL of chloroform was added. The tubes were shaken very vigorously for 15 sec and kept at room temperature for 2 min. To clearly separate the aqueous and organic phases, the samples were brought to 4,000 rpm in the centrifuge and the upper (aqueous) phase was transferred to a clean 1.5 mL tube. To precipitate the DNA in the aqueous phase, 700 μL of cold (4°C) isopropanol was added, and the tubes were mixed gently and stood for 15 min on ice. During this time the DNA descends to the lower half of the microtube so as much isopropanol as possible was removed and replaced with 600 μL of 80% ethanol. The tubes were mixed by gentle inversion to wash the DNA. The ethanol removed the brown contaminants that coloured the DNA. Ethanol washes were repeated until as much colour as possible had been removed from the DNA pellet. The ethanol was discarded; the DNA was allowed to air dry and was then resuspended in 50-100 μL TE buffer, pH 8.

Extraction buffer:	**10 mL**
CTAB	0.2 g
PVP40	0.1 g
5 M NaCl	2.8.mL
0.5 M EDTA	400 μL
1 M Tris-HCl	1 mL
Milli-Q H_2O	5.6 mL

2.4.4 Quantification of DNA

The quantity of the extracted DNA was measured based on the corrected A_{260} value using a spectrophotometer (Ultraspec 3000®, Pharmacia Biotech). The concentration of the extracted DNA was calculated by assuming that one A_{260} unit equalled 50 μg of double stranded DNA.

Comparisons between DNA extraction protocols and DNA concentration and purity are drawn in Chapter Four, specifically **Table 4.1**.

2.5 RNA extraction methods

Several methods were trialled to extract RNA from *Metrosideros* vegetative and floral tissues, and *Arabidopsis* vegetative tissue.

2.5.1 'Hot Borate' maxi preparation

The procedure of Wilkins and Smart (1996) was followed as described below. Before beginning, all solutions and instruments had to be RNase-free. This was done by wrapping all glassware and metal objects in foil and baking at 200°C for 4 h, treating all solutions (except those containing Tris) with DEPC and by soaking all plastic tubes overnight in 3% hydrogen peroxide then rinsing with RNase-free water.

The required amount of extraction (XT) buffer (3.5 mL per gram of tissue) was

placed in a 50 mL Falcon tube and 10 mM dithiothreitol (DTT), 2% (w/v) polyvinylpyrrolidone (PVP-40) and 1% Nonidet P-40 (NP-40) were added and heated in a water bath to 80°C. The frozen tissue was ground to a fine powder in a chilled mortar using a chilled pestle and liquid nitrogen. Once the liquid nitrogen had evaporated, the ground tissue was transferred to a room temperature mortar and pestle with a chilled metal spatula. The hot XT buffer was added and the tissue homogenised for a further 1-2 min. The homogenate was transferred to an Oak Ridge tube standing on ice that contained 105 μL of 20 mg mL^{-1} Proteinase K (Roche). The samples were mixed gently and incubated in a shaking incubator at 42°C for 1.5 h at 100 rpm. To the homogenate, 280 μL 2 M KCl was added, mixed gently and incubated on ice for 1 h. To remove debris, the homogenate was centrifuged at 10,000 rpm for 20 min at 4°C in the Sorvall RC5C centrifuge using the SS34 rotor. The supernatant was then filtered though sterile miracloth into a clean Oak Ridge tube.

To selectively precipitate the RNA, 1/3 vol of 8 M LiCl was added to the filtered supernatant and incubated overnight at 4°C. The next day the precipitated RNA was collected by centrifugation at 10,000 rpm for 20 min at 4°C. The pellet was washed in 4 mL of cold 2 M LiCl and gently dispersed. The samples were centrifuged at 10,000 rpm for 20 min at 4°C. This washing step was repeated at least two more times to remove pigments from the RNA pellet. The pellet was resuspended in 2 mL 10 mM Tris-HCl, pH 7.5, by warming to room temperature and by gentle vortexing. Any insoluble material was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant containing the RNA was transferred to a 15 mL glass Corex tube and 1/10 vol of 2 M potassium acetate, pH 5.5, was added and incubated on ice for 15 min. Polysaccharides and insoluble materials were removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a clean 15 mL Corex tube and the RNA precipitated by adding 2.5 vol 100% ethanol and by incubating at -20°C overnight.

The next day, the RNA was pelleted by centrifugation at 9,000 rpm for 30 min at 4°C and the ethanol was discarded. The pelleted RNA was gently washed with 1 mL of cold 70% ethanol, centrifuged for a further 5 min and the ethanol discarded. Residual ethanol was removed under vacuum and the pellet resuspended in 300 μL of DEPC-treated milli-Q water. The solution was transferred to a sterile, RNase-free 1.5 mL microtube. The RNA was treated with *DNase1* (Roche) and kept at -20°C until required for RT-PCR.

For long term storage, the RNA was precipitated by adding 1/10 vol of 3 M sodium acetate, pH 6, and 2.5 volumes of cold 100% ethanol and stored -80°C.

Hot Borate Extraction (XT) buffer:

0.2 M sodium borate decahydrate (Borax)
30 mM EDTA
3% (w/v) SDS

Add components to pre-warmed Milli Q water in an RNase-free Schott bottle to dissolve. Adjust pH to 9 using 5 M sodium hydroxide. Treat with DEPC (1 mL L^{-1}) and autoclave.

Just before use add 10 mM DTT, 1% Nonidet P-40 and 2% (w/v) PVP-40.

2.5.2 Modified 'Hot Borate' mini preparation

This protocol was developed by modifying the procedure of Wilkins and Smart (1996) (Section 2.5.1). For every 100 mg of *Metrosideros* tissue, 35 μL 100 mM DTT, 70 μL 10% PVP-40 solution and 3.5 μL Nonidet–NP40 was added to 350 μL of XT buffer (described in Section 2.5.1) in a 1.5 mL microtube and equilibrated to 65°C. Tissue (100 mg) was ground into a fine powder using a mortar and pestle and liquid nitrogen. Without thawing, but once the liquid nitrogen had evaporated, the ground powder was transferred into the XT buffer with a chilled metal spatula and shaken vigorously for 15 s. Proteinase K stock solution (20 mg mL^{-1}) was added to the sample, and the tubes were incubated at 42°C for 45 min in a water bath and

mixed gently every 15 min. Potassium chloride (2 M, 28 μL) was added; the solution was swirled gently to mix and incubated on ice for 20 min. The samples were then centrifuged in the refrigerated Eppendorf Benchtop 5417R Centrifuge for 20 min at 10,000 rpm at 4°C. The supernatant was transferred to a clean 1.5 mL microtube and 1/3 volume of 8 M lithium chloride was added, mixed gently and incubated at -20°C for 1 h. The tubes were centrifuged for 20 min at 10,000 rpm at 4°C to collect the RNA. The pellet was washed in 500 μL 2 M lithium chloride and centrifuged at 10,000 rpm for 10 min at 4°C. This wash was repeated as many times as required to remove any colour from the RNA pellet. The pellet was resuspended in 200 μL 10 mM Tris-HCl pH 7.5 by warming to room temperature and gently vortexing. The samples were centrifuged for 10 min at 10,000 rpm at 4°C to remove any insoluble material. The supernatant containing the RNA was transferred to a clean 1.5 mL microtube and precipitated by adding 500 μL cold 100% ethanol and incubation at -80°C for 20 min. The RNA was pelleted by centrifugation at 9,000 rpm for 15 min at 4°C, and the ethanol was discarded. The pellet was air dried, and then resuspended in 30 μL of DEPC-treated water

2.5.3 Tri reagent mini preparation

Metrosideros vegetative apices (200 mg) were ground to a fine powder using a mortar and pestle and liquid nitrogen. Using a chilled metal spatula the ground tissue was transferred to a 1.5 mL microtube. TRI™_ reagent (Molecular Research Centre, Inc) (1.3 mL) was pipetted into the tube and mixed with the ground tissue. The tubes were then vortexed vigorously to shear the cells open. The tubes were centrifuged at 12,000 rpm for 2 min at 4 °C. The supernatant was transferred to a clean 1.5 mL microtube and stored at room temperature for 5 min. To the supernatant, 260 μL of chloroform was added. The tubes were shaken vigorously for 15 s, left at room temperature for 10 min, and then centrifuged at 12,000 rpm for 15 min at 4°C to separate the aqueous phase, interphase and organic phases. The upper (aqueous) phase was transferred into a clean 1.5 mL microtube. To this 320 μL of isopropanol and 320 μL of high salt precipitation solution (SPS) were added. The tubes were

mixed well and stood at room temperature for 8 min. The samples were centrifuged for 8 min at 10,000 rpm at 4°C. The supernatant was discarded and the RNA pellet washed in 500 μL of 70% ethanol. The RNA was centrifuged at 7,000 rpm, 4°C, for 5 min and the ethanol removed and the pellet air dried. The RNA pellet was dissolved in 30 μL of DEPC-treated water.

SPS (for the removal of polysaccharides and proteoglycans).

0.8 M sodium citrate

1.2 M NaCl

Make up to 100 mL with DEPC-treated water.

2.5.4 RNA quantification

The quality and concentration of each RNA preparation was assessed by determining the A_{230}, A_{260}, A_{280}, and A_{320} values of a 1/100 dilution (1 A_{260} = 40 μg RNA mL^{-1}) in a spectrophotometer (Ultraspec 3000®, Pharmacia Biotech) and calculated from a corrected A_{260}. High purity RNA was assessed as being over 1.8 – 2.0.

Comparisons between RNA extraction protocols and DNA concentration and purity are drawn in Chapter Four, specifically **Table 4.2**.

2.6 *DNase1* treatment of RNA

To remove genomic DNA from the RNA preparations, 30 μg RNA, 1.5 μL 20 U μL^{-1} *DNase1* (Roche), 2 μL 100 mM DTT and *DNase1* buffer to a final volume of 100 μL was pipetted into a 200 μL microtube. The *DNase1* reaction was performed in a ThermoHybaid gradient PCR machine (ThermoElectron Corporation, Germany) at 37°C for 30 min, then 75°C for 10 min.

***DNase 1* buffer**:

100 mM sodium acetate

5 mM $MgSO_4$

2.7 Reverse transcriptase polymerase chain reaction (RT-PCR)

The same RT-PCR protocol was repeated for all RT-PCR reactions and was as follows. To a 200 μL microtube, 1 μg of RNA, 1 μL 50 pmol μL^{-1} oligo dT_{15} and DEPC-treated Milli-Q water to a volume of 10.2 μL were added and heated at 65°C for 5 min, then placed on ice for 5 min to cool. To this mixture was added 4 μL 5x RT buffer (Roche), 2 μL 100 mM DTT, 2 μL 10 mM dNTP mixture (Roche), 0.8 μL RNA Secure (Ambion) and 1 μL Expand Reverse Transcriptase (Roche) to make a final reaction volume of 20 μL. The reaction mix was left at room temperature for 10 minutes and then incubated at 42°C for 45 min. The reaction was halted by cooling on ice and the newly synthesised cDNA was used immediately in PCR reactions.

2.7.1 RT-PCR of *M. excelsa* and *M. collina* equivalents of *LEAFY*

A genomic sequence similar to that of the *Arabidopsis LFY* sequence was first isolated by McKenzie *et al.* (1997). The same primers were used in this project to examine the expression of *MEL* in *M. excelsa* and *M. collina*.

The primer sequences were:

LFY forward primer:	5' GCGAATTCACIAAYCARGTITTYMGIYAYGC 3'
LFY reverse primer:	5' CGGATCCGTGICKIARIYKIGTIGGIACRTA 3'

The PCR reaction was performed as follows:

10 x PCR buffer	5 μL
50 mM $MgCl_2$	2.5 μL
2.5 mM dNTP mixture	5 μL
cDNA	3 μL
LFY forward primer (10 pmol μL^{-1})	2 μL
LFY reverse primer (10 pmol μL^{-1})	2 μL
Water	30.3 μL
Platinum Taq Polymerase (5 U μL^{-1}) (Invitrogen)	0.2 μL

2.7.2 RT-PCR of *M. excelsa* and *M. collina* equivalents of *APETALA1*

The *Metrosideros* partial equivalent of the *Arabidopsis AP1* gene (*MESAP1*) was isolated by Sreekantan (2002) using primers designed using the *AP1* sequence of *Brassica oleraceae*. These primers were used to examine the expression of *AP1* in *M. excelsa* and *M. collina*.

The primer sequences were:

AP1 forward primer: 5' CAAGCTTGAAGAGGATAGAG 3'

AP1 reverse primer: 5' GCTTAAGAGCAGTATCAAGCTG 3'

The PCR reaction was performed as follows:

10 x PCR buffer	5 μL
50 mM $MgCl_2$	2.5 μL
2.5 mM dNTP mixture	5 μL
cDNA	3 μL
AP1 forward primer (10 μM)	5 μL
AP1 reverse primer (10 μM)	5 μL
Water	24.3 μL
Platinum Taq Polymerase (5 U μL^{-1}) (Invitrogen)	0.2 μL

2.7.3 RT-PCR of *ACTIN*

The primer sequences were as follows:

ACTIN forward primer: 5' GCGAATTCTTCACCACYACHGCYGARCG 3'

ACTIN reverse primer: 5' CGGATCCCCRAYCCARACAACTGTAYTTC3'

The PCR reactions were performed as follows:

10 x PCR buffer	5 μL
50 mM $MgCl_2$	2.5 μL
2.5 mM dNTP mixture	5 μL
cDNA	3 μL
ACTIN forward primer (10 μM)	5 μL
ACTIN reverse primer (10 μM)	5 μL

Water	24.3 μL
Platinum Taq Polymerase (5 U μL^{-1})(Invitrogen)	0.2 μL

The PCR protocol for *MEL*, *MCL*, *MSAP1*, *MTAP1* and *ACTIN* amplification was as follows:

94°C	4 min	
94°C	30 sec	35 cycles
45°C	30 sec	
72°C	1 min	
72°C	10 min	
4°C	holding temperature	

To visualise the results of the RT-PCR, 10 μL of PCR products were mixed with 2 μL of loading dye and loaded onto 1.5% agarose gels. The amplified fragments were visualised by staining with ethidium bromide and photographing under UV light.

2.8 Polymerase chain reaction (PCR) of *FLOWERING LOCUS C (FLC)*

The *FLC* primers were designed from an alignment of *Brassica napus FLC 1-5* and *A. thaliana FLC* sequences.

The PCR reaction was performed as follows using 1μL of genomic DNA isolated from *A. thaliana*, *M. excelsa* or *M. collina*:

10 x PCR buffer	5 μL
50 mM $MgCl_2$	2.5 μL
2.5 mM dNTP mixture	5 μL

Genomic DNA	1 μL
FLC forward primer (10 pmol μL^{-1})	2 μL
FLC reverse primer (10 pmol μL^{-1})	2 μL
Water	32.3 μL
Platinum Taq Polymerase (5 U μL^{-1})	0.2 μL

FLC-1 primers were designed to amplify a sequence from exon 2 to exon 7 to produce a product that was 1873 bp.

FLC-1 forward primer	5' CATGCTGATCATCTTAAAGCC 3'
FLC-1 reverse primer	5' CAACAAGCTTCAACATTAGTTC 3'

The PCR protocol performed as follows:

94°C	4 min	
94°C	30 sec	} 35 cycles
55°C	30 sec	
72°C	1 min	
72°C	10 min	
4°C	holding temperature	

FLC-2 primers were designed to amplify a sequence from exon 2 to exon 5 to produce a product that was 557 bp.

FLC-2 forward primer:	5' CTGGTCAAGATCCTTGATCGATATGG 3'
FLC-2 reverse primer:	5' ATCAGCTTCGGCTCCCYCAAGAT 3'

The PCR protocol was as follows:

94°C	4 min	
94°C	30 sec	35 cycles
65°C	30 sec	
72°C	1 min	
72°C	10 min	
4°C	holding temperature	

FLC-3 primers were designed to amplify a sequence from exon 2 to exon 4 and produce a fragment that was 399 bp.

FLC-3 forward primer: 5' GATCCTTGATCGATATGG 3'
FLC-3 reverse primer: 5' CATTTGATTCCACAAGCTT 3'

The PCR protocol was as follows:

94°C	4 min	
94°C	30 sec	35 cycles
45°C	30 sec	
72°C	1 min	
72°C	10 min	
4°C	holding temperature	

To visualise the results of the PCR, 10 μL of PCR products were mixed with 2 μL of loading dye and loaded onto 0.6% agarose gels. The amplified fragments were visualised by staining with ethidium bromide and placing under UV light. All thermal cycling reactions were performed in a ThermoHybaid gradient PCR machine (ThermoElectron Corporation, Germany).

2.9 Sequencing of isolated DNA and cDNA fragments

For sequencing of the PCR products, entire 50 μL PCR reactions were run in double wells on 0.8% agarose gels. The gels were stained with ethidium bromide and visualised with UV. DNA bands of the correct size were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen) and quantified using High DNA Mass Ladder (Invitrogen). Once each PCR-derived fragment was purified and quantified, the required amount of each purified cDNA or genomic DNA fragment was pipetted into a 200 μL microtube with 2 μL BigDye™ Terminator Version 3.1 dye (Applied Biosystems) and 3 μL of 5x sequencing buffer (Applied Biosystems). A ten-fold dilution of each primer was prepared to a final concentration of 1pmolμl^{-1} and 3.2 μL of either the forward or reverse primer was added. Milli-Q water was added to each reaction to a final volume of 20 μL per reaction. The sequencing was carried out at the Allan Wilson Centre Genome Service, Massey University, Palmerston North, New Zealand, using the ABI.3730 DNA Analyzer (Applied Biosystems). Sequences and electropherograms were viewed and analysed using the biological sequence alignment editor, BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.10 Restriction enzyme digests of genomic DNA from *Metrosideros*

Genomic DNA (30 μg per reaction) from *M. excelsa* and *M. collina* was prepared using the modified CTAB method and used in three separate digests with *Bam*HI, *Eco*RI and *Hind*III restriction enzymes (Invitrogen) as these enzymes do not cut within the fragments of *MEL*, *MESAP1* or *MTTFL1*. Into each digest was placed 30 μg of either *M. excelsa* or *M. collina* DNA, 5μL of *Bam*HI, *Eco*RI or *Hind*III, and 3 μL of the respective enzyme buffer to a 1 x concentration. Each digest was made up to a total volume of 30 μL with water. The digests were incubated at 37°C for 4 h.

Control tubes with no enzyme were treated the same way as the digests.

2.11 Southern blotting

The AlkPhos Direct Kit (Amersham Pharmacia Biotech) was used for probe labelling and hybridisation of the membranes. The volume of the gel box was calculated and 0.8% agarose made with 1x TAE. Ethidium bromide at a concentration of 0.5 μg mL was added to the melted agarose. Once the agarose gel had set 25 μL of each genomic digest together with 4 μL gel loading dye was run on the agarose gel. The gel was run at 60 V, stained with ethidium bromide and visualised under UV light.

The gel was placed in a shallow container containing 0.25 M HCl and depurinated for 15 min after the colour of the loading dye had changed. The HCl was discarded and the gel was rinsed with sterile Milli-Q water. Denaturing solution was poured into the container and the gel was incubated at room temperature for 30 min at 30 rpm. The denaturing solution was discarded and the gel was rinsed with sterile water. The gel was rinsed in neutralising buffer, which was then discarded. New neutralisation buffer was added and the gel was incubated at room temperature for 15 min at 30 rpm. The neutralisation buffer was discarded and this step was repeated with fresh neutralisation buffer, then the gel was rinsed with sterile Milli-Q water. The DNA was transferred onto Hybond-N positively charged membrane (Amersham Life Science) overnight using 20x SSC. The next morning the membrane was rinsed with 2x SSC and dried at room temperature. The DNA was then immobilised on the membrane by crosslinking using UV light.

Denaturing solution: (2 L)

1.5 M sodium chloride	175.32 g
0.5 M sodium hydroxide	40 g
Make up to 2 L with dd-H_20	

Neutralising solution: (2 L)

1.5 M sodium chloride	175.32 g
0.5 M Tris-HCl, pH 7.2	121.14 g
1 μM EDTA	0.75 g
Make up to 2 L with dd-H_20	

20x SSC: (2 L)

3 M sodium hydroxide	350.6 g
0.3 M tri-sodium acetate	176.46 g
Make up to 2 L with dd-H_20	

2x SSC: Use a 1/10 dilution of 20x SSC and dd-H_20.

0.25 M HCl: (1 L)

Add 9.115 g of concentrated HCl to 500 mL of sterile dd-H_20 water and make up to 1 L with more sterile dd-H_20 water.

2.11.1 Probe synthesis:

The probes used for the Southerns were cDNA fragments of *MEL*, *MTAP1* and *MTTFL1* already contained within pGEM-T or pGEM-T Easy (Promega) plasmids in *E. coli* DH5$_\alpha$ cells. These constructs had been prepared previously by Marian McKenzie and were stored as glycerol stocks at -80°C.

