Molecular studies of flowering in *Metrosideros excelsa* *(Myrtaceae)*

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Abstract

Molecular and anatomical studies were conducted on *Metrosideros excelsa* to determine if the current genetic models for flowering with regard to inflorescence and floral meristem identity genes in annual plants applied for a woody perennial. Microscopy studies revealed that floral initiation as cymule primordia began in May. Cymules began to develop by August and by October all the floral organs were fully differentiated. *MEL, MESAP1* and *METFL1*, the partial orthologues of *LEAFY*, *APETALA1* and *TERMINAL FLOWER1* respectively, were then isolated from *M. excelsa* buds through RT-PCR. RT-PCR analysis and expression Southerns showed that *MEL* and *MESAP1* were present at low levels as early as March, and that they were both upregulated at the time cymule primordia were initiated and again during floral organogenesis. As *AP1* is considered an indicator of floral determination, the expression of both *MEL* and *MESAP1* as early as March indicated that floral commitment had occurred by then. The results from microscopy studies supported this conclusion. Studies on juvenile *M. excelsa* plants revealed that GA_3 application caused upregulation of *MEL* but not *MESAP1* indicating that meristem competence was also probably required to promote flowering in *M. excelsa* as has been suggested for *Arabidopsis* (Weigel and Nilsson, 1995).

In situ hybridisation studies revealed that *MEL* expression shifted from the apex of the distal axillary bud in May to cymule primordia in early June and subsequently to the sepals, petals, anthers and the gynoecium and ovules in the later stages of floral development. *MESAP1* expression was seen in young floral meristems, but during the later stages of floral development it was confined to the sepals, petals and the perianth region, which is typical of a Class A gene. *METFL1* was expressed throughout the period of inflorescence development. It was expressed in the inflorescence meristem and not in the floral meristems, as is the case with *TFL1* in *Arabidopsis*. Thus the key floral and inflorescence meristem identity genes in the woody perennial *M. excelsa* showed similar spatial expression patterns as their equivalents in herbaceous plants. However, there were differences in temporal expression patterns such as the bimodal pattern of expression seen with *MEL* and *MESAP1*.

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Chapter 1. Introduction

1.1 Overview

Metrosideros excelsa (Soland. ex Gaertn) commonly known as pohutukawa or the New Zealand Christmas tree is endemic to New Zealand. Because of its spectacular red flowers *M. excelsa* has been identified as having significant ornamental value and has been included in the Public Good Science Fund Native Ornamental Plants Programme, and the work reported in this thesis was carried out at Massey University under subcontract CO2X0015 to the New Zealand Institute for Crop and Food Research Ltd. Previous work on flowering in *Metrosideros* species was confined to floral morphology (Dawson, 1968a) and floral development (Orlovich et al., 1996). Schmidt-Adam et al. (1999) studied the floral biology and breeding system of *M. excelsa* and Schmidt-Adam and Gould (2000) described the phenology of inflorescence development in *M. excelsa*. However, none of these studies tracked the development of the flowers from floral initiation to organ differentiation and development. Neither had the signals involved in flowering in these plants been studied, nor was the time frame from floral induction to evocation and flowering clearly understood.

Flowering is influenced by interacting genetic, environmental and physiological factors. Little is known of how these factors influence flowering in *M. excelsa*. During the present work a calendar of floral development in *M. excelsa* was developed and attempts made to correlate the developmental events with genetic and environmental factors.

There are two overriding patterns of reproductive development in angiosperms (Meeks-Wagner, 1993). In the first case, vegetative meristems are directly transformed into determinate floral meristems. In the second case, the vegetative (V) meristems are first transformed into inflorescence (I) meristems, which then generate the floral (F) meristems. This meristem activity of overlapping developmental stages has been described as the V > I > F progression (Meeks-Wagner, 1993). The inflorescence meristem does not produce floral organs directly. It also does not generate leaves. Instead of leaves it may

produce floral meristems subtended by bracts, or a mixture of floral and further inflorescence meristems in the axils of the bracts in the model plant *Arabidopsis thaliana.* In the woody perennial *M. excelsa*, it is seen that the inflorescence is indeterminate (Dawson, 1968a), terminating in a vegetative bud that remains quiescent during flowering and can resume vegetative growth after the flowering period (Sreekantan et al., 2000). In this thesis, the key floral and inflorescence meristem identity genes, *LEAFY (LFY)*, *APETALA1 (AP1)* and *TERMINAL FLOWER1 (TFL1)* were studied as these are significant genetic factors influencing flowering in several herbaceous plants (Ma, 1998).