Using aseptic technique, *E. coli* DH5$_\alpha$ cells from the glycerol stocks were streaked on to LB plates containing ampicillin (75 μg mL$^{-1)}$ and grown overnight at 37°C. A single colony was picked and suspended in 5 mL Luria broth containing ampicillin (75 μg mL^{-1}). The bacteria was grown overnight at 37°C in a shaking incubator at 225 rpm. This protocol was used for the production of plasmids containing the *MEL*,

MTAP1 and *MTTFL1* fragments and each culture was grown in triplicate.

The plasmid preparation was performed as described in Sambrook and Joseph (2001). The next day, 1.5 mL of each culture was transferred to 1.5 mL microtubes and the bacteria pelleted by centrifuged for 30 sec at 13,000 rpm and 4°C. The LB medium was aspirated to leave the bacterial pellet as dry as possible. The bacterial pellet was completely dispersed in 350 μL STET buffer, pH 8, and 25 μl of fresh 10 mg mL^{-1} Lysozyme (Sigma) added. The cells were lysed by vortexing for 3 sec. The bacterial lysate was placed in a boiling water bath for exactly 40 sec then centrifuged for 15 min at 13,000 rpm and 4°C. The supernatant was transferred to a clean 1.5 mL microtube. The plasmid DNA was precipitated by adding 40 μL of 2.5 M sodium acetate, pH 5.2, and 420 μL of isopropanol. The solution was mixed and kept at room temperature for 5 min. The DNA was recovered by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatant was aspirated and the pellet air dried then rinsed with 1 mL of cold 70% ethanol. The ethanol was removed and the pellet air dried for 10-15 min. The DNA was resuspended in 50 μL TE, pH 8, that contained *RNase* A (Roche) and vortexed briefly before storing at -20°C until required for the labelling reaction.

STET Buffer, pH 8: Sambrook and Joseph (2001)

10 mM Tris-HCl, pH 8
0.1 M NaCl
1 mM EDTA, pH 8
5% (v/v) Triton X-100
There is no need to autoclave

The probes for the Southerns were amplified from purified plasmids using the primers for *MEL* and *MESAP1* that were described in Sections 2.7.1 and 2.7.2 respectively. The *MTTFL1* probes were amplified using the following primers that were designed using the *B. oleraceae TFL-1* sequences by Sreekantan (2002):

MTTFL1 forward primer	5' GGTTATGACAGACCCAGATGT 3'
MTTFL1 reverse primer	5' CGAACCTGTGGATACCAATG 3'

The PCR reaction mixtures for *MEL* and *MTAP1* were previously described in Sections 2.7.1.and 2.7.2 respectively but contained 1 μL of a 1/100 dilution of purified plasmid instead of cDNA. The annealing temperature was increased to 52°C for all PCR reactions. *MTTFL1* was amplified using the same reaction mix and protocol as described for *MTAP1* but used 5 μL of each 10 μM *MTTFL1* primer. The entire product of each PCR reaction was run on a 1.5% agarose gel and the bands excised and purified using the QIAquick Gel Extraction Kit (Qiagen) and quantified using High DNA Mass Ladder (Invitrogen).

2.11.2 Probe Labelling

The labelled probes were prepared using the reagents and methods supplied in the AlkPhos Direct Kit (Amersham Pharmacia Biotech). The probes were diluted to a concentration of 10 ng μL^{-1} with water supplied and 10 μL placed into a 1.5 mL microtube and denatured by boiling for 5 min. The DNA was immediately cooled on ice and the contents briefly centrifuged to the bottom of the tube. Ten μL of reaction buffer was added to the cooled DNA and mixed thoroughly but gently. Two μL of labelling reagent was added and the tube mixed again. To the mixture, 10 μL of the diluted cross-linker working solution was added and the solutions mixed thoroughly and centrifuged to the bottom of the tube. The labelling reaction was incubated at 37°C for 30 min. The probe now labelled with alkaline phosphatase enzyme was kept on ice until required for hybridisation.

2.11.3 Probe hybridisation and stringency washes

The required volume of AlkPhos Direct hybridisation buffer (0.125 mL per cm^2 of membrane) was placed in hybridisation tubes and heated to 60°C. The blots were placed into the hybridisation buffer and pre-hybridised for 15 min at 60°C in a hybridisation oven. A small aliquot of warm hybridisation buffer was removed from

the hybridisation tubes and added to the labelled probes then mixed gently. This mixture was then pipetted into the hybridisation buffer remaining in the hybridisation tubes. Care was taken to avoid placing the probe directly on the membrane. The blots were hybridised overnight at 60°C in a hybridisation oven. The next morning, stringency washes were done. The required amount of primary wash buffer (used in excess at 2-5 mL per cm^2 of membrane) was heated to 60°C. The blots were transferred to this solution and washed for 10 min at 60°C at 30 rpm. The primary wash buffer was discarded and the wash repeated with fresh primary wash buffer at 60°C for 10 min. The blots were placed in clean containers and an excess of secondary wash buffer was added. The blots were washed at room temperature for 5 min. The secondary wash buffer was discarded and the wash repeated. The blots could be kept in secondary wash buffer for up to 30 min before detection.

Hybridisation Buffer:

0.5 M NaCl

4% (w/v) blocking reagent (Amersham Pharmacia Biotech)

Add to the hybridisation buffer provided (Amersham Pharmacia Biotech) and stir at room temperature for 1-2 h.

Primary Wash Buffer:

2 M urea

0.1% SDS

50 mM M Na phosphate, pH 7

150 mM NaCl

1 mM $MgCl_2$

0.2% blocking reagent (Amersham Pharmacia Biotech)

Secondary Wash Buffer:

1 M Tris base

2 M NaCl

2.11.4 Detection of labelled probes

Each blot was treated separately. The excess secondary wash buffer was drained

from the blot and placed on Saran Wrap. The detection reagent, CDP-*Star*™ (Amersham Biosciences) was pipetted onto the blot at a volume of 30-40 μL per cm^2 membrane and left for 2-5 min. The excess detection reagent was drained off and the membrane wrapped in Saran wrap. Care was taken to ensure there were no air bubbles or excess reagent present between the membrane and the Saran wrap. The blots were placed on a tray in the LAS 1000 Intelligent Dark Box (Fuji) and the chemiluminescent signal generated by the labelled probes hybridised to the target sequences on the membrane was detected and the image captured using Fujifilm Image Gauge software (Imaging Research AIS). The length of exposure ranged from 1h – 4 h, the optimum exposure time being 1 h.

2.12 Histological techniques

2.12.1 Processing and embedding of *M. collina* samples

M. collina floral buds of different sizes (1 mm – 9 mm) were collected from adult plants and stored at -80°C after being frozen immediately in liquid nitrogen.

Procedure: Carnoy's Fluid and Histoclear

The buds were taken from the -80°C freezer and placed into 15 mL Falcon tubes containing the fixative Carnoy's Fluid that had been equilibrated to -20°C. The samples in fixative were allowed to come to room temperature before being fixed under vacuum for two hours to remove air bubbles. The buds were kept at room temperature overnight then rinsed in 70% ethanol. The buds were subjected to a dehydration series using ethanol and tertiary butyl alcohol (TBA) (AnalaR®)changes before wax infiltration with Paraplast (Oxford® Labware) as shown in the flowchart in **Figure 2.3**.

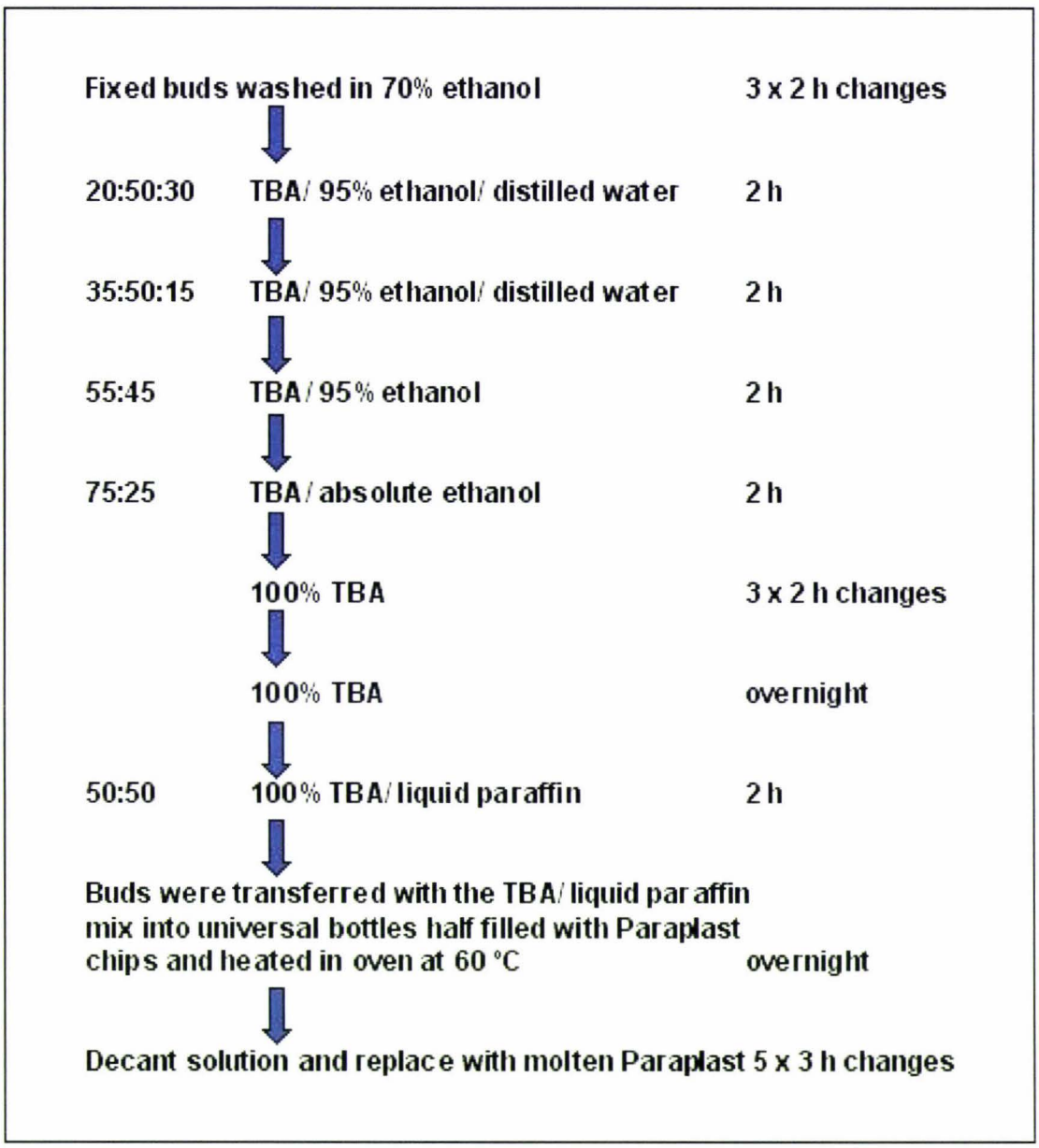


Figure 2.3 Flow chart for tissue dehydration and infiltration for paraffin embedding of *M. collina* buds required for histological studies. The buds were subjected to a dehydration series using ethanol and tertiary butyl alcohol (TBA) changes before wax infiltration with Paraplast.

The buds were embedded into wax blocks using the Leica EG1160 embedding machine, left at 4°C overnight and sliced to a thickness of 10 μm using the Leica RM2145 microtome. The wax ribbons were floated on water at 42°C, then mounted onto polysine-coated microscope slides (Biolab Scientific) and left to dry overnight on the Leica HI1210 slide warmer at 42°C.

Carnoy's fluid:

60ml 100% ethanol
10 mL glacial acetic acid
30 mL chloroform

2.12.2 Schedule for staining slides

The slides were stained using Safranin Red and Fastgreen according to a staining schedule adapted from a method by Johansen (1940) by Liz Nickless, Massey University in Palmerston North (**Figure 2.4**). After staining, the slides were drained for 10 min and 30 mm x 60 mm cover slips (Biolab Scientific) were mounted over the sample sections using DPX mounting fluid (BDH). The slides were dried in the fume hood overnight before being viewed under the light microscope.

Safranin Red: 1% safranin in 70% ethanol

Picric acid: 0.5% picric acid in 95% ethanol

Fastgreen: (150 mL)

Clove oil	15 mL
Methoxyethanol	15 mL
95% ethanol	90 mL
Glacial acetic acid	30 mL
Fastgreen	0.3 g
Histoclear/Absolute ethanol	50: 50
Clove oil/Absolute ethanol/Histoclear	50: 25: 25
Histoclear/Absolute ethanol	50: 50

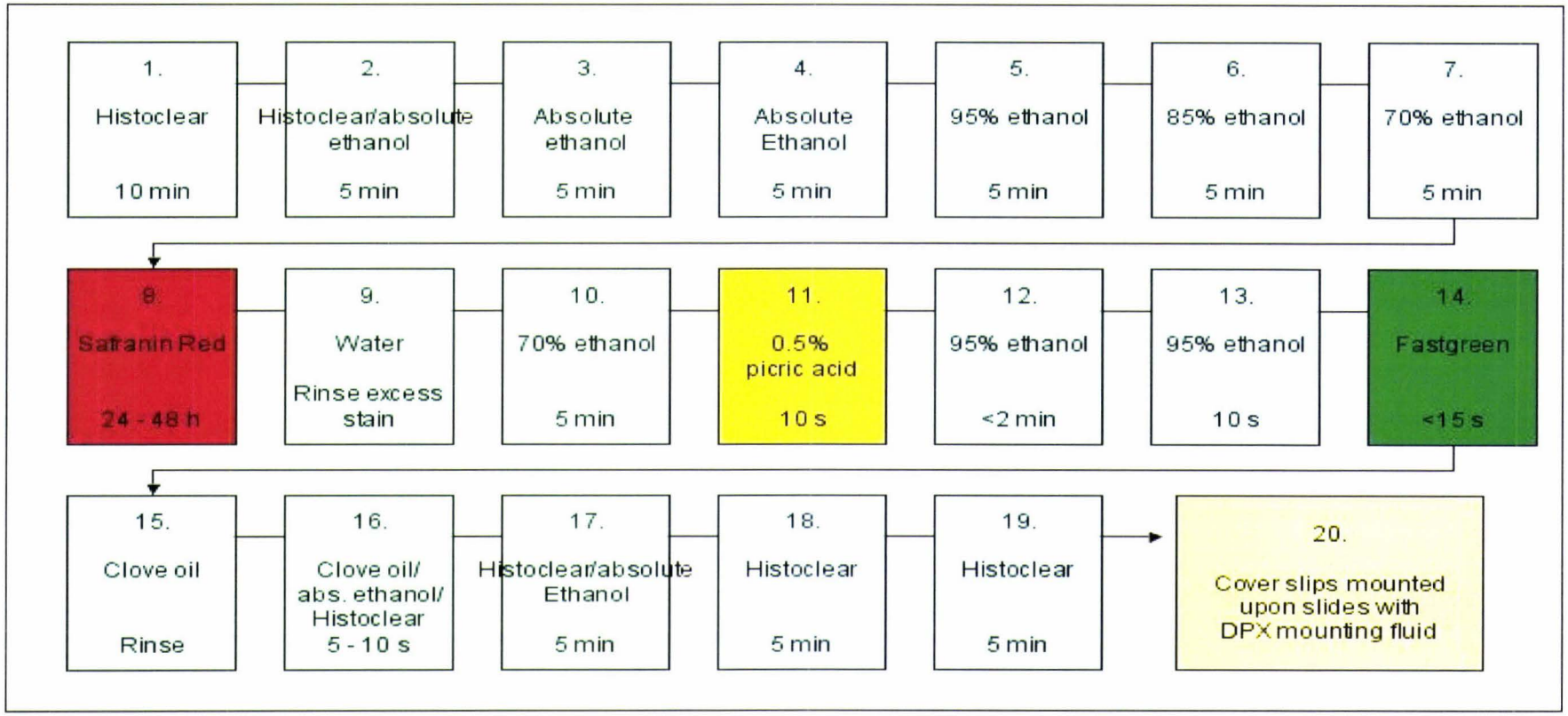


Figure 2.4 Schedule for staining *M. collina* bud sections. The bud sections were stained using Safranin Red and Fastgreen according to a staining schedule adapted from a method by Johansen (1940) by Liz Nickless, Massey University in Palmerston North.

CHAPTER THREE

Growth and Development of *Metrosideros*

3.1 Introduction

Sreekantan *et al.* (2004) showed that the application of GA_3 to juvenile *M. excelsa* (Scarlet Pimpernel) caused upregulation of *LFY* expression and an increase in shoot elongation, but not upregulation of *AP1* expression or early flowering. To extend this observation and to determine the specificity of the genetic response, micropropagated, seedling and adult *M. excelsa* (Scarlet Pimpernel) plants, and micropropagated and adult *M. collina* cv. Tahiti plants were subjected to a variety of growth regulator and hormone treatments. The morphological response that treated plants exhibited was used to show that the hormones and growth regulators were being absorbed into the plant.

M. collina produces vegetative and floral buds throughout the year. It is difficult to differentiate between floral and vegetative buds in early developmental stages, as they outwardly appear the same. All buds develop with red bud scales that are shed as the vegetative or floral organs emerge during bud break. Measurements of *M. collina* buds were taken to complement histological studies of bud development. It was expected that bud size would be a good marker to differentiate between floral and vegetative buds of *M. collina* cv. Tahiti, and to determine whether *M. collina* has a regular pattern to flowering. In comparison, *M. excelsa* flowers only once a year in the summer, and floral and vegetative buds differ morphologically from an early developmental stage (Sreekantan *et al.*, 2001).

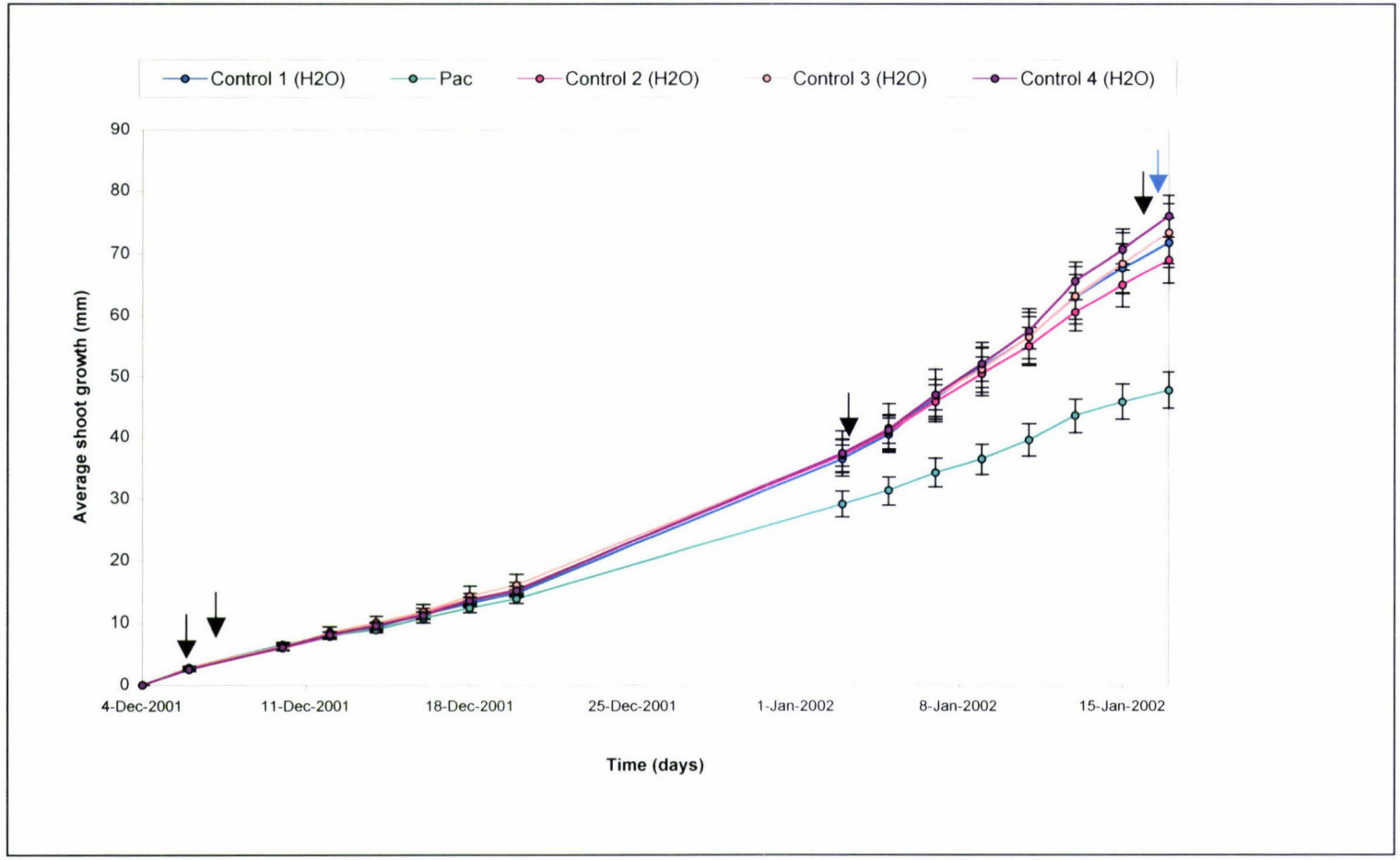
3.2 The effect of plant growth regulators and plant hormones on plant growth

Micropropagated *M. excelsa* plants were first treated with only water or paclobutrazol over a 51 d treatment period from 04 December 2002, until 24 January 2003, until a significant growth decrease was observed in the plants treated with paclobutrazol. The average shoot growth of paclobutrazol-treated *M. excelsa* plants was 47.7 mm, while the average shoot growth of water-treated *M. excelsa* plants was approximately 70 mm (**Figure 3.1**).

The observed morphological response to paclobutrazol and other growth regulators and hormones is presented in **Figure 3.2**. The photo shown in **Figure 3.2** was taken one month after the last stem growth measurements were taken. These plants had been left to grow after sample collection. This photo shows the effect that the applications of plant hormones and growth regulators had on the morphology of the micropropagated *M. excelsa* plants.

The water-treated plants represent the normal growth habit of micropropagated *M. excelsa* (**Figure 3.2 A**). The plants treated with the cytokinin, 6-benzylaminopurine (6-BAP), and with abscisic acid (ABA) (**Figure 3.2 B and F,** respectively) show a comparable growth habit to the water-treated plants . The plants treated with 0.05 mg mL^{-1} GA_3 and 0.5 mg mL^{-1} GA_3 (**Figures 3.2 C** and **3.2 D**, respectively) show elongated stems. These plants grew significantly taller than plants in the other treatment groups, which was indicative of increased stem growth stimulated by the GA_3 applications. The internodes of these plants became elongated, when compared to the water-treated control plants, which gave the plants a spindly appearance. The leaves of these plants were also elongated when compared to the water-treated control plants. The only difference between the two GA_3 treatments was that the plants that had received the application of 0.5 mg mL^{-1} GA_3 displayed a lack of chlorophyll in

Figure 3.1 Effects of paclobutrazol on plant growth. Fifteen micropropagated *M. excelsa* plants were treated with the growth inhibitor, paclobutrazol, until a significant growth inhibition was observed in comparison to the other four groups (fifteen plants each) that had only been treated with water. The black arrows represent respective paclobutrazol treatment dates of 10 mg a.i. L^{-1} soil drench on December 6th, 2001, a backup foliar application of 100 mg a.i. L^{-1} on December 7th, 2001, a soil drench on January 3rd 2002, at 10 mg a.i. L^{-1}, and a final soil drench on January 22nd 2002. The other groups of plants were treated on January 22nd 2002, with water, GA_3, cytokinin, or abscisic acid. The blue arrow represents the collection of young, apical, vegetative tissue on January 24th 2002, for subsequent RNA extraction.


Control 1 (H2O)
Pac
Control 2 (H2O)
Control 3 (H2O)
Control 4 (H2O)
Average shoot growth (mm)
0
10
20
30
40
50
60
70
80
90
4-Dec-2001
11-Dec-2001
18-Dec-2001
25-Dec-2001
1-Jan-2002
8-Jan-2002
15-Jan-2002
Time (days)

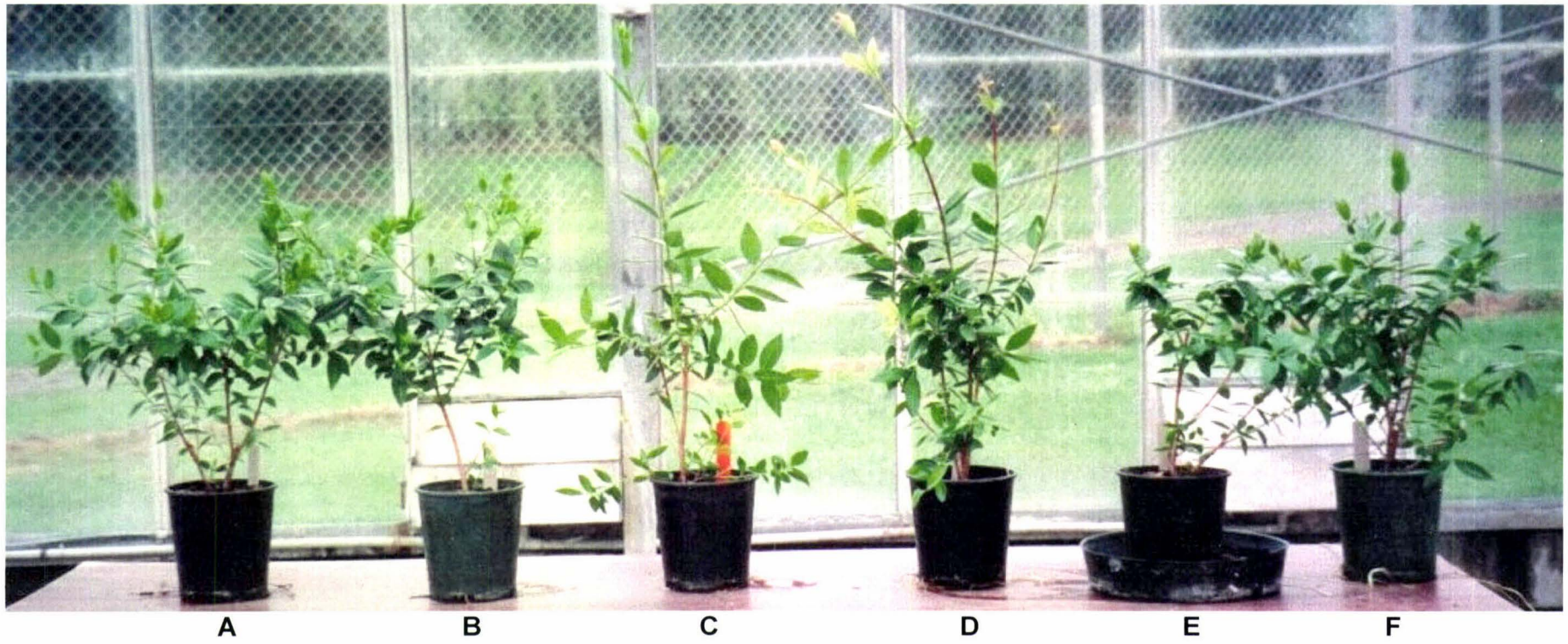


Figure 3.2 Observed morphological response of micropropagated *M. excelsa* plants to paclobutrazol, and other plant growth regulators and hormones one month after the last application and subsequent sample collections. The difference in plant growth of paclobutrazol-treated plants is significant compared to the water-treated control plants. **A)** Water-treated, **B)** 0.5 mg mL^{-1} 6-BAP, **C)** 0.05 mg mL^{-1} GA_3, **D)** 0.5 mg mL^{-1} GA_3, **E)** 10 mg a.i. L^{-1} paclobutrazol, **F)** 0.5 mg mL^{-1} ABA.

their most apical of leaves, as their leaves were pale green – yellow in colour. However, the leaves of the plants that had received 0.05 mg mL^{-1} GA_3 were visually assessed to be as green as the leaves of the water-treated control plants. There was no subsequent floral development seen in any of the micropropagated plants treated with either concentration of GA_3.

The micropropagated plants treated with paclobutrazol (**Figure 3.2 E**) exhibited greatly reduced shoot growth compared to the shoot growth of the water-treated plants over the same growth period. These plants became stunted in appearance. Their growth was inhibited by the paclobutrazol and as a consequence, their internodes were shortened and their vegetative apices were smaller and 'cabbage-like' in comparison to the water-treated control plants.

Seedling and adult *M. excelsa*, and micropropagated and adult *M. collina* were also treated with plant growth regulators, but these groups were limited to 0.5 mg mL^{-1} GA_3 application and water application only. The seedling *M. excelsa* and micropropagated *M. collina* plants treated with 0.5 mg mL^{-1} GA_3 also showed an increase in internode growth, similar to that observed for the micropropagated *M. excelsa* plants treated with 0.5 mg mL^{-1} GA_3 shown previously in **Figure 3.2 D**. The adult *M. excelsa* and *M. collina* plants treated with 0.5 mg mL^{-1} GA_3 did not show increased internode length. There was no hastening of floral development seen in any of the *Metrosideros* plants treated with GA_3. It was observed that the GA_3-treated adult *M. collina* plants did not produce flower buds as often as untreated plants. This is possibly because GA_3 inhibits flowering, or because the shoot tips had been removed during sample collection. Analyses of gene expression are presented in **Chapter Four**.

3.3 Bud growth measurements of adult *M. collina* cv. Tahiti

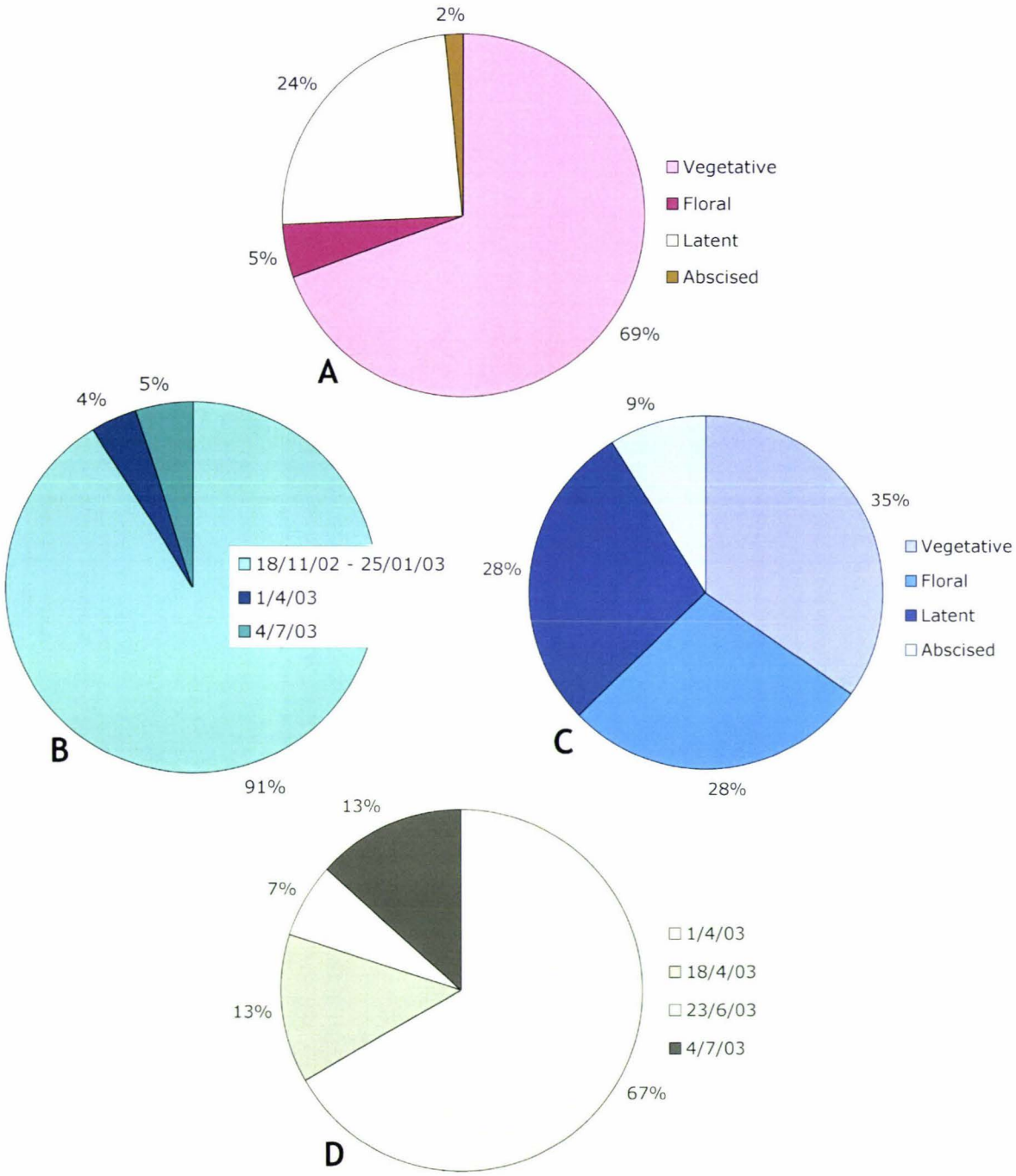
In order to establish the pattern of flower formation in *M. collina*, the growth of a selection of adult *M. collina* vegetative and floral buds was monitored at weekly intervals from 26/09/02 until 08/07/03. Thirty shoots, each holding two buds were measured across their widest part with calipers. Two shoots each had apical buds that had not abscised so held three buds per shoot. In total, 62 buds were present on the 30 shoots.

Over the first growing period, 62 buds were monitored and labelled as Set 1 buds. **Figure 3.3 A** displays the proportions of bud types that developed in Set 1. Out of these 62 buds, 24% (15/62) of buds were latent and their development did not continue further than 1- 3 mm in diameter. There were two buds that were first categorised as latent but later developed rapidly into small vegetative buds. One bud disappeared, either by insect consumption or bud abscission. Three buds (5%) developed into floral shoots. The fate of becoming a determinate (floral) meristem marked the end of the growth period for these shoots. Forty-three buds (69%), in total, continued on with an indeterminate growth pattern and produced vegetative organs and new buds, which went on to make up Set 2 buds.

The Set 2 buds became visible, and quantifiable, at various time points including 04/11/02, 09/12/02, 16/12/02, and 06/01/03. The shoots that produced the Set 2 buds each developed one apical bud with two axillary buds either side of it. The apical bud usually abscised during the first stages of growth, but it was observed that if the apical bud did not abscise and produced vegetative buds in the next set, the new apical bud did not abscise either.

Of the possible 86 buds that could have developed in Set 2, had all 43 Set 1 vegetative buds successfully produced buds, 78 Set 2 buds did develop. **Figure 3.3 B** shows when the majority of Set 2 buds developed from Set 1 vegetative shoots, and

Figure 3.3 Proportions of *M. collina* bud type development and dates of bud development. **A**) The proportions of bud types that developed in Set 1. These buds matured and were responsible for producing Set 2 buds. **B**) The majority (91%) of Set 2 buds developed from Set 1 vegetative buds between 18/11/02 and 25/01/03. **C**) The proportions of final bud type of Set 2 buds. The vegetative Set 2 buds produced Set 3 buds. **D**) Dates when Set 3 buds were quantifiable, the majority (67%) being quantifiable on 01/04/03.


2%
24%
5%
69%
Vegetative
Floral
Latent
Abscised
A
4%
5%
91%
18/11/02 - 25/01/03
1/4/03
4/7/03
B
9%
35%
28%
28%
Vegetative
Floral
Latent
Abscised
C
13%
7%
13%
67%
1/4/03
18/4/03
23/6/03
4/7/03
D

the proportions of final bud type in Set 2 are shown in **Figure 3.3 C**. Of these 78 Set 2 buds, 70 buds (91%) were first visible in the summer months between 18/11/02 and 25/01/03 and developed further. Three buds (4%) started developing at a later point during autumn on 01/04/03 and another four buds (5%) during the beginning of winter on 04/07/03 (**Figure 3.3 B**). Of these 78 buds, 22 buds (28%) stayed a small size, 3 mm and smaller, and were labelled as latent; 22 buds (28%) had flowered by 08/07/03 and seven buds (9%) abscised. Twenty-seven (35%) buds developed vegetative organs and went on to produce the third set of buds (**Figure 3.3 C**).

Measurements for Set 2 and Set 3 began when the two axillary buds at the shoot apex were visible to the eye and measured less than 1 mm in diameter.

Set 2 vegetative shoots produced 30 Set 3 buds that were still being measured at the end of the study, which had to be stopped because of heavy frost. The majority of these buds, 20/30 or 67%, were first of a measurable size on 01/04/03. Ten other buds became measurable on 18/04/03, 23/06/03, and 04/07/03, as shown in **Figure 3.3 D**. On average, Set 3 buds were not larger than 3 mm in diameter by the time the study ended.

The relationship between bud diameter (mm) and the percentage of bud break over time is presented in **Figures 3.4** and **3.5**. **Figure 3.4** shows the percentage of total bud break in each of the three sample sets, while **Figure 3.5** shows the percentage of Set 1 vegetative bud break and the percentage of Set 2 floral bud break.

For both **Figures 3.4** and **3.5**, bud diameter was defined as the distance in millimetres (mm) from one side of the bud to the other at the widest point, as measured with calipers. These values were determined from the grouped average of the average measurements for the two axillary buds on each shoot at each time point.

Percentage bud break was determined by calculating the percentage of buds that had

not broken at the particular measuring time point. These buds were still quantifiable because they had not broken yet. As the study continued the percentage bud break increased with increasing bud diameter, as the number of quantifiable buds decreased. Bud break was defined as occurring when the second outermost pair of bud scales had separated by 1 mm.

In **Figure 3.4**, the first data point for Set 1 begins with an average bud diameter below 4 mm in diameter because these buds were already in various stages of growth when the study started. As stated previously, 15 shoots were measured from when their buds were approximately 2 mm in diameter and 15 shoots were measured from when their buds were 5 mm or larger in diameter.

The data for **Figure 3.4** was gathered from all buds used in the study, both vegetative and floral, which developed in Set 1, Set 2, and Set 3.

As shown for Set 1 buds, 100% vegetative bud break was reached during December 2002, approximately four months after the beginning of the study. However, some buds (30%) had begun to break as early as September 2002, but it is unknown as to when the development of these buds was initiated as these buds were already developing when the study was started. Bud diameter did not increase further than 8 mm before 100% bud break was reached.

Set 2 vegetative buds were first visible in January 2003. These buds were produced from the vegetative buds of Set 1. Bud break began (20%) in February 2003, and continued, with increasing bud diameter, until March 2003. Bud break was rapid, as shown by the steep slope of the plotted data. This occurred when bud diameter was also increasing rapidly past 4 mm in diameter. Bud measurements for Set 2 did not increase past 10 mm in diameter as 100% vegetative bud break was reached approximately at the beginning of April 2003.

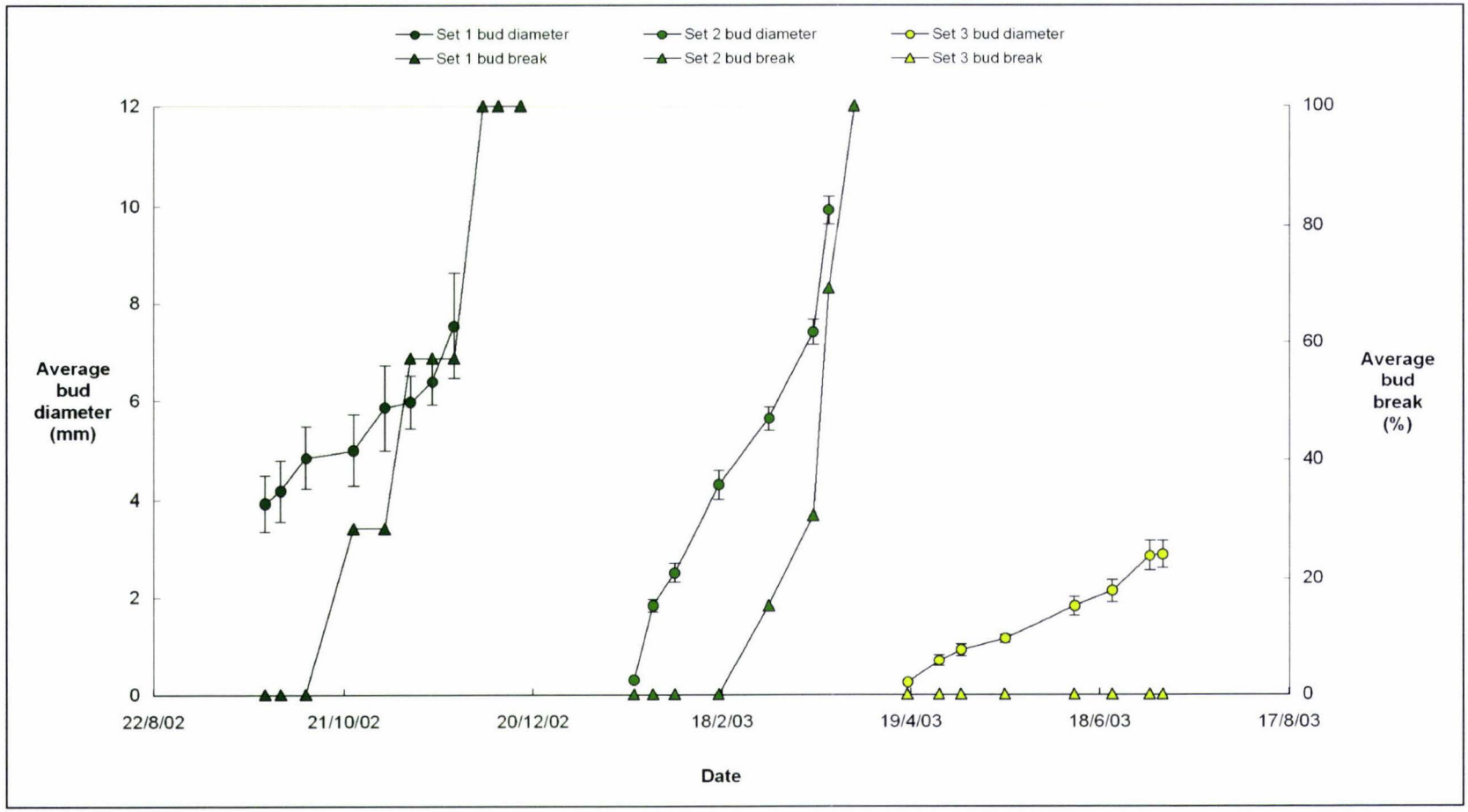


Figure 3.4 Percentage of adult *M. collina* vegetative bud break in each of the three sample sets in relation to bud diameter.