Gibberellins (GAs) have been shown to enhance expression of floral meristem identity genes such as *LFY* and to promote flowering in herbaceous, long day plants (Blasquez et al., 1997). In woody angiosperms, GAs have been found to be inhibitory to flowering (Looney et al., 1985; Looney and Pharis, 1986; Oliveira and Browning, 1993; Clemens et al., 1995). The present study analyses the role of the floral meristem identity genes *LFY*, *AP1* and *TFL1*, their temporal and spatial expression patterns in relation to the progression of floral development in *M. excelsa*, and how *LFY* and *AP1* interact with GAs. The current chapter reviews the literature available on flowering, as it pertains to this study.

1.2 Pathways to flowering

The transition from vegetative to reproductive growth is one of the most dramatic events in the life cycle of flowering plants. It is regulated by a complex genetic network that monitors the developmental state of the plants as well as environmental conditions such as light and temperature (Koornneef et al., 1998; Simpson et al., 1999). Plants adapt their flowering time to the environment in which they grow for successful reproduction. From a large number of investigations on several plant species, two conclusions have now been derived. Firstly, during the transition to reproductive development, the vegetative shoot meristem undergoes a complex restructuring to form a reproductive meristem that gives rise to floral structures (Colasanti and Sundaresan, 1996). Thus, the shoot apex is the target for a floral stimulus. Secondly, signals that influence the transition to flowering could originate distant from the shoot apex in organs such as leaves, and are transported to

the apex to trigger the transition to flowering (King and Zeevart, 1973; Lang et al., 1977).

Genetic analyses of flowering time mutants have identified about 80 genes placed in

multiple genetic pathways that control the floral transition (Araki, 2001). Figure 1.1 shows

the different pathways and the key genes involved. The hierarchy of the genes follows the

pattern that the flowering time genes influence the inflorescence and floral meristem

identity genes, which in turn influence the organ identity genes resulting in the formation

of flowers.
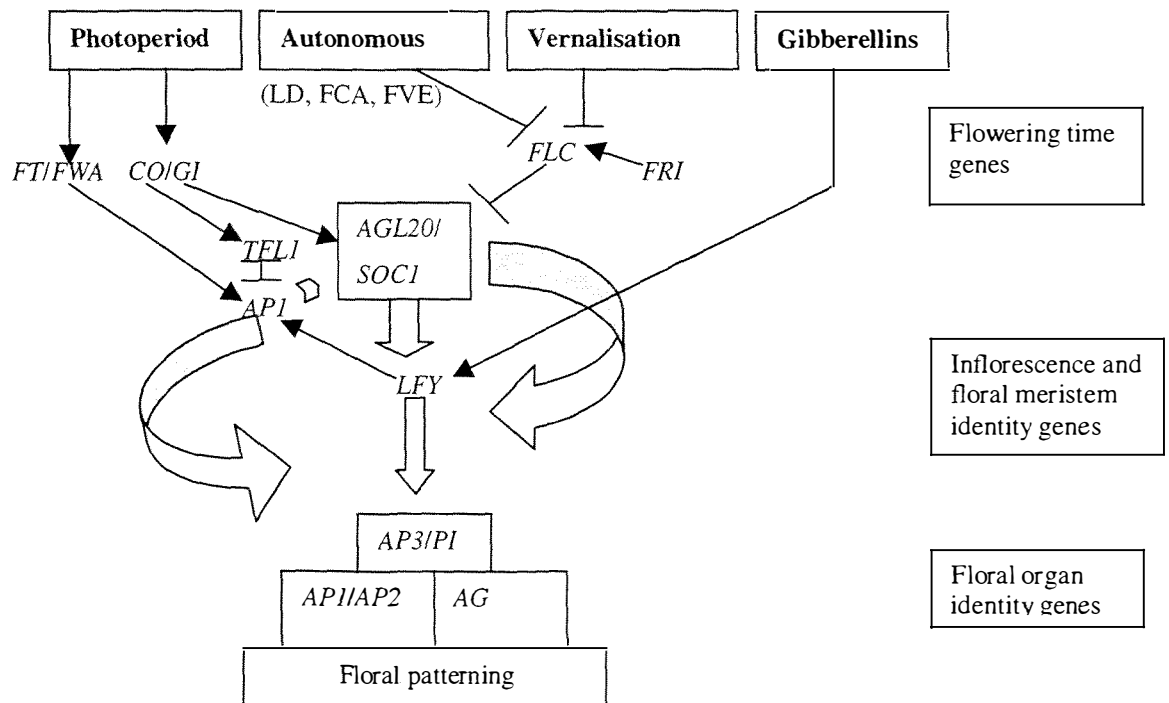
Figure 1.1 Pathways to flowering in *Arabidopsis thaliana*. Modified from Reeves and Coupland (2000).