Set 3 buds were first visible on April 19th, 2003. Bud measurements did not continue past July 2003, as a heavy frost destroyed all the new, soft tissue that was slowly developing in the colder temperatures associated with this time of year. There was no sign of bud break (0%) in Set 3 buds. Their average bud diameter was approximately 3 mm by the end of the study. This data was not conclusive as the proportion of vegetative and floral buds in Set 3 could not be determined before the end of the study.

However, from the Set 3 data gained before the frost, it is possible to say that bud growth was very slow compared to the growth of Set 2 buds, which was indicated by the extremely rapid growth during the warmer months of December 2002 until February 2003. From Set 3 and Set 1 data it is possible to surmise Set 3 buds would have continued developing to reach the starting point of Set 1 bud development from which measurements were taken from in August 2002. This supports the possibility of two distinct bud development cycles in each year.

The relationship between bud diameter, bud break, and time for Set 1 vegetative buds and Set 2 floral buds that developed from the Set 1 vegetative buds is presented in **Figure 3.5**. The Set 1 bud data is taken from buds in Set 1 that developed into vegetative shoots, not floral.

The Set 2 floral bud data (**Figure 3.5**) shows that the growth of floral buds until bud break occurs takes longer than the growth of Set 2 vegetative buds, shown in the **Figure 3.4**. Set 2 vegetative bud development was initiated in Jan/Feb 2003 and continued until bud break in March 2003 (3 months). Set 2 floral development was initiated in December 2002 and continued until the end of June 2003 (6 months).

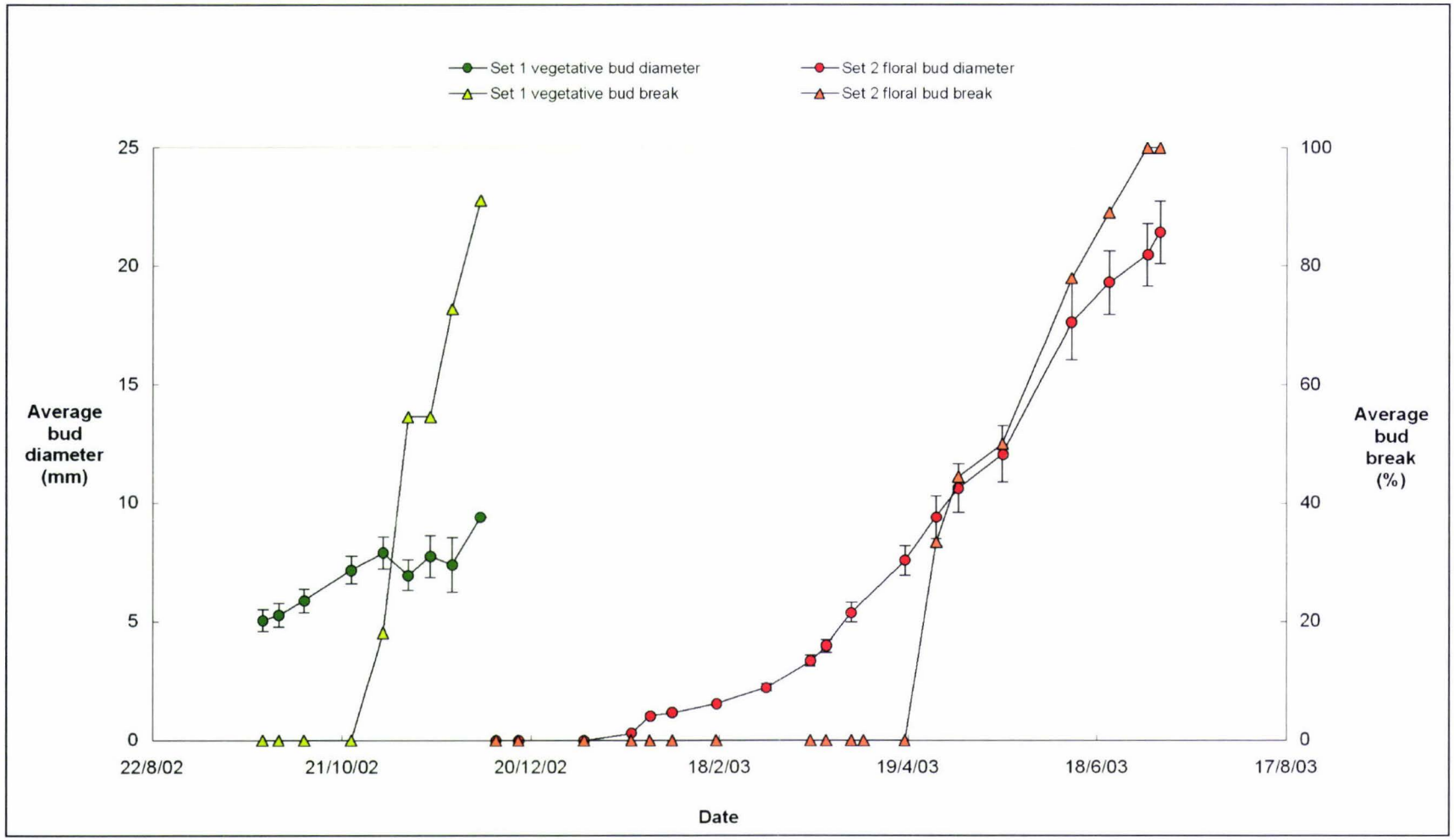


Figure 3.5 Percentage of adult *M. collina* Set 1 vegetative bud break and the percentage of Set 2 floral bud break in relation to bud diameter.

3.4 Histological analysis of bud growth and development of *M. collina* cv. Tahiti.

On 10 March 2003, a series of buds were collected from adult *M. collina* cv. Tahiti plants. A total of 40 buds were collected in eight size categories, five buds in each category. The size categories were <2 mm, 3 mm, 4 mm, 5 mm, 6 mm, 7 mm, 8 mm, and 9 mm. The buds were frozen in liquid nitrogen and prepared for histological analysis to determine if bud size has an influence on when a floral bud can be distinguished from a vegetative bud, and to develop a bud developmental sequence for both vegetative and floral *M. collina* cv. Tahiti buds

Vegetative and floral buds develop alongside each other. At its apex, one shoot may hold two vegetative buds, two floral buds, or one vegetative and one floral bud together. New bud growth is first visible at the shoot apex when the buds are approximately 0.7 mm across their widest part. **Table 3.1** presents the percentage of vegetative and floral buds present in the group of *M. collina* buds collected for histological analysis on the 10th of March 2003.

3.4.1 Vegetative bud development

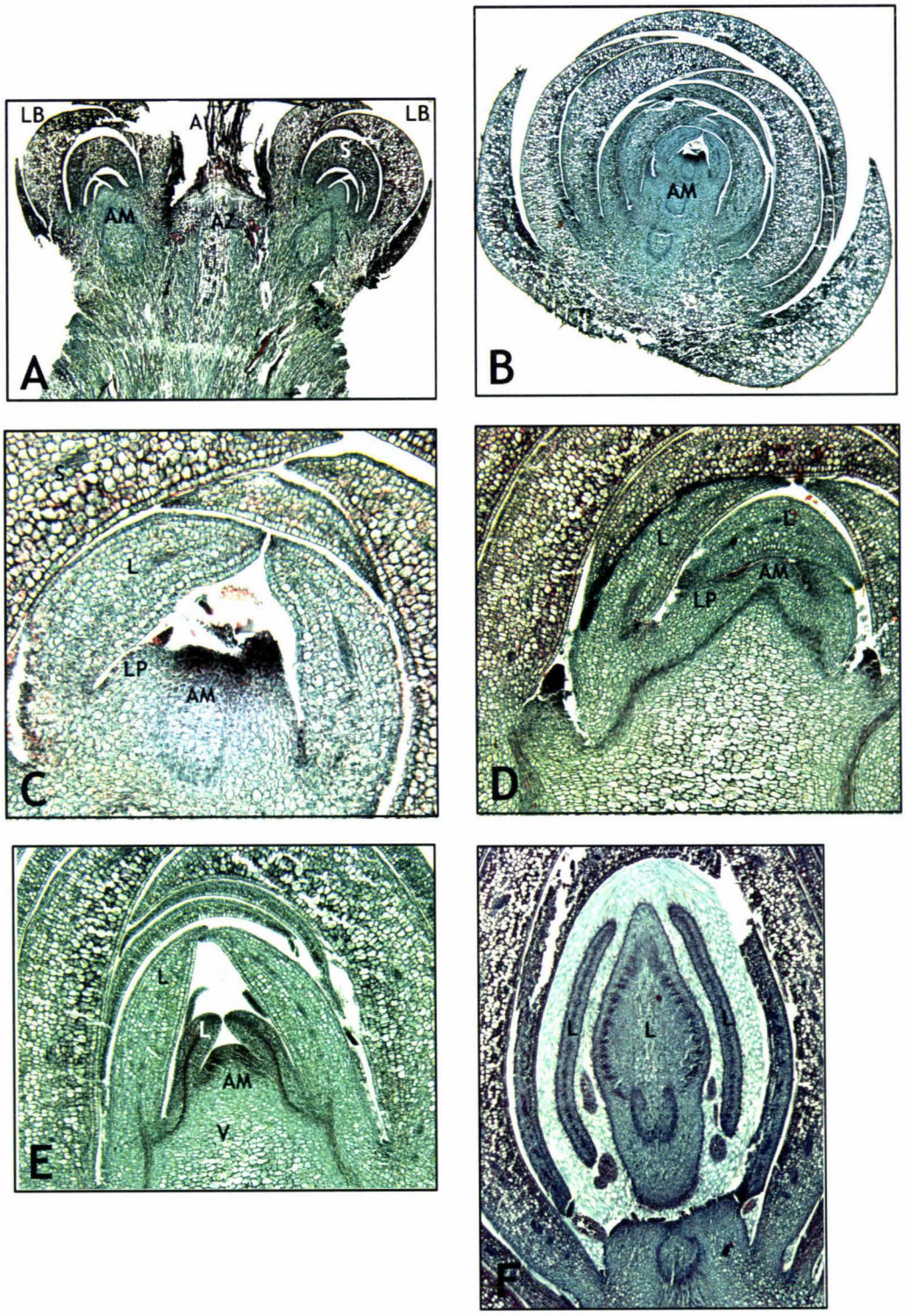
The sequence of bud development for *M. collina* cv. Tahiti vegetative buds in relation to bud size is displayed in **Figures 3.6 A – F**. When the buds are less than 2 mm in diameter (**Figure 3.6 A**), the apical meristem and bud scales of each lateral bud is visible. Also seen in **Figure 3.6 A** is the abscission zone and an apical bud that is incompletely abscised.

At 3 mm in diameter (**Figure 3.6 B** and **3.6 C**), the budding of a new leaf primordium is observed. **Figure 3.6 C** is a higher magnification image of the meristem seen in **Figure 3.6 B** to show the leaf primordia and apical meristem in more detail.

Table 3.1 Percentage of vegetative and floral buds present in the group of *M. collina* buds collected for histological analysis on the 10th of March, 2003.

Bud size	Vegetative	% Vegetative	Floral	% Floral
<2 mm	5	100	0	0
3 mm	2	40	3	60
4 mm	3	60	2	40
5 mm	2	40	3	60
6 mm	1	20	4	80
7 mm	1	20	4	80
8 mm	1	20	4	80
9 mm	0	0	5	100

Figure 3.6 Histological analysis of vegetative bud development of adult *M. collina*. A sequence of vegetative bud development in relation to bud size was developed. **A**) Apical meristem and scales visible in lateral buds at less than 2 mm in bud diameter, 4 x mag. Also visible are partially abscised apical bud and its abscission zone. **B** and **C**) Apical meristem and leaf primordia visible at 3 mm in diameter, 10 x mag. and 40 x mag., respectively. **D**) Older leaves develop from leaf primordia at 4 mm in diameter, and vascular traces also present (**E** – 10 x mag.). **F**) Fully developed leaves present prior to bud break in 5 mm in diameter vegetative bud, 40 x mag. A = partially abscised apical bud, AM = apical meristem, AZ = abscission zone of apical bud, L = leaf, LB = lateral bud, LP = leaf primordia, S = bud scale, V = vascular trace.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At 4 mm in diameter (**Figure 3.6 D** and **3.6 E**), the vegetative meristem is actively producing leaf primordia, which develop into leaves as the shoot matures. Vascular traces are also seen (**Figure 3.6 E**). When the buds reach 5 mm in diameter (**Figure 3.6 F**) and above, leaves are fully developed and the meristem produces more leaves from leaf primordia, until vegetative bud break occurs.

Vegetative buds can start to break as early as reaching 4 mm in diameter. While most buds develop uniformly and start to break between 7 - 10 mm, some buds develop faster and are therefore smaller than others and so break 'early'. Very few vegetative buds grow larger than 10 mm in diameter before bud break occurs.

3.4.2 Floral bud development

The sequence of floral bud development for *M. collina* cv. Tahiti is displayed in **Figures 3.7 A – H**. There were no buds smaller than 3 mm in diameter with distinguishable floral characteristics. At 3mm in diameter (**Figure 3.7 A** and close up **Figure 3.7 B**) the apical meristem, cymule primordia and bud scales are present in the floral bud.

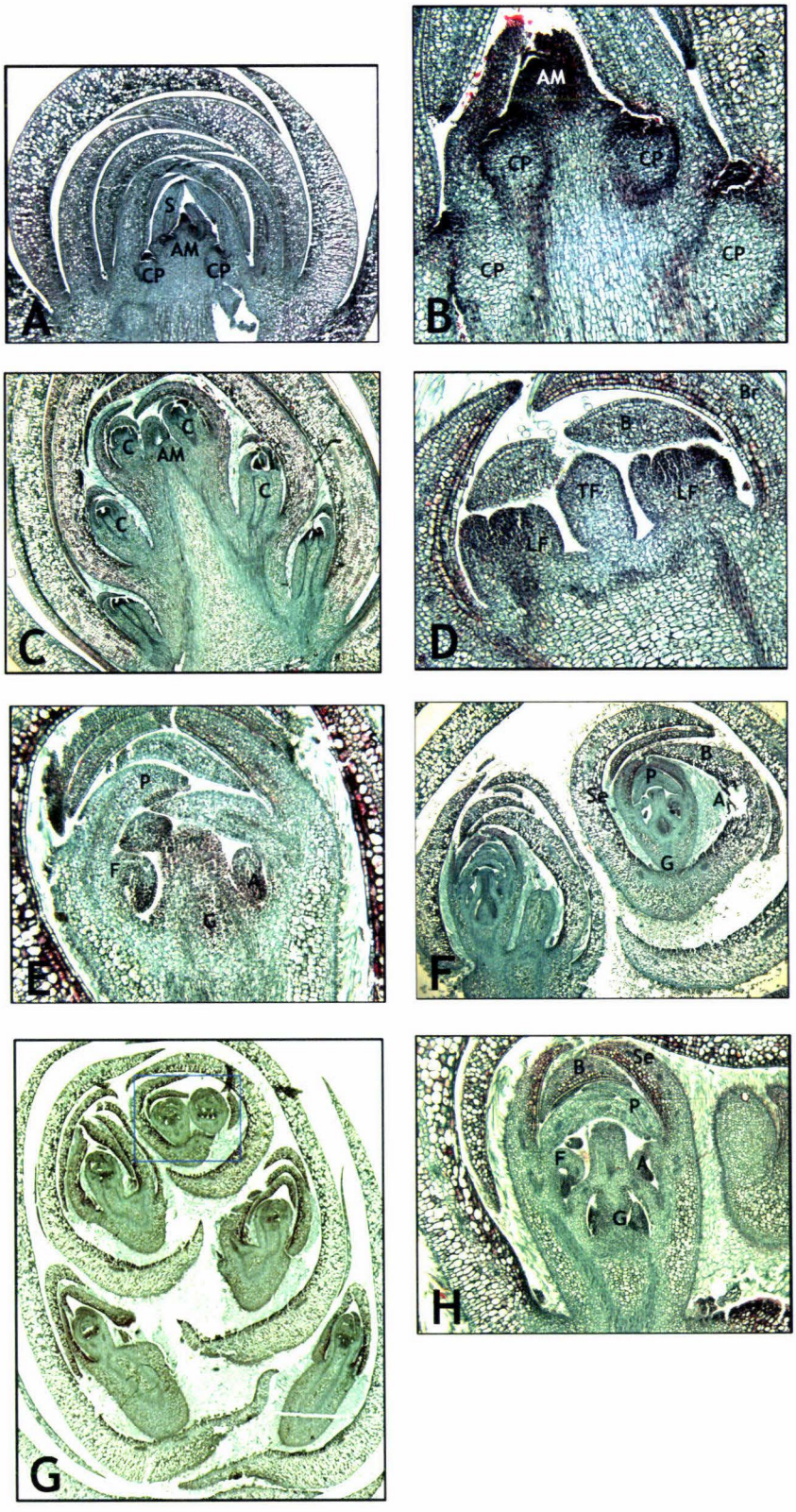
When the buds reached 5 mm in diameter (**Figure 3.7 C**), cymule primordia had differentiated into cymules. Each cymule was comprised of two lateral flowers flanking a terminal flower. It was observed that five to seven cymules were usually present in a section. Six cymules were visible in this bud. **Figure 3.7 D** is a higher magnification image of a single cymule primordium. Two lateral flowers and one terminal flower are seen in more detail. Bracteoles and bracts surround the developing flowers. As the bud matures, the stem that the cymules are attached to elongates while the cymules develop. Significant elongation of this stem is seen when the bud reaches 5 mm wide. At 7 mm in diameter (**Figure 3.7 E**), each lateral flower has differentiated into floral organs. The gynoecium, anthers, filaments, and petals can be observed and these structures continue to develop fully in the 8 and 9 mm buds.

When the buds reached 8 mm in diameter (**Figure 3.7 F**), the aforementioned floral organs continue to develop. Sepals are also visible. Two flowers in one cymule are pictured here. At 9 mm in diameter (**Figure 3.7 G**), the whole bud is shown. The blue outline indicates a single cymule, which contains two visible flowers. Five cymules are visible in this section.

As for vegetative buds, most floral buds follow this developmental program, but some are smaller and can develop faster and flower at a smaller size because they produce fewer cymules.

At a higher magnification one flower in a cymule of a 9 mm bud (**Figure 3.7 H**), the aforementioned floral structures are seen in better detail. The gynoecium including carpel, filaments and anthers, and petals, bracts and sepals are also still visible and further developed. The organs are fully distinguishable under a light microscope at this stage. As the floral buds mature, and move towards bud break, the cymules separate on their individual stems and the bud finally opens. The lateral buds further develop to produce large red blooms.

Figure 3.7 Histological analysis of floral bud development of *M. collina*. A sequence of floral bud development was established in relation to bud size and the presence of certain anatomical structures from buds collected on 10th March 2003. **A)** Cymule primordia budding from scale axes when buds are 3 mm in diameter 20 x magnification. **B)** Magnification of initial floral structures seen in A at 40 x mag. **C)** When bud reaches 5 mm in diameter; undifferentiated lateral and terminal flowers are observable inside each cymule (100 x mag.). **D)** 5 mm bud viewed at 10 x mag. Six cymules are visible. **E)** Differentiated filaments, petals and gynoecium are observed in one flower at 7 mm bud diameter (100 x mag.). **F)** Two flowers present in one cymule at 40 x mag. Gynoecium further developed than in E). **G)** One 9 mm floral bud showing five cymules. The box in **G)** surrounds one mature cymule in a 9 mm floral bud. There are three flowers usually present in one cymule, in this case only two are visible (10 x mag.). **H)** Well developed gynoecium, anthers, filaments, petals, bracts and bracteoles are present in 9 mm bud (100 x mag.). A = anther, AM = apical meristem, CP = cymule primordia, B = bracteole, Br = bract, C = cymule, F = filament, G = gynoecium, LF = lateral flower, P = petal, S = bud scale, Se = sepal, TF = terminal flower.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3.5 Discussion

3.5.1 Morphological and physiological changes resulted from applications of GA_3 and paclobutrazol

As shown by **Figure 3.1**, a significant decrease in shoot length and plant height resulted from multiple applications of paclobutrazol to micropropagated *M. excelsa* while the adult plants treated with GA_3 did not produce flower buds or show stem elongation. Whereas micropropagated and seedling *M. excelsa*, and micropropagated M. collina treated with GA_3 did exhibit elongated internodes compared to their water-treated partners (**Figure 3.2**), but neither produced floral buds prematurely. It was important to have observed a physiological response to determine whether the plant absorbed the plant growth regulator or hormone that was applied. The morphological and physiological changes that resulted from paclobutrazol and GA_3 treatment confirmed this. Changes in gene expression in response to plant regulator and hormone uptake are discussed in **Chapter Four**.

A distinction was made between micropropagated and seedling *M. excelsa* plants because seedling plants are 'true' juveniles. Micropropagation of perennial plants causes reversion of adult tissue to the juvenile phase (Brand and Lineberger, 1992). Juvenile characteristics of micropropagated *M. excelsa* are apparent as the leaves are more elongated than mature leaves, and there is an absence of hairs from the lower surface (Dawson, 1968). Previous research also performed on micropropagated *M. excelsa* showed that the application of GA_3 did not hasten flowering, although stem elongation occurred (Sreekantan *et al.*, 2004).

When GA_3 was exogenously applied to long day responsive *Arabidopsis* plants, even in conditions unfavourable for flowering, stem elongation occurred and the transition from vegetative to floral phase was hastened (Bagnall, 1992). This is unlike short day responsive perennials such as *Metrosideros* (Clemens *et al.*, 1995), *Eucalyptus*

(Moncur *et al.*, 1994), and grape (Bradley *et al.*, 1960) where GAs have been shown to inhibit flowering. Noteworthy, is the fact that when GA is applied to short day responsive *Arabidopsis* plants, no transition to floral meristem is observed (Pharis and King, 1985).

In previous studies with GAs and woody perennial plants (i.e. apples and grapes) it was discovered that the application of some GAs delayed the transition to flowering (Looney *et al.*, 1985; Bradley *et al.*, 1960) and, therefore, fruit production. Previous research with *M. collina* also indicated that GA had an inhibitory effect on the transition to flowering as the application of florigenic GAs delayed flowering and inhibited bud break (Clemens *et al.*, 1995). In this study, the lack of floral bud production observed on adult *M. excelsa* and *M. collina* plants after GA_3 application correlates with this.

Growth retardants such as paclobutrazol have been used to decrease the levels of endogenous GAs in *Eucalyptus* and promote flowering. This was used in combination with cold temperatures since treated plants kept in warmer temperatures did not flower (Moncur and Hasan, 1994). However, in this case, juvenile *M. excelsa* plants treated with paclobutrazol did not exhibit hastened floral development.

3.5.2 *M. collina* bud development may be linked to temperature

It was clearly shown in **Figure 3.4** that as vegetative and floral buds matured, defined as an increasing bud diameter, the amount of bud break also increased. Floral buds indicate a determinate growth habit that terminates shoot growth and inhibits further development of the particular shoot. This is unlike the growth habit for vegetative buds, which is indeterminate, as a vegetative bud will develop and elongate into a shoot with its own axillary buds. It was not possible to relate bud break directly to bud diameter from **Figures 3.4** and **3.5**, even though these figures show that bud break increases as bud diameter increases. It was not a direct correlation because the 20% of buds that had started breaking, in **Figure 3.4**, would not have been 4 mm in

diameter when they broke, but would have been larger in diameter.

Since the majority (91%) of Set 2 buds developed during the summer (between 18/11/02 and 25/01/03 – **Figure 3.3 B**), this leads to the suggestion of a 'summer development to winter flowering' program. Warm temperatures appear to be conducive to bud development, whether floral or vegetative. However, there were more buds that developed into floral buds in Set 2 (28%) than in Set 1 (5%) (Compare **Figure 3.3 C** to **A**). Therefore, warm temperatures and/or long photoperiods could be inductive for floral development and flowering as they are for the continuation of *M. excelsa* floral development after stalling.

The colder temperatures from April 2003 to July 2003 slowed the growth of the Set 3 buds. These buds were initiated in the cold months and may have broken around the same times as the Set 1 buds in the next year. If this is the case then there is a clear division of two bud developments for *M. collina* per year. Measurements over a longer time period would be more conclusive. The buds in Set 2 (**Figure 3.5**) that became floral were initiated much earlier than the buds in Set 2 that became vegetative (December 2002 for floral buds, compared with July 2003 for vegetative buds). It is possible that the Set 3 buds initiated in April would be floral, not vegetative.

This indicated that the 'decision to be floral' is probably 'made' at bud initiation. This 'decision' could be temperature dependent if the early buds were initiated during warm temperatures, compared with the later bud initiation in cooler temperatures, which promoted the development of vegetative buds. Or this 'decision' could have been made earlier by the Set 1 vegetative buds to produce floral buds, so floral buds were the first ones to develop in Set 2. It is possible that warmer temperatures could be responsible for initiating floral bud growth as *M. collina* buds were observed to flower in warmer temperatures. In colder temperatures, it was observed that the plant delayed flowering until warm temperatures occurred. During winter, in a week that

was particularly warm, certain floral buds that appeared to be stalled in their development at approximately 20 mm flowered.

Set 2 floral bud data (**Figure 3.5**) shows that while floral bud initiation is earlier than the initiation of vegetative buds, the development and bud break takes longer because floral buds grow to a larger size before breaking. Also, more specific organs and structures must develop (**Figure 3.7**).

Floral bud growth in Set 2 increased steadily from buds that were less than 1 mm at initiation until they reached a size approximately between 20 and 25 mm, at which point the cymules in the bud are fully developed and separated (protruding on their individual stems waiting to flower) just prior to flowering (stamens showing). Flowering could occur earlier than 20 mm. Bud break began rapidly when small floral buds (40%), with no more than three cymules, flowered. The rest of the buds flowered from this point.

M. collina appeared to not require a stimulus to promote flowering as it went through two 'cycles' of bud development. However, warm temperatures could play a key role in promoting flowering. Bud development may be slower in the colder months compared to the rapid floral development observed in the warmer months (**Figure 3.4**, Set 2 buds).

3.5.3 *M. collina* bud developmental sequence

A temporal developmental calendar of bud development cannot be attained for *M. collina* as for *M. excelsa* (Sreekantan *et al.*, 2004) because *M. collina* does not respond seasonally to photoperiod like *M. excelsa*. Instead, a way of predicting bud developmental stage by measuring bud diameter was devised. However, bud developmental stage did not relate well to bud size due to some buds developing rapidly and flowering at a smaller size. The developmental stages of both vegetative and floral *M. collina* buds have been successfully documented.

At 1-2 mm at the widest part of the bud, the buds cannot be distinguished morphologically as floral or vegetative. This stage appears to be a very rapid developmental stage in *M. collina* as there were no floral buds found in this stage of development when analysed histologically. The first sign of floral organ initiation was observed when bud diameter had reached 3mm (**Figure 3.7a** and close up **Figure 3.7b**) and cymule primordia were more developed when compared to *M. excelsa*. There were many buds at this stage of development, which indicates this stage is a prominent stage of development in *M. collina*. This is unlike the first stage of *M. excelsa* floral bud development, which was anatomically discernible by May and involved the development of floral initials in the axils of the bud scales. The initials were stalled in their development over the winter chilling period after which rapid organogenesis continues (Sreekantan *et al.*, 2001). At the time the *M. collina* buds were collected for histological studies, **Figure 3.5** shows that the average bud diameter of floral buds was approximately 3 mm.

Unlike *M. excelsa* whose floral buds develop at a particular time of year and are morphologically different to the vegetative buds, the floral buds of *M. collina* appear to develop alongside the vegetative buds and are indistinguishable until they reach around 5 mm in size, on average. At this stage, the cymules can be felt through the bud scales as hard, circular protrusions, compared with the vegetative buds at this stage, which feel flat and soft.

If floral inductive signals are perceived in the young expanding leaves, then *M. collina* may flower through out the year because it has continuous vegetative flushes alongside floral development. *M. excelsa* only has an autumn vegetative flush which correlates with autumn floral initial development.

During histological studies, all bud stages were observable. This is unlike *M. excelsa,* which exhibits highly synchronous bud development. *M. collina* appears to

be similar to *M. excelsa* in that the floral buds may stall briefly in their development during cold temperatures, but *M. collina* floral buds stall at random developmental stages and only until a warmer temperature is perceived.

CHAPTER FOUR

Isolation, identification, and expression of floral meristem identity genes in *Metrosideros*

4.1 Introduction and hypotheses

Previous research into flowering in *Metrosideros* has described the isolation of partial *LFY* and *AP1* orthologues from *M. excelsa*, named *MEL* and *MESAP1*, respectively. *MEL* expression has been shown to be upregulated by GA_3 application in juvenile *M. excelsa* plants. However, flowering was not induced in response to the GA_3 application. *MEL* expression during floral organogenesis was also similar to that of *Arabidopsis* (Sreekantan *et al.*, 2004).

This chapter describes the isolation and identification of floral meristem genes, *MCL* and *MTAP1*, from *M. collina*. These are homologues of *MEL* and *MESAP1*, respectively, from *M. excelsa*. The homology of these isolated nucleotide sequences and putative amino acid sequences and their evolutionary relationships to other amino acid sequences is also discussed. The expression of *MEL* and *MESAP1* in *M. excelsa*, and *MCL* and *MTAP1* in *M. collina*, in response to the application of GA_3 and other growth regulators and plant hormones, and the differences between these genes and their homologues in *Arabidopsis* is also described.

Hypotheses tested in this section of work were that the floral meristem identity gene homologues of *LFY* and *AP1* in *M. excelsa* and *M. collina* would be upregulated in adult and juvenile *Metrosideros* in response to GA_3 application, and the response would be GA specific. Also, the *Arabidopsis* model for the transition from vegetative to floral development would be useful for analysing gene expression during floral transition in *Metrosideros* species.

4.2 Development and optimisation of protocols

4.2.1 Comparison of DNA extraction methods

The CTAB maxi preparation (Reichardt and Rogers, 1994) (**Section 2.4.1**) was a good method for extracting large amounts of DNA from *M. excelsa* and *M. collina* vegetative tissue. Although this protocol included steps for the removal of phenolic compounds and polysaccharides present in *Metrosideros* leaves, the DNA preparations resulting from this method were brown in colour and of high viscosity. Altering the NaCl concentration should have been sufficient to remove the polysaccharides and phenolics from the DNA solution before the final centrifuge to pellet the DNA.

In this protocol, the DNA is not washed with ethanol until after the final centrifugation, which is too late to remove contaminating compounds associating with the DNA. Therefore, other DNA extraction methods were trialled.

The cesium chloride density centrifugation method of Richards (1999) (**Section 2.4.2**) did not yield any DNA. The DNA did not accumulate as a band in the cesium chloride gradient and, therefore, could not be recovered from the tube using the hypodermic needle and syringe.

The CTAB mini preparation protocol (**Section 2.4.3**) was recommended by Andrew Clarke from the Allan Wilson Centre, Institute of Molecular Biosciences, Massey University, Palmerston North. This method was modified slightly to suit *Metrosideros* tissue and proved to be the best method for extracting DNA from both *Metrosideros* and *Arabidopsis*. In this method, the DNA was washed repeatedly in ethanol until as much colour as possible had been removed from the *Metrosideros* DNA. Washes of the DNA in 80% ethanol have proved very useful for removing contaminants and reducing the viscosity of the solution.

A comparison of DNA yield and quality using the aforementioned DNA extraction protocols are shown in **Table 4.1.**

4.2.2 Comparison of RNA extraction methods

The 'Hot Borate' maxi RNA extraction method of Wilkins and Smart (1996) (**Section 2.5.1**) was a good method to prepare a large amount of *Metrosideros* RNA from a large amount (1 g) of starting material. Requiring a large amount of starting material can bee a disadvantage. Another drawback of this method was the length of time (3 days) needed to complete the RNA extraction.

Other methods that took shorter times, used less equipment and had fewer steps were tested. The 'Hot Borate' mini preparation (**Section 2.5.2**) was modified from Wilkins and Smart (1996) for use with smaller amounts of sample and more samples could be completed in one day. This method produced high quality RNA from both *Metrosideros* and *Arabidopsis* vegetative tissue and was subsequently used for RT-PCR. Compared to the 'Hot Borate' maxi preparation (Wilkins and Smart, 1996) this method was the preferred method as it yielded enough RNA for the subsequent cDNA synthesis and took less time.

TRI™-reagent (Molecular Research Centre, Inc) (**Section 2.5.3**) was also used to extract RNA from *Metrosideros* tissue. No RNA was extracted successfully from *Metrosideros* using this reagent.

A comparison of RNA yield and quality of RNA extracted from the above methods are shown in **Table 4.2.**

Table 4.1 A comparision of quantity and quality of various DNA extraction methods.

DNA extraction method	DNA quantity $\mu g\ \mu L^{-1}$	DNA quality
CTAB maxi prep (Reichardt & Richards, 1994)	2.42	1.8
Cesium chloride density centrifugation	0	0
Modified CTAB mini prep	1.23	2.0

Table 4.2 A comparison of quantity and quality of various RNA extraction methods.

RNA extraction method	RNA quantity $\mu g\ \mu L^{-1}$	RNA quality
'Hot Borate' maxi prep (Wilkins & Smart, 1996)	4.6	1.7
Modified 'Hot Borate' mini prep	2.1	2.0
TRI-Reagent	0	0

4.3 Nucleotide sequences

4.3.1 *LFY*-like sequences

A partial *LFY* sequence was first isolated from *M. excelsa* by McKenzie *et al.* (1997) by RT-PCR using degenerate primers and named *MEL*. The primer sequences were:

LFY forward primer	5' GCGAATTCACIAAYCARGTITTYMGIYAYGC 3'
LFY reverse primer	5' CGGATCCGTGICKIARIYKIGTIGGIACRTA 3'

Using the same primers, a partial orthologue of *LFY* was isolated from adult *M. collina* cv. Tahiti vegetative tissue using RT-PCR (**Figure 4.1**). It has been named *M. collina LFY-like* (*MCL*). After sequencing, the *MCL* nucleotide fragment was determined to be 249 bp. However, this fragment runs close to the 300 bp marker in the 1kb$^+$ ladder. The nucleotide sequence for *MCL* is given below.

M. collina LFY-like sequence (MCL) 249 bp:

CGGGGGGGGGGGTGGGCATTGAAGATAACGTCGATGTCCCAGTTGGCCCTGCGGCCGGC
GATTGTAACCAGGGGGTGGTAGCAGGCTTGCCACTAGGCGCAAACGTTCTCCCCGCGCTC
CTTGAAGCTCTTGCGGAGGGCGTTGGAGGCGTGCAGGGCGTAGCAGTGGACGTAGTGCCT
CATCTTCGGCTGGTTTATGTACCTTGCTCCCGCCTTCTTCGCTTCCCCAAACACCTGATTCG
GAAATCCC

The results of a nucleotide BLAST (BLASTn) separated this sequence into two uneven sections. The first 140 nucleotides showed homology to other *LFY*-like sequences. There was a gap of between two and 10 nucleotides, then the remaining nucleotides showed homology to other *LFY*-like sequences. The two part sequences showed 94% and 97% homology to *MEL* (*M. excelsa*), 91% and 95% homology to *ELF1* (*E. globulus*), and 94% and 81% to *LFY1* (*E. grandis*). The nucleotide gap can also be observed as a gap in the amino acid sequence in **Figure 4.2**.

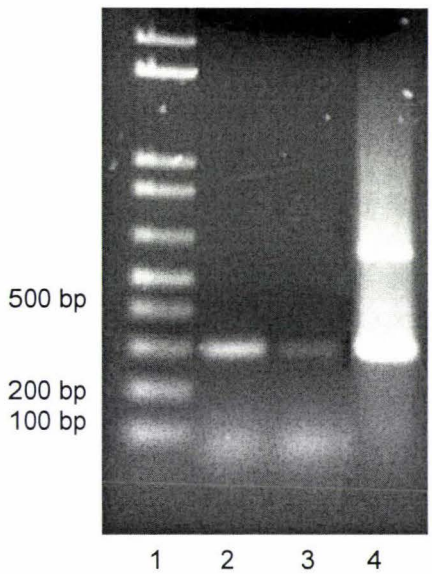


Figure 4.1 Isolation of a partial *LFY* equivalent from *M. collina*. Using degenerate primers, a partial orthologue of *LFY* was isolated from adult *M. collina* cv. *Tahiti* vegetative tissue using RT-PCR. It has been named *M. collina LFY-like* (*MCL*). After sequencing, the *MCL* nucleotide fragment was determined to be 249 bp. However, this fragment runs close to the 300 bp marker in the 1kb$^+$ ladder. Lane 1: 1 kb$^+$ ladder; lanes 2 and 3: duplicate RT-PCR reactions using *M. collina* RNA; lane 4: positive control (*MEL* fragment cloned into pGEM-T Easy by McKenzie *et al.* 1997).

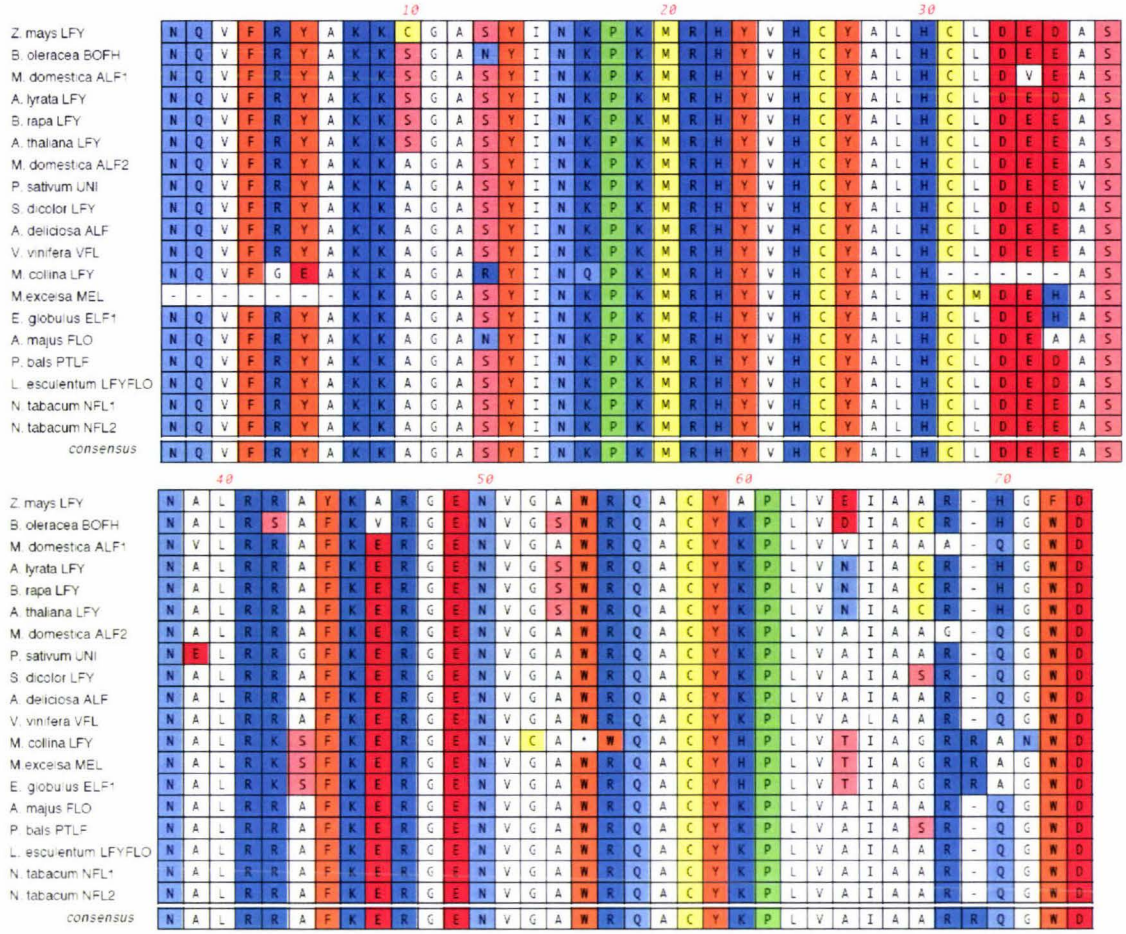


Figure 4.2 Comparison of deduced amino acid sequence of the putative LFY-like sequence from *M. collina* and LFY sequences from other plant species. The alignment was produced using the MacVector program (Accelrys). Identical amino acid residues are coloured the same colour in each sequence. Dashed lines indicate gaps introduced by the program to achieve maximal alignment.

4.3.2 *AP1*-like sequences

A partial equivalent of the *Arabidopsis AP1* gene was isolated from adult *M. collina* vegetative tissue (**Figure 4.3**) using RT-PCR with degenerate primers that had been designed to isolate the *MESAP1* fragment from *M. excelsa* (Sreekantan *et al.*, 2001). The primer sequences were:

AP1 forward primer	5' CAAGCTTGAAGAGGATAGAG 3'
AP1 reverse primer	5' GCTTAAGAGCAGTATCAAGCTG 3'

The nucleotide sequence for MTAP1 is shown below.