Several genes have been discovered in the photoperiod pathway and include *CO*, *FD*, *FE*, *FHA*, *FT* and *GI* (Zhao et al., 2002). Similarly, several genes have also been discovered in the autonomous pathway. They include *FCA*, *FY*, *FPA*, *FVE*, *LD* and *FLD* (Zhao et al., 2002). Like many other plants, flowering in *Arabidopsis* is also accelerated by an extended period of cold treatment called vernalisation. Because vernalisation can suppress the effects of mutations in genes of the autonomous pathway and is independent of photoperiod, it is thought to operate through a separate pathway. The photoperiod pathway and the vernalisation pathway mediate signals from the environment, whereas the autonomous pathway probably monitors endogenous cues from the developmental state.

Conversely, mutations that block synthesis of the hormone gibberellin abolish flowering in short days, but have on their own only a minor effect in long days. Genes involved in GA biosynthesis and GA signal transduction have been suggested to form a distinct promotion pathway (Koornneef et al., 1998; Simpson et al., 1999). Understanding where and how these pathways are integrated is a prerequisite for understanding why similar environmental or endogenous cues can elicit contrasting flowering responses in different plants (Blasquez and Weigel, 2000).

Physiological and genetic studies by Bernier et al. (1993) of the physiological signals that induce flowering focused on results obtained with two model experimental plants, *Sinapis alba* and *Arabidopsis thaliana*. It was found that the different environmental stimuli that controlled flower initiation, e.g. photoperiod and temperature, were perceived by different parts of the plant. Thus, the apical meristem integrated one or more long-range signals before switching from vegetative to reproductive development. The nature of these signals is not yet fully determined, but results showed that a number of different agents could control the transition to flowering, including carbohydrates, auxins, gibberellins, cytokinins and calcium. In the long day plant *S. alba*, the fluxes and levels of these chemicals were profoundly altered by photoperiodic stimulation, and the participation of all parts of the plant in the rapid exchange of signals following changes in the light/dark regime indicated that the control of flowering transition was multifactorial. Additionally, the study of metabolic and flowering mutants of *Arabidopsis* showed that this complex

control was multigenic. In *Arabidopsis*, floral induction leads ultimately to the upregulation of floral meristem identity genes such as *LFY*, indicating that floral inductive signals are integrated upstream of *LFY* (Blasquez and Weigel, 2000).

1.2.1 Photoperiod and flowering

Some of the first studies on the mechanism of flowering originated more than 70 years ago when Garner and Allard (1920) discovered that some plants flower in response to specific environmental signals, notably daylength. Long day (LD) plants do not flower unless the photoperiod is longer than some critical value, whereas short day (SD) plants require a photoperiod shorter than a critical value if they are to flower. These two groups differ not so much in terms of the absolute length of the day as in their response to the changing length of day relative to night (Fosket, 1994).

Grafting experiments on several species have shown that daylength is detected in the leaves and a signal transmitted from there to the shoot apex (King and Zeevaart, 1973; Lang et al., 1977). The shoot apical meristem (SAM) of *Arabidopsis* plants grown for 30 days under SD conditions ceased producing leaf primordia and started producing flower primordia within a few hours of being shifted to LD conditions. The rapidity with which the first flowers developed led Hempel and Feldman (1995) to propose that in *Arabidopsis* the signal