M. collina AP1-like sequence (MTAP1) 303 bp:

CTTTGAAGCTTAGGATCAAGTTCTTCTCCCATCAGATTCTTTTGATTTTTCTGCAAAATCTC
CATCCTGGCCTTGAGTTTTGCATGTTCCAAAGTCCAGTTCCCATTGGTTTCGGCGTTGTTTG
TGAGAATCTGTCTCTCTGCATATGAATATCTTTCATAGCGCTCAAGGATCTTCTCCATGCA
AGCATCGGTGGCGTACTCGAAGAGCTTGCCCTTGGTGGAGAAGACGATGAGGGCGACCTC
CGCGTCGCAGAGGACGGAGATCTCGTGGGCCTTCTTCAGCAGCCCACATCTCGAATTC

This sequence had 100% nucleotide sequence homology to *MESAP1* (*M. excelsa*), 94% homology to *EAP2L* and *EAP2S* (*E. globulus*), and 91% homology to *EAP1* (*E. globulus*).

4.4 Amino acid sequences

4.4.1 LFY-like sequences

The translated amino acid sequence for the predicted MCL fragment is given below:

1 NQVFGEAKKAGARYINQPKMRHYVHCYALHASNALRKSFKERGENVCAWQ
ACYHPLVTIAGRRANWDIDVIFNAHP 76

A comparison of the amino acid sequence of the MCL fragment with other *LFY*-like amino acid sequences using a translated BLAST (BLASTx) (GenBank) showed that

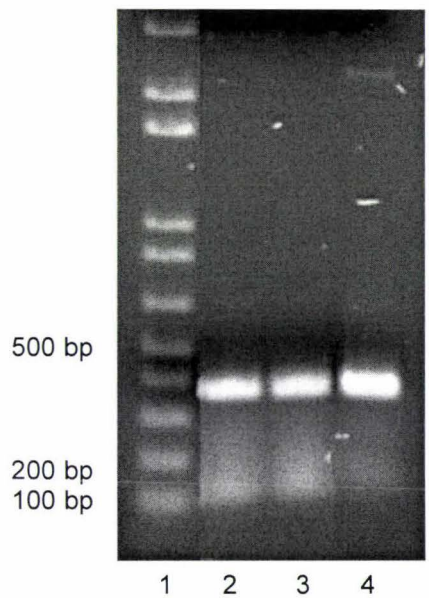


Figure 4.3 Isolation of a partial *AP1* equivalent from *M. collina*. A partial equivalent of the *Arabidopsis AP1* gene was isolated from adult *M. collina* vegetative tissue using RT-PCR with degenerate primers that had been designed to isolate the *MESAP1* fragment from *M. excelsa*. Lane 1: 1 kb^+ ladder; lanes 2 and 3: duplicate RT-PCR reactions using *M. collina* RNA; lane 4: positive control (*MESAP1* fragment cloned into pGEM-T Easy by Sreekantan *et al.* 2001).

it shared 84% amino acid identity with MEL (*M. excelsa*), 82% identity with ELF1 (*E. globulus*), 81% identity with LFY/FLO (*E. grandis*). The amino acid identity and similarity with other species is shown in **Table 4.3**.

An alignment of the translated MCL fragment with LFY-like amino acid sequences from other species is shown in **Figure 4.2**. The alignment was produced using the MacVector program (Accelrys).

The putative amino acid sequence of the MEL fragment is dissimilar to that of other LFY-like sequences in that it has an amino acid change from leucine to methionine at the 32nd position (Sreekantan *et al*, 2004). The MCL amino acid fragment is different to the MEL fragment. There are gaps from the 31st to the 35th amino acid. There are however, similarities between sequences from the Myrtaceae family members, *Metrosideros* and *E. globulus*. At the 43rd position in the MCL sequence, there is a change from an alanine to a serine. At the 64th position in the MCL sequence there is a threonine. At this position in other orthologues the amino acid ranges from E, V, N, A. Members of the Brassica family in the alignment all have asparagine. But only the Myrtaceae family members have the threonine (**Figure 4.2**).

4.4.2 *API*-like sequences

The predicted amino acid sequence for the MTAP1 amino acid fragment is given below:

1 RCGMLKKAHEISVLFDAEVXLIVFSTKGKLFEYATDPCMEKILESYERYS
YAERQILTNNAETNGNWTLEHAKLKARREFLQKNQKNVMEKNL 93

When putting this sequence into a translated BLAST (GenBank) to compare amino acid identity and similarity of this sequence with AP1-like sequences from other species, the MTAP1 sequence showed 100% identity with MESAP1 (*M. excelsa*), 94% identity with both EAP2L and EAP2S (*E. globulus*), and 80% identity with

Table 4.3 The percent amino acid identity and similarity of the MCL fragment and other LFY-like amino acid sequences using a translated BLAST (BLASTx) (GenBank).

gi number	Translated sequence	% Identity	% Positives
gi|3098307	*E. globulus* ELF1	82	84
gi|49615781	*E. grandis* LFY1	81	82
gi|2258406	*M. excelsa* MEL	84	85
gi|166430	*A. majus* FLO	74	79
gi|7658237	*L. esculentum* LFY/FLO	74	79
gi|23193454	*V. vinifers* VFL	73	79
gi|6010659	*A. deliciosa* ALF	74	79
gi|28912458	*P. tremuloides* LFY	74	79
gi|29423802	*S. dicolor* LFY/FLO	74	79
gi|18424517	*A. thaliana* LFY	83	89
gi|20563244	*A. lyrata* LFY	83	89

MADS5 (*B. pendula*). The percent amino acid identity and similarity of the MTAP1 sequence with other AP1-like sequences is shown in **Table 4.4**.

An alignment of the predicted MTAP1 amino acid sequence and other AP1-like sequences was produced using MacVector (Accelrys) and is shown in **Figure 4.4**.

The putative MTAP1 amino acid also shows similarities between Myrtaceae family members, *Metrosideros* and *E. globulus* EAP2S and EAP2L. The amino acid at the 2nd position in the MTAP1 sequence is a cysteine, compared to serine, aspartate, or glycine in this position in other species. At the 34th position there is a change from a serine to an alanine in these Myrtaceae sequences. Threonine instead of an alanine or serine at position 58, asparagine instead of a glutamic acid or aspartic acid at position 60, a grouping of alanine at position 61, a group of threonine residues at position 63, a group of asparagine at position 66 also seen in *Arabidopsis thaliana* and *Arabidopsis lyrata*, *Antirrhinum*, and *Brassica*, a grouping of methionine residues instead of isoleucine, leucine and valine (hydrophobic) at position 78. A change specific to *Metrosideros* is an alanine in *Metrosideros* at position 37, which is a cysteine in *Eucalyptus*, and a serine in other sequences. Position 54 shows an arginine in all sequences including *Metrosideros*, but exclusive of *Eucalyptus*, which has serine (**Figure 4.4**).

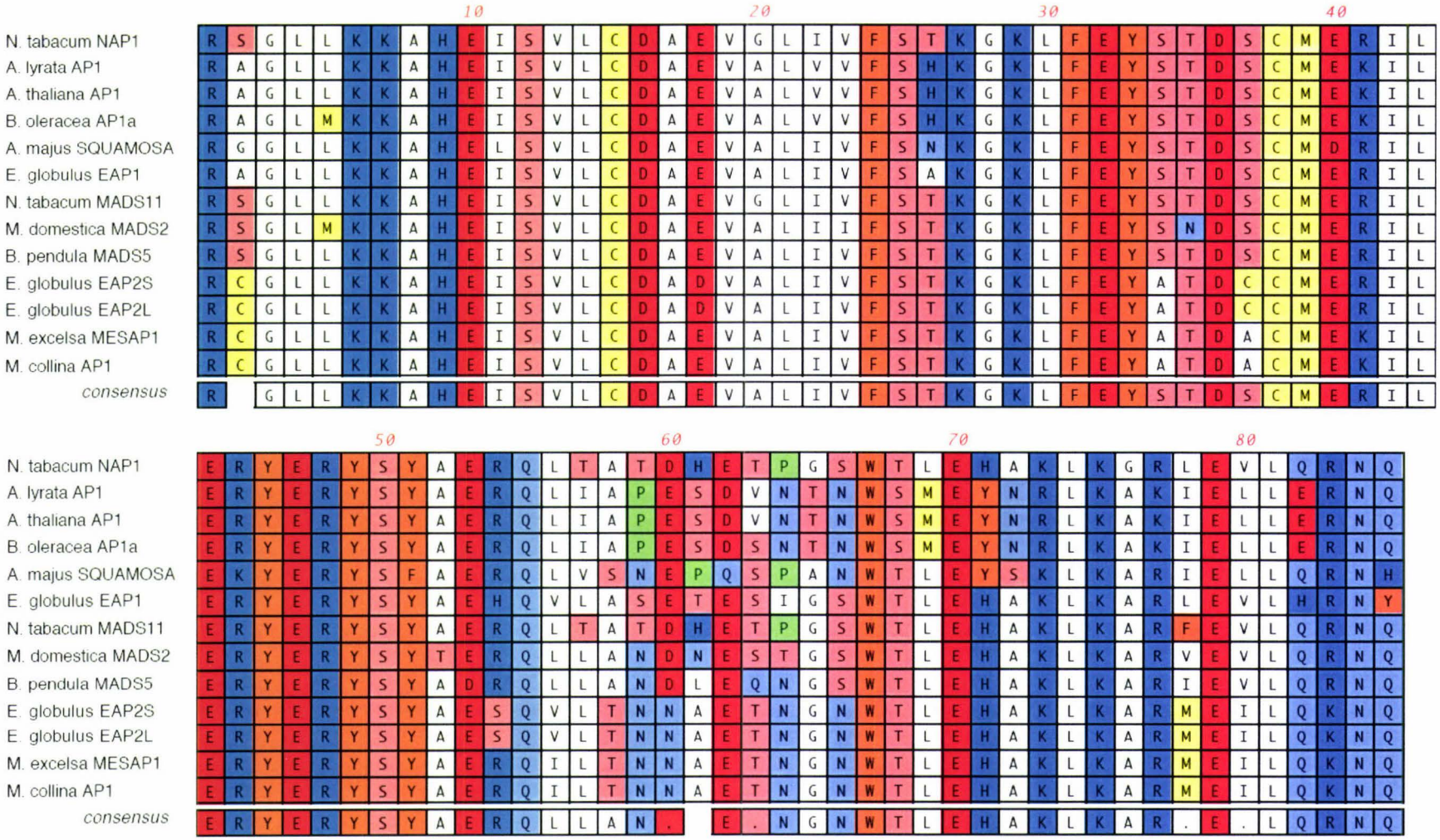
4.5 Phylogenetic relationships

To establish the evolutionary relationships between putative amino acid sequences isolated from *M. collina* and published sequences of these genes from other plant species, phylogenetic trees were produced using ClustalW (http://www.ebi.ac.uk). Comparisons were made using alignments of partial amino acid sequences extracted from GenBank with a BLAST search using the putative *MCL* and *MTAP1* translated sequences. The phylogenetic trees were constructed using the Neighbour Joining (NJ) method and show bootstrap values that enable the estimation of confidence that

Table 4.4 The percent amino acid identity and similarity of the MTAP1 sequence with other AP1-like sequences using a translated BLAST (BLASTx) (GenBank).

gi number	Translated sequence	% Identity	% Positives
gi27542946	*M. excelsa* MESAP1	100	100
gi11120557	*E. globulus* EAP2L	94	97
gi11037010	*E. globulus* EAP2S	94	97
gi1483232	*B. pendula* MADS5	80	94
gi3947985	*M. domestica* MADS2	76	92
gi27373049	*N. tabacum* MADS11	77	89
gi10946429	*E. globulus* EAP1	75	89
gi4102111	*N. tabacum* NAP-1	76	88
gi46949180	*V. vinifera* AP1	73	92
gi16052	*A. majus* SQUA	70	91
gi3646320	*M. domestica* MADS	71	89
gi207993702	*A. lyrata* AP1	68	90
gi16162	*A. thaliana* AP1	68	90

Figure 4.4 Comparison of deduced amino acid sequence of the putative AP1-like sequence from *M. collina* and AP1 sequences from other plant species. The alignment was produced using the MacVector program (Accelrys). Identical amino acid residues are coloured the same colour in each sequence. Dashed lines indicate gaps introduced by the program to achieve maximal alignment.


10
20
30
40
N. tabacum NAP1
A. lyrata AP1
A. thaliana AP1
B. oleracea AP1a
A. majus SQUAMOSA
E. globulus EAP1
N. tabacum MADS11
M. domestica MADS2
B. pendula MADS5
E. globulus EAP2S
E. globulus EAP2L
M. excelsa MESAP1
M. collina AP1
consensus
50
60
70
80
N. tabacum NAP1
A. lyrata AP1
A. thaliana AP1
B. oleracea AP1a
A. majus SQUAMOSA
E. globulus EAP1
N. tabacum MADS11
M. domestica MADS2
B. pendula MADS5
E. globulus EAP2S
E. globulus EAP2L
M. excelsa MESAP1
M. collina AP1
consensus

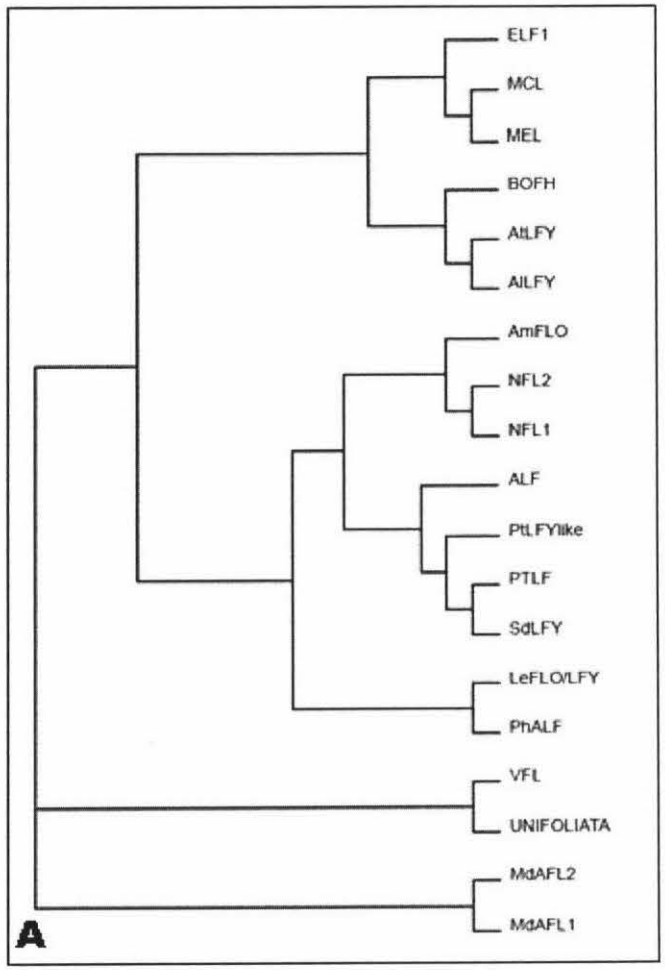
can be assigned to particular nodes in a phylogenetic tree.

The phylogenetic trees of LFY-like and AP1-like proteins are shown in **Figures 4.5** and **4.6**, respectively. Phylogenetic trees based on sequences from both annual and perennial plants are shown in **Figures 4.5** and **4.6 A** while trees based on sequences from perennial plants only are shown in **Figures 4.5** and **4.6 B**.

Phylogenetic analysis of the MCL and MTAP1 fragments from *M. collina* was performed to compare the evolutionary relationships between these sequences to orthologous sequences in other plant species. The phylogenetic tree of MCL provides evidence to support the close relationship between *Metrosideros* LFY- and AP1-like amino acid sequences with orthologues from other plants. **Figure 4.5 A** presents a phylogenetic tree of LFY-equivalents from a range of species, both annual and perennial. MEL and MCL are grouped here with ELF1 from *E. globulus*, another member of the Myrtaceae family. This supports evidence that *Metrosideros* and *Eucalyptus* are closely related species. **Figure 4.5 B** shows a tree of LFY equivalents from perennial species only. Again, MEL and MCL are grouped with ELF1, but also with the LFY-equivalent from *A. lyrata*, a perennial *Arabidopsis* species. See **Appendix II** for abbreviations of LFY orthologues.

MTAP1 and MESAP1 are also grouped together in **Figures 4.6 A** and **B**. **Figure 4.6 B** is a phylogenetic tree of annual and perennial AP1 sequences. MESAP1 and MTAP1 are grouped with EAP2L and EAP2S (refer to similarities in putative amino acid sequence **Figure 4.4**). In **Figure 4.6 B**, a phylogenetic tree of perennial sequences only, MESAP1 and MTAP1 are grouped together, but on the same root at EAP2L and EAP2S, indicating high similarity. See **Appendix II** for abbreviations of LFY orthologues.

Figure 4.5 The phylogenetic relationship of the putative LFY-like sequence from *M. collina* and LFY sequences from other plant species. CLUSTALW was used to produce the trees. **A)** Phylogenetic tree based on sequences from both annual and perennial species. **B)** Phylogenetic tree based on perennial sequences only. See **Appendix II** for abbreviations of LFY orthologues.


ELF1
MCL
MEL
BOFH
AtLFY
AlLFY
AmFLO
NFL2
NFL1
ALF
PtLFYlike
PTLF
SdLFY
LeFLO/LFY
PhALF
VFL
UNIFOLIATA
MdAFL2
MdAFL1
A

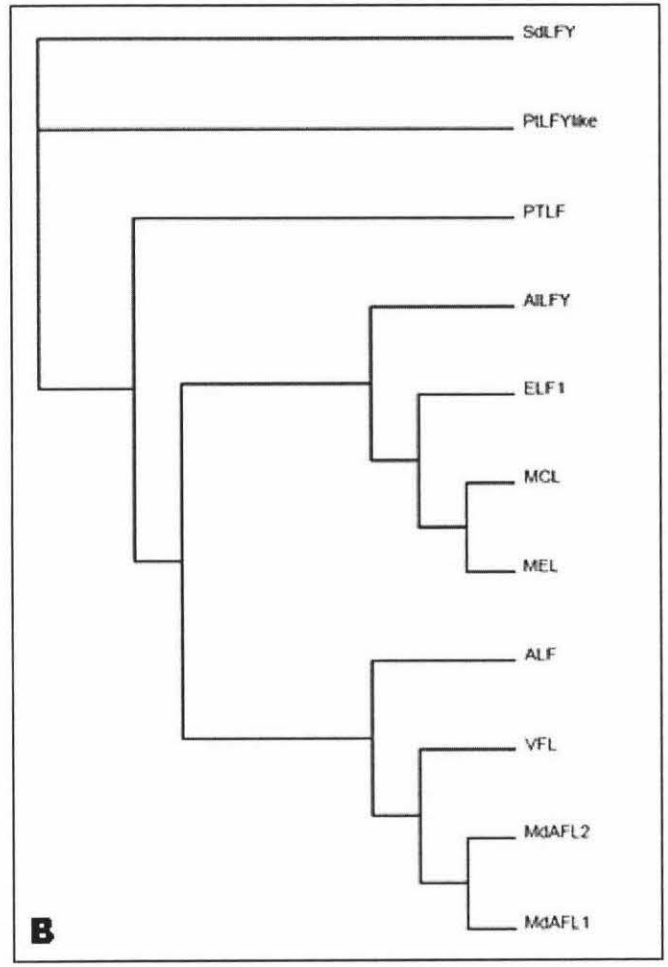
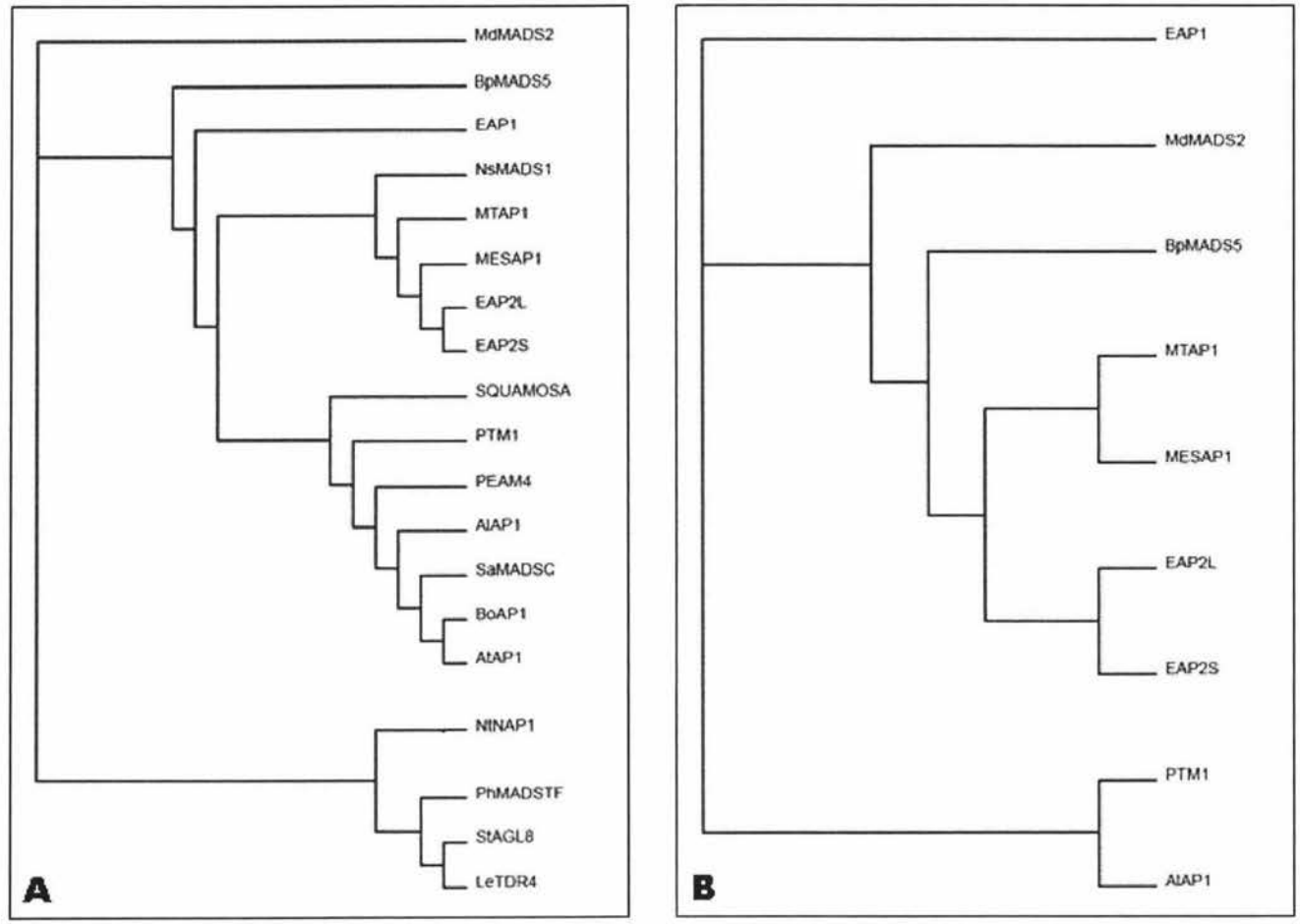

SdLFY
PtLFYlike
PTLF
AtLFY
ELF1
MCL
MEL
ALF
VFL
MdAFL2
MdAFL1
B

Figure 4.6 Phylogenetic relationship of the putative AP1-like sequence from *M. collina* and AP1 sequences from other plant species. CLUSTALW was used to produce the trees. **A)** A phylogenetic tree based on sequences from both annual and perennial species. **B)** A phylogenetic tree based on perennial sequences only. See **Appendix III** for abbreviations of AP1 orthologues.


MdMADS2
BpMADS5
EAP1
NsMADS1
MTAP1
MESAP1
EAP2L
EAP2S
SQUAMOSA
PTM1
PEAM4
AtAP1
SaMADSC
BoAP1
AtAP1
NtNAP1
PhMADSTF
StAGL8
LeTDR4
A
EAP1
MdMADS2
BpMADS5
MTAP1
MESAP1
EAP2L
EAP2S
PTM1
AtAP1
B

4.6 Southern blotting of *M. excelsa* and *M. collina* genomic DNA

4.6.1 Detection of *LFY*-, *AP1*-, and *TFL-1*-like genes

The aim of this was to develop a protocol for non-radioactive detection of Southern membranes in the lab as ^{32}P-labelled probes had been used previously. This non-radioactive method used probes labelled with alkaline phosphatase. The blotted membranes were exposed in the Fuji LAS 1000 Intelligent Dark box for various developing times. The positive controls were *MEL*, *MTAP1* and *TFL-1* fragments that had been amplified from pGEM-T vectors by PCR. The positive controls that transferred onto the nitrocellulose membranes produced strong signals after two hours of exposure (**Figure 4.7**). After four hours of exposure, the signals detected from the positive control bands were extremely strong but still no signal was detected from the genomic DNA.

4.7 Gene expression response to growth regulator and hormone applications in *Metrosideros*.

The expression of *MEL* and *MESAP1*, and *MCL* and *MTAP1*, from *M. excelsa* and *M. collina*, respectively, was analysed in response to the application of plant growth regulators and plant hormones. The *ACTIN* gene was used as loading control.

4.7.1 Expression of *MEL* and *MESAP1* in micropropagated, seedling, and adult *M. excelsa*.

The expression of *MEL* and *MESAP1* in *M. excelsa* micropropagated, seedling, and adult plants in response to the exogenous application of various plant growth regulators and plant hormones is shown in **Figure 4.8**. There was no expression of *MEL* in micropropagated *M. excelsa* in the water control (lane 2) or in plants treated with paclobutrazol (lane 3). The plants treated with 0.05 mg mL^{-1} GA_3 (lane 4), 0.5

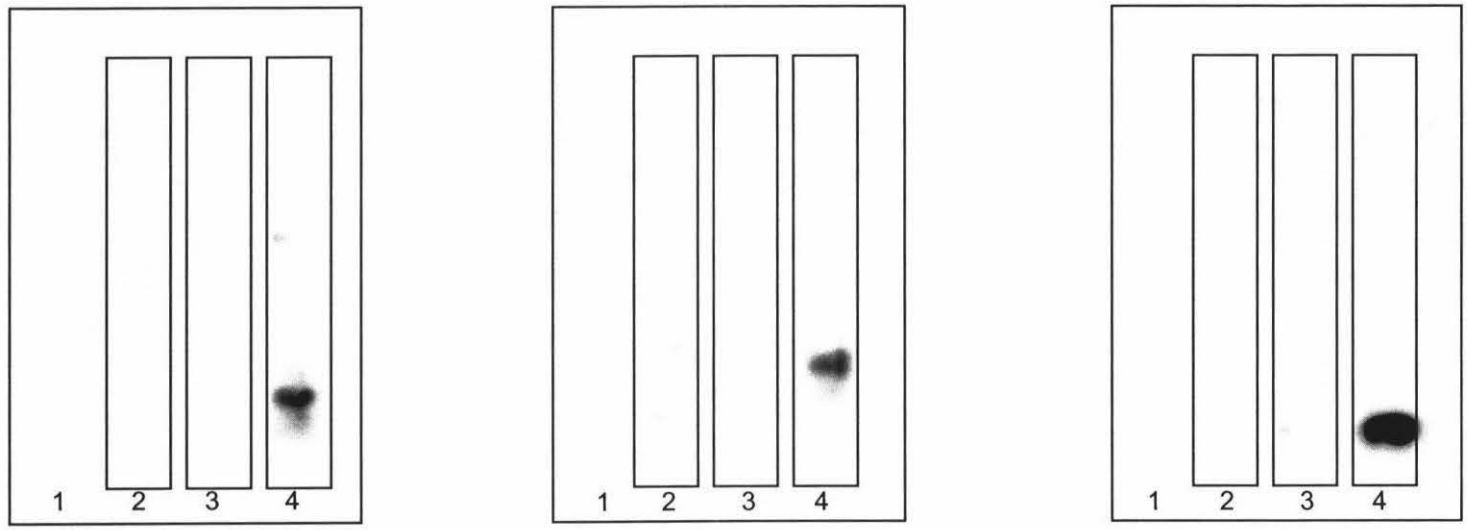


Figure 4.7 Southern blots of *M. excelsa* and *M. collina* DNA with probes made from A) *LFY*-, B) *AP1*-, and C) *TFL1*-like genes. No signal was detected in the lanes containing genomic DNA. In each blot, lane 1: 1kb^{+} ladder; lane 2: *M. excelsa* DNA; lane 3: *M. collina* DNA; lane 4: positive control (pGEM-T Easy vector containing *MEL*, *MESAP1* and *MTTFL1* fragments, respectively.

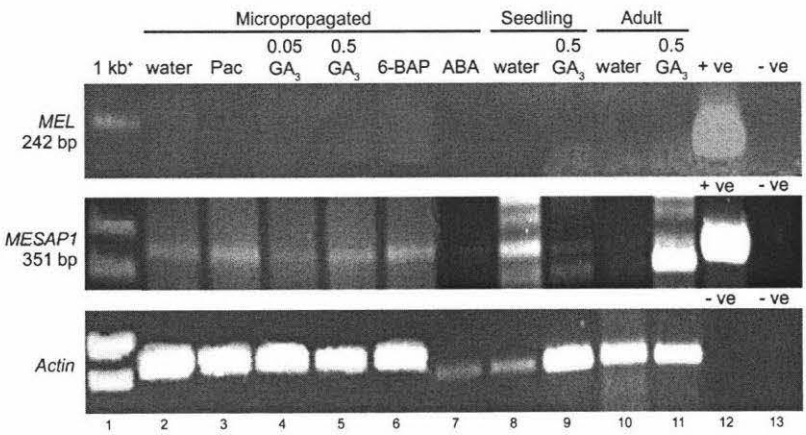


Figure 4.8 Expression of *MEL* and *MESAP1* in response to the exogenous applications of growth regulators and plant hormones to adult, seedling, and micropropagated *M. excelsa*. Lane 1: 1 kb^+ ladder; Lane 2: *M. excelsa* micropropagated control; Lane 3: *M. excelsa* micropropagated paclobutrazol-treated; Lane 4: *M. excelsa* micropropagated 0.05 mg mL^{-1} GA_3-treated; Lane 5: *M. excelsa* micropropagated 0.5 mg mL^{-1} GA_3-treated; Lane 6: *M. excelsa* micropropagated 0.5 mg mL^{-1} 6-BAP-treated; Lane 7: 0.5 mg mL^{-1} ABA-treated *M. excelsa* micropropagated; Lane 8: *M. excelsa* seedling control; Lane 9: *M. excelsa* seedling 0.5 mg mL^{-1} GA_3-treated; Lane 10: *M. excelsa* adult control; Lane 11: *M. excelsa* adult 0.5 mg mL^{-1} GA_3-treated; Lane 12: positive control for *MEL* and *MESAP1*, or negative control for RT reaction in *Actin* gel; Lane 13: negative control for PCR.

mg mL^{-1} GA_3 (lane 5), 0.05 mg mL-1 6-benzylaminopurine (6-BAP) (lane 6) and 0.05 abscisic acid (ABA) (lane 7) showed no significant *MEL* expression, just some smearing. There was no *MEL* expression seen in seedling (lanes 8 and 9) or adult *M. excelsa* samples (lanes 10 and 11) whether treated with water or GA_3.

There was *MESAP1* expression in all the *M. excelsa* micropropagated samples except for the ABA treated sample (lane 7). There was stronger expression seen in the 0.5 mg mL^{-1} GA_3 sample (lane 4) than the 0.05 mg mL^{-1} GA_3 treated sample (lane 5).

The water treated *M. excelsa* seedling (true juvenile) samples showed *MESAP1* expression while there is less *MESAP1* expression in the GA_3 treated seedling sample. There was no *MESAP1* expression seen in either of the adult samples (lanes 10 and 11). The very bright band seen in the GA_3 treated sample (lane 11) is not *MESAP1*, but a completely different gene.

The second, smaller band that appeared on the 1% agarose gels of *MESAP1* (**Figure 4.8**, lanes 9 and 11) had a small amount of high sequence homology to *M. excelsa AP1* (*MESAP1*) partial coding sequence found in GenBank (AY170871) with 23/23 identical nucleotides.

Other band	240	CCTTCTTCAGCAGCCCACATCTC	262
MESAP1	58	CCTTCTTCAGCAGCCCACATCTC	36

However, this sequence also corresponded to regions in *Mus musculus* hypothetical protein D030051D21, mRNA (cDNA clone MGC:76458 IMAGE:30437501), (GenBank Accession number BC076567), Human DNA sequence from clone RP11-218L14 on chromosome X (GenBank Accession number BX276110), and *Lycopersicon esculentum* clone 134978F, mRNA sequence (GenBank Accession number BT013309).

4.7.2 Expression of *MCL* and *MTAP1* genes in micropropagated and adult *M. collina.*

As shown in **Figure 4.9**, there was no expression of *MCL* in micropropagated *M. collina* vegetative tissue. However, *MCL* was expressed in water treated adult vegetative tissue, but not in GA_3 treated tissue. The same results were observed for *MTAP1* with the exception of the GA_3-treated micropropagated tissue (lane 3), which corresponds with the *MTAP1* expression in *M. excelsa* micropropagated plants (**Figure 4.8**). This result is contrary to Sreekantan (2003). There was no *MTAP1* expression adult GA_3 treated *M. collina.*

4.7.3 Temporal expression of *MCL* and *MTAP1* in adult *M. collina*

MCL was expressed in water-treated adult *M. collina* plants up to day 6 (lane 4) after water treatment. *MTAP1* was expressed at days 4 and 6 (lanes 3 and 4, respectively) after treatment with water (**Figure 4.10**). There was no *MCL* expressed in GA_3-treated adult *M. collina*. *MTAP1* was expressed from day 6 (lane 4) (**Figure 4.11**).

4.8 Discussion

4.8.1 *MCL* and *MTAP1* were isolated from *M. collina* cv. Tahiti

According to sequence information the *LFY*-like homologue, *MCL*, and the *AP1*-like homologue, *MTAP1*, were successfully isolated from *M. collina*. These sequences both show high nucleotide homology and amino acid identity to *MEL* and *MESAP1*, from *M. excelsa*, respectively, as isolated by Sreekantan *et al.* (2004). MTAP1 showed 100% amino acid identity to the MESAP1 translated sequence, while MCL showed 84% identity to MEL due to a gap in the MCL sequence (**Figure 4.3**). The MADS box regions of MESAP1 and MTAP1 were identical.

There were similarities and differences at the amino acid level between the *Metrosideros* translated sequences of MEL and MCL, and MESAP1 and MTAP1.

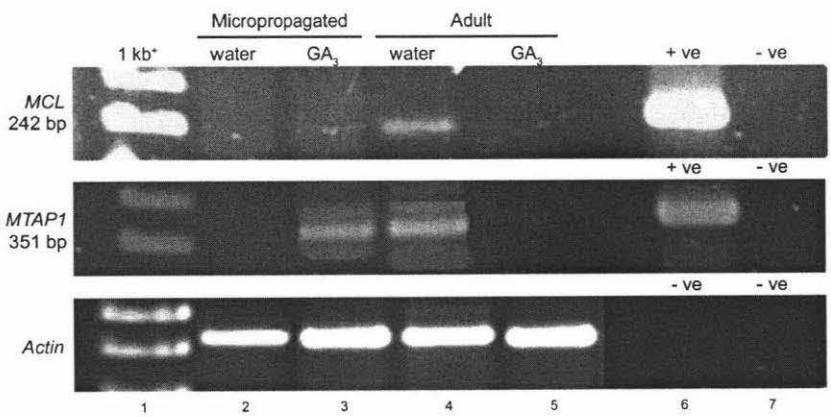


Figure 4.9 Expression of *MCL* and *MTAP1* genes in response to the exogenous application of GA_3 to micropropagated and adult *M. collina*. Lane 1: 1 kb^+ ladder; Lane 2: micropropagated control; Lane 3: micropropagated GA_3-treated; Lane 4: adult control; Lane 5: adult GA_3-treated; Lane 6: negative control for PCR; Lane 7: *MCL* and *MTAP1* positive controls, or negative control for RT reaction in *Actin* gel.

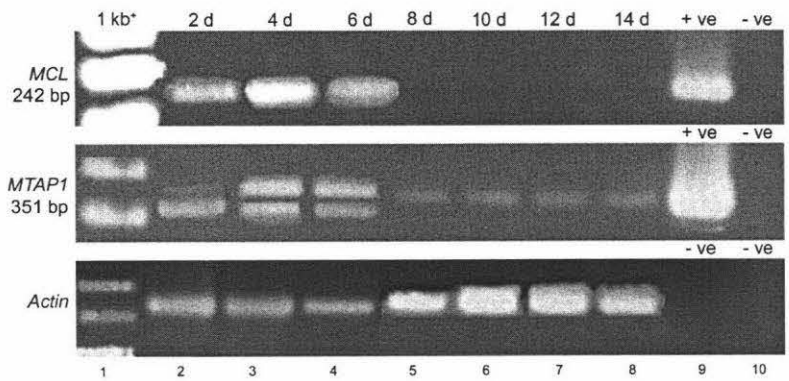


Figure 4.10 Temporal expression of *MCL* and *MTAP1* in adult *M. collina* in response to the exogenous application of water. Lane 1: 1 kb$^+$ ladder; Lanes 2 – 8: 2 days to 14 days subsequent to GA_3 application; Lane 9: *MCL* and *MTAP1* positive controls, or negative control for RT reaction in *Actin* gel; Lane 10: negative control for PCR.

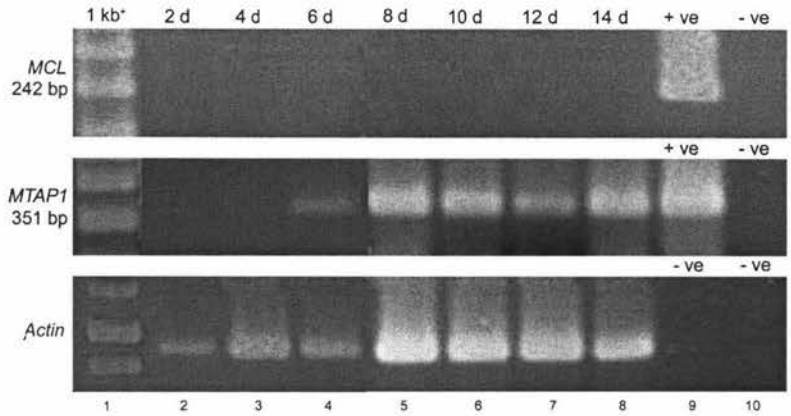


Figure 4.11 Temporal expression of *MCL* and *MTAP1* in adult *M. collina* response to the exogenous application of GA_3. Lane 1: 1 kb^+ ladder; Lanes 2-8: 2 days to 14 days subsequent to GA_3 application; Lane 9: *MCL* and *MTAP1* positive controls, or negative control for RT reaction in *Actin* gel; Lane 10: negative control for PCR.

There are also similarities and differences between sequences from plants in the same family, and from unrelated plants. Amino acid changes have consequences in protein folding and function, as essential hydrogen bonds between amino acids can be lost.

Sreekantan *et al.* (2004) phylogenetic trees of *MEL* and *MESAP1* showed similar results to the phylogenetic trees of *MCL* and *MTAP1* (**Figures 4.5** and **4.6**, respectively). *MEL* branched off with *ELF1*, and *MESAP1* branched off with *EAP2 L* and *EAP2 S* (Sreekantan *et al.*, 2004). The high relatedness shown in the phylogenetic trees of *MCL* and *MTAP1* (**Figures 4.5** and **4.6**, respectively) supports the view that *MCL* and *MTAP1* are closely related to *LFY* and *AP1* equivalents from *M. excelsa* and *E. globulus*.

It was established by Southern blot that one homologue of each of *MEL*, *MESAP1*, and *METFL1* were present in the genome of *M. excelsa* (Sreekantan, 2002). In other perennials there are one or two *LFY*-like or AP1-like equivalents.
The numbers of *MEL*, *MTAP1* and *MTFL1* homologues in *M. collina* was not determined successfully using Southern blots. There were no bands detected in *M. excelsa* or *M. collina* DNA, but the positive controls were detected. To determine whether the DNA had transferred from the gel to the membrane successfully, the blotted gel could have been exposed to UV light. If DNA was still present in the gel, ethidium bromide staining would have been seen, indicating that the DNA did not transfer from the agarose gel to the nitrocellulose membrane.

The positive control for each gene was detected on the membrane because the sequence was complimentary to the probe, so hybridisation between the AlkPhos-labelled probe and the DNA sequence occurred.

4.8.2 *MEL* and *MCL* were downregulated by GA_3 application

As described in **Chapter Three**, GA_3 treatment produced the morphological response of stem elongation in juvenile *Metrosideros* plants. This response indicates that GA_3

was absorbed into the plant and had an effect. Paclobutrazol-treated plants showed retarded stem growth, which indicated that paclobutrazol had also been taken into the plants. It had previously been shown that GA_3 application upregulated *MEL* expression in juvenile *M. excelsa* plants (Sreekantan *et al.*, 2004). The following gene expression experiments were performed to replicate and extend the findings of Sreekantan *et al.* (2004), and ascertain that *MEL* (and *MCL*) were responsive to GA_3. Also to determine if the response in gene expression is specific to GA_3 application, and to juvenile phase in micropropagated plants.

As shown in **Figure 4.8**, the adult GA_3 treated samples may not have had any *MESAP1* expression because they had no *MEL* expression, and would therefore not have been competent to flower. That there is *MESAP1* expression in the entire juvenile samples was not expected, as there has been no reported *AP1* expression in juvenile plants to date. Although the *MESAP1* band from the juvenile *M. excelsa* RT-PCR was sequenced and proved to be homologous to *MESAP1* (Sreekantan *et al.*, 2002), expression Southerns (probing of a nitrocellulose membrane to which gel-run RT-PCR products had been transferred) would have been advantageous in confirming the identity of the two bands that resulted from *MTAP1* RT-PCR.

In **Figure 4.9** there was no expression of *MCL* in GA_3-treated juvenile or adult samples. This supports GA-repression of flowering in woody perennials. In the adult GA_3-treated samples there is no *MCL* or *MTAP1* expression. If *MCL* were repressed by GA application then *MTAP1* expression would not get upregulated and adult plants would not be competent to flower. *MTAP1* expression is shown in the GA_3-treated micropropagated sample and is equally unexplainable since it was sequenced and found to show high homology to *MESAP1*.

These results that show juvenile samples expressing *MESAP1* or *MTAP1* should be repeated as they are not at all in agreement with Sreekantan *et al.* (2004) who

reported upregulated *MEL* expression but no subsequent *MESAP1* expression in GA_3-treated micropropagated *M. excelsa*.

MCL from *M. collina* was expressed in adult water-treated plants (**Figure 4.10**) but not in GA_3 -treated plants (**Figure 4.11**) in March. It is possible that since these plants were adult that they could be expressing *MTAP1* at baseline levels, which is why *MTAP1* expression is shown in response to water treatment. The GA_3 application could have downregulated *MCL* expression, and ultimately *MTAP1* expression. These results indicated that GA_3 application led to *MCL* being downregulated. It was expected that *MCL* would be upregulated in juvenile *M. collina* in response to GA_3 application as Sreekantan *et al.* (2002) reported *MEL* upregulation upon GA_3 application in micropropagated *M. excelsa*. The work presented in this section supports previous research that GA inhibits flowering in woody perennials. Sreekantan *et al.* (2004) also reported that *MEL* expression was downregulated when endogenous GA_1 was absent when *M. excelsa* bud development has stalled. It can be concluded that the application of GA_3 interferes with floral development by disrupting the normal patterns of *MCL* expression in adult *M. collina*. That *MCL* is not upregulated by GA_3 indicated a significant difference between *M. collina* and *Arabidopsis*, where GA_3 application upregulates *LFY* due to the *LFY* promoter having *cis* elements that respond separately to GA_3, as well as to photoperiod (Blazquez and Weigel, 2000).

CHAPTER FIVE

Isolation of the *Flowering Locus C* (*FLC*) gene from *Metrosideros*

5.1 Introduction and hypotheses

The *FLC* gene is an important flowering time gene that has been studied mainly in winter annual *Arabidopsis* and *Brassica* species. Vernalisation is a key regulator of FLC and flowering, as vernalisation is thought to suppress *FLC* expression thereby lifting the FLC-induced repression of flowering on downstream floral promoters such as *LFY* (Michaels and Amasino, 2001).

FLC was considered to be an interesting gene to study in *Metrosideros* species as *M. excelsa* and *M. collina* appear to have different requirements for floral promotion. *M. excelsa* requires chilling of the floral initials over winter before flowering in the summer (Sreekantan *et al.*, 2001), while *M. collina* appears to have no specific stimulus and flowers more than one time each year (J. Clemens, pers. comm.).

In this chapter, the initial isolation and sequence analysis of *FLC*-like sequences will be discussed. Hypotheses tested with this research were that *FLC* will be present in the *Metrosideros* genome and will be a major factor in the differences of floral promotion between *M. excelsa* and *M. collina*.

5.2 Initial isolation and analysis of *FLC*-like sequences

Three pairs of primers, FLC-1, FLC-2, and FLC-3, were designed from an alignment of the five *Brassica napus FLC* gene sequences and the *Arabidopsis FLC* gene sequence. The FLC-1 primers produced two PCR products from the *Arabidopsis*

DNA used to test the primers initially (**Figure 5.1**). The FLC-3 primers produced three PCR products from the *Arabidopsis* DNA (**Figure 5.2**). The FLC-2 primers did not produce any products. Unfortunately, this initial research into *FLC* did not isolate *FLC*-like sequences from *Metrosideros* DNA, just the *Arabidopsis* DNA used to test the primers.

5.2.1 Nucleotide sequences

The FLC-1 primers were designed to amplify a sequence from exon 2 to exon 7 of the *FLC* gene to produce a product that was 1873 bp.

FLC-1 forward primer	5' CATGCTGATCATCTTAAAGCC 3'
FLC-1 reverse primer	5' CAACAAGCTTCAACATTAGTTC 3'

After PCR with the FLC-1 primers, two bands were visible on the 1% agarose gel containing ethidium bromide (**Figure 5.1**). After sequencing of both the large and small DNA fragments, the nucleotide sequences were determined to be 938 nucleotides and 516 nucleotides in size, respectively. Each sequence was submitted into BLASTn (GenBank) to establish whether the DNA sequence showed any homology to any gene sequences in GenBank.

The large DNA sequence (fragment 1) showed homology to:
Arabidopsis thaliana genomic DNA, chromosome 5, BAC clone:F15O5, *Arabidopsis thaliana MADS affecting flowering 4 variant III* (*MAF4*) mRNA, and other *Arabidopsis MAF4* variants.

The smaller DNA fragment (fragment 2) showed homology to:
Arabidopsis thaliana flowering time protein (*FLC*) gene, *Arabidopsis thaliana* cultivar C24 MADS box protein *FLOWERING LOCUS F* (*FLF*) gene, *Arabidopsis thaliana* flowering locus C protein mRNA *Brassica oleraceae FLC3* (*FLC3*) gene exons 2 through 7, and *Brassica rapa FLC5* (*FLC5*) gene, exons 2 through 7.

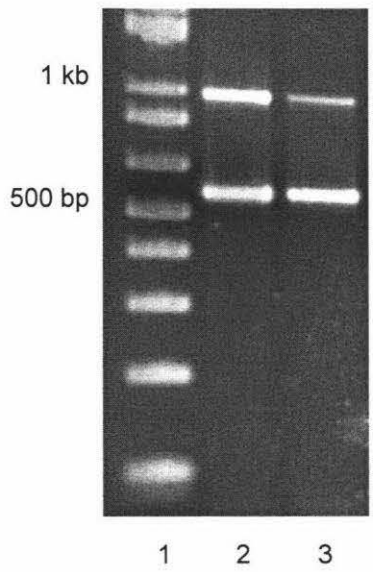


Figure 5.1 Isolation of *FLC* from *Arabidopsis*.
After PCR with the FLC-1 primers, two bands were visible on the 1% agarose gel containing ethidium bromide. The larger band was estimated to be approximately 1 kb in size. The smaller band was approximately 550 bp. Lane 1: 1 kb+ ladder; Lanes 2 and 3: Duplicate RT-PCR reactions using *Arabidopsis* DNA.

The amount of homology of both the large and small DNA fragments that were isolated using the FLC-1 primers is shown in **Table 5.1**.

FLC-2 primers were designed to amplify a sequence from exon 2 to exon 5 to produce a product that was 557 bp.

FLC-2 forward primer: 5' CTGGTCAAGATCCTTGATCGATATGG 3'
FLC-2 reverse primer: 5' ATCAGCTTCGGCTCCCYCAAGAT 3'

No PCR products were produced from *Arabidopsis* DNA using these primers.

FLC-3 primers were designed to amplify a sequence from exon 2 to exon 4 and produce a fragment that was 399 bp.

FLC-3 forward primer: 5' GATCCTTGATCGATATGG 3'
FLC-3 reverse primer: 5' CATTTGATTCCACAAGCTT 3'

After PCR with *Arabidopsis* DNA using the FLC-3 primers, three DNA bands were visible in the 1% agarose gels containing ethidium bromide (**Figure 5.2**). The largest band (fragment 1) was 1080 bp in size. It did not show significant homology to relevant sequences in GenBank. The medium band (fragment 2) was 556 bp in size and showed homology to multiple sequences of *Arabidopsis* gamma-glutamyltranspeptidase mRNA. The small band (fragment 3) was 363 bp in size. It showed homology to the following sequences:
BK000546 *Arabidopsis thaliana* flowering time protein (*FLC*) gene, AF116528 *Arabidopsis* thaliana cultivar C24 MADS box protein *FLOWERING LOCUS F* (*FLF*) gene, and *Brassica oleracea FLC3* (*FLC3*) gene, exons 2 through 7.

Table 5.1 The homology of both the large and small DNA fragments isolated using the FLC-1 primers.

Fragment	Accession number	Name	Sequence homology
large	AB026633	*Arabidopsis thaliana* genomic DNA	913/915 (99%)
	AY231452	*Arabidopsis thaliana* MADS affecting flowering 4 variant III	251/251 (100%)
small	BK000546	*Arabidopsis thaliana* flowering time protein (*FLC*) gene, complete cds	478/502 (95%)
	AF116528	*Arabidopsis thaliana* cultivar C24 MADS box protein *FLOWERING LOCUS F* (*FLF*) gene	478/502 (95%)
	AF537203	*Arabidopsis thaliana flowering locus C* protein mRNA	98/101 (97%)
	AY115673	*Brassica oleracea FLC3* (*FLC3*) gene	111/123 (90%)
	AY115675	*Brassica rapa FLC5* (*FLC5*) gene	100/115 (86%)

Table 5.2 The homology of both the medium and small DNA fragments isolated using the FLC-3 primers.

Fragment	Accession number	Name	Sequence homology
medium	AK175882	*Arabidopsis* gamma-glutamyltranspeptidase mRNA	201/220 (91%)
small	BK000546	*Arabidopsis thaliana* flowering time protein (*FLC*) gene	312/340 (91%)
	AF116528	*Arabidopsis thaliana* cultivar C24 MADS box protein *FLOWERING LOCUS F* (*FLF*) gene	312/340 (91%)
	AY115673	*Brassica oleracea FLC3* (*FLC3*) gene	105/123 (85%)

The amount of homology of the three DNA fragments that were isolated using the FLC-3 primers is shown in **Table 5.2.**

5.2.2 Amino acid sequences

When the large sequence produced from the FLC-1 primers was translated, submitted into a translated BLAST (BLASTx) and matched to sequences in the protein database, the sequences to show amino acid identity and similarity were from *Arabidopsis* MAF4 and FLC-like 2 (FCL2) proteins.

When the small sequence above was translated and submitted into a BLASTx it showed sequence similarity to the *Brassica rapa* and *Brassica oleracea FLC1* genes.

gi27497559| *FLC1* [*Brassica rapa*]

Identities = 25/34 (73%), Positives = 28/34 (82%)

gi27436693 *FLC1* [*Brassica oleracea*]

Identities = 25/34 (73%), Positives = 28/34 (82%)

In a BLASTx, the fragments isolated using the FLC-3 primers did not show any similarity to relevant amino acid sequences, even though they showed significant similarity to the FLC gene at the nucleotide level.

5.3 Discussion

Unfortunately, due to time constraints, this research into *FLC* was not continued, as initial results from *Metrosideros* PCR were not promising. The primers used to isolate an *FLC*-like sequence from *Metrosideros* DNA were not successful, although they did isolate regions of the *Arabidopsis* control DNA. Based on results from sequencing of the isolated *Arabidopsis* fragments, similarity was found to *Arabidopsis FLC*, *B. rapa FLC1* and *FLC5*, *B. oleraceae FLC1* and *FLC3*,

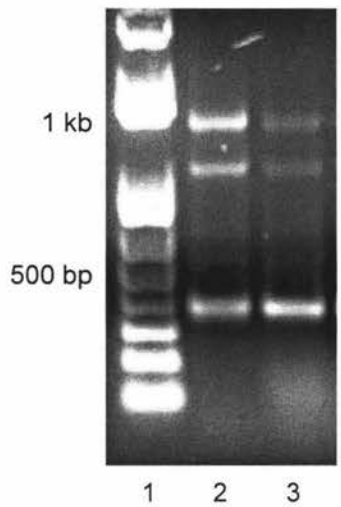


Figure 5.2 Isolation of *FLC* from *Arabidopsis*. After PCR with the FLC-3 primers, three bands were visible on the 1% agarose gel containing ethidium bromide. The larger band was estimated to be approximately 1 kb in size. The middle band was approximately 800 bp and the smaller band approximately 400 bp. Lane 1: 1 kb+ ladder; Lanes 2 and 3: Duplicate RT-PCR reactions using *Arabidopsis* DNA.

Arabidopsis MADS Affecting Flowering 4 (*MADS4*), and *Arabidopsis FLC-like 2* (*FCL2*).

It is possible that a functional *FLC* gene is not present in *M. excelsa* or *M. collina* as neither required vernalisation as a stimulus to promote flowering, unlike the winter annual ecotypes of *Arabidopsis*. *M. excelsa* requires a period of chilling of bud initials (Sreekantan *et al.*, 2001), but not vernalisation, for continued floral development in the spring. Flowering in *M. collina* was observed to stall briefly when temperatures decreased, but did not appear to respond to any 'cold stimulus' to either promote the change in phase from vegetative to floral meristem, as required for winter ecotypes of *Arabidopsis* (Michaels and Amasino, 2000), or for continued bud development in spring as required for *M. excelsa* (Henriod *et al.*, 2000).

CHAPTER SIX

Final Discussion

6.1 Final Discussion

Differences in floral promotion have evolved between short day- and long day-responsive plant species, and between annual and perennial species with respect to involvement by gibberellins, daylength and temperature. Sreekantan *et al.* (2002) linked floral gene expression to floral development and seasonality in *M. excelsa* by describing a bimodal pattern of expression for *MEL* and *MESAP1* during floral initiation and organogenesis over the different seasons of the year. The expression of these genes was upregulated during the beginning of floral initiation, downregulated during winter when development is stalled and upregulated again during floral organogenesis in the spring. Endogenous GA_1 was shown to be absent during the period when *MEL* is downregulated. Flowering in *M. excelsa* generally fitted with the models of flowering developed using *Arabidopsis* and *Antirrhinum* (Sreekantan *et al.*, 2004). Bimodal expression of kiwifruit (*A. deliciosa*) homologues of *LFY* and *AP1*, (*ALF* and *AAP1*), respectively, was also observed (Walton *et al.*, 2001), the difference being that kiwifruit floral development takes place over two seasons. However, the period when the vine was dormant coincided to little or no *ALF* expression.

The developmental path of both vegetative and floral buds of *M. collina* cv. Tahiti was characterised, and it was shown that bud size could not be used to predict bud developmental stage. It was also shown that *M. collina* had two flowering periods in a year and that flowering appeared to stall briefly when temperatures decreased. Flowering time and floral meristem identity genes were investigated with respect to floral initiation and development in two *Metrosideros* species, and in comparison to

the model developed using *Arabidopsis*. In Chapter Four, it was shown that *MCL* and *MTAP1* were successfully isolated from *M. collina* vegetative tissue. These genes could then be investigated along with previously isolated *MEL* and *MESAP1* from *M. excelsa*. Studies with plant growth regulators and hormones showed that *MEL* and *MCL* were not expressed in response to GA_3 application to *Metrosideros*, regardless of species or phase. This correlated with earlier research that documented GA_3 application inhibiting flowering in perennials.

A search was made for an equivalent *FLC* gene in *Metrosideros* to find out if it had any function in promoting flowering. While isolation of an *FLC* sequence from *Arabidopsis* DNA was successful using PCR, *FLC*-like sequences from either *Metrosideros* species were not isolated. Since *M. excelsa* and *M. collina* have different requirements for chilling, and those requirements differ to both vernalisation and dormancy mechanisms, the distinction is made between chilling for flower development, and vernalisation, the former being required by *M. excelsa* buds to continue floral organogenesis, and the latter being required for floral initiation in winter annual ecotypes of *Arabidopsis* (Michaels and Amasino, 2000). A functional *FLC* gene appears not to be present in either *Metrosideros* species, as neither requires vernalisation to promote flowering. This is similar to the summer ecotypes of *Arabidopsis* that have null alleles at the *FLC* (and *FRI*) loci (Michaels and Amasino, 2000). These results suggest that *FLC* has no role in chilling during floral development of *M. excelsa*. Neither does *FLC* have any role in the brief stalling of bud development observed in *M. collina* in cold periods.

M. collina has previously been thought to have an irregular bud initiation and development pattern. It was observed during this study that buds developed faster in warmer weather compared with development in colder temperatures. This was not seasonal though, since colder temperatures in the summer also appeared to briefly delay flowering. *M. collina* did not appear to require this chilling, but only stalled in its path to flowering during colder temperatures. *M. collina* being of tropical origin

may be more responsive to slight changes in temperature or day length. Buds could stall in their development until the plant perceives a certain warm temperature, then floral development occurs rapidly. Neither *M. excelsa* nor *M. collina* are deciduous. It was documented that *M. excelsa* has two major vegetative flushes in a year, an autumn flush that corresponds with floral bud initiation, and a spring flush that corresponds with the continuation of floral development. It is thought that the autumn flush of leaves is required to perceive the signals that cause the floral initials to develop (Sreekantan *et al.*, 2001). Unlike *M. excelsa, M. collina* produced vegetative flushes throughout the year.

6.2 Future Directions

To completely determine whether *M. collina* does exhibit two distinct periods of flowering in one year, more comprehensive measurements of bud growth should be taken in conjunction with temperature measurements over a longer time frame. The fragments of *MEL*, *MCL*, *MESAP1* and *MTAP1* should be extended using 3' and 5' RACE (Rapid amplification of cDNA ends) so the full sequences can be isolated and compared with *Arabidopsis*. To determine whether temperature fluctuations do cause brief delays to flowering of *M. collina*, the expression levels of *MCL* and *MTAP1* could be monitored daily and compared with temperature records. Real-time PCR could be used to examine expression levels quantatively.

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APPENDIX I

General Lab Solutions

DEPC treatment of water:

Add 1 mL DEPC per L of water.

Shake well to dissolve DEPC overnight at 37°C.

Autoclave for and shake while still hot.

Ethidium bromide stain for agarose gels:

Make stock concentration of 10 mg mL^{-1} in water. Use working concentration of 0.5 $\mu g\ mL^{-1}$. Store stock at 4°C in dark.

Xylene cyanol/bromophenol gel loading dye:

25% (w/v) xylene cyanol

25% (w/v) bromophenol blue

Dissolve the powdered dyes in desired volume of ddH_20.

10 x TAE: (1 L) (pH 8.5)

Tris	48.4 g
EDTA	7.4 g
Glacial acetic acid	11.4 mL
Distilled water to 1 L	
Use at 1x concentration.	

TE buffer:

10 mM Tris base pH 8

1 mM EDTA (Na salt)

LB broth: (1 L)

NaCl	10 g
Tryptone	10 g
Yeast extract	5 g
Make up to 1 L with ddH_20	

LB agar: (1 L)

NaCl	10 g
Tryptone	10 g
Yeast extract	5 g
Agar granules	20 g
Make up to 1 L with ddH_20	

APPENDIX II

Abbreviations of LFY orthologues used in Figure 4.5.

Abbreviation	Orthologue name
ELF1	*E. globulus* LFY 1
MCL	*M. collina* cv. Tahiti LFY-like
MEL	*M. excelsa* LFY-like
BOFH	*B. oleracea* FLO
AtLFY	*A. thaliana* LFY
AlLFY	*A. lyrata* LFY
AmFLO	*A. majus* FLORICAULA
NFL2	*N. tabacum* FLO/LFY-like 2
NFL1	*N. tabacum* FLO/LFY-like 1
ALF	*A. deliciosa* LFY/FLO
PtLFYlike	*P. tomemtosa* LFY-like
PTLF	*P. trichocarpa* LFY/FLO
SdLFY	*S. dicolor* LFY-like
LeFLO/LFY	*L. esculentum* FLO/LFY
PhALF	*P. hybrida* LFY/FLO-like
VFL	*V. vinifera* FLO/LFY
UNIFOLIATA	Mutation in PEAFLO, the *P. sativa* LFY/FLO orthologue
MdAFL2	*M. domestica* apple FLO/LFY 2
MdAFL1	*M. domestica* apple FLO/LFY 1

APPENDIX III

A list of abbreviations of AP1 orthologues used in Figure 4.6.

Abbreviation	Orthologue name
MdMADS2	*M. domestica* MADS2
BpMADS5	*B. pendula* MADS5
EAP1	*E. globulus* AP1-like
NsMADS1	*N. sylvestris* MADS-box protein MADS1
MTAP1	*M. collina* cv. Tahiti AP1-like
MESAP1	*M. excelsa* AP1-like
EAP2L	*E. globulus* AP2L
EAP2S	*E. globulus* AP2S
SQUAMOSA	*A. majus* SQUAMOSA
PTM1	*P. tremuloides* MADS-box gene 1
PEAM4	*P. sativa* AP1/SQUA homologue
AlAP1	*A. lyrata* AP1
SaMADSC	*S. alba* MADSC
BoAP1	*B. oleraceae* AP1
AtAP1	*A. thaliana* AP1
NtNAP1	*N. tabacum* AP1
PhMADSTF	*P. hybrida* MADS transcription factor
StAGL8	*S. tuberosum* AGL8
LeTDR4	*L. esculentum* TDR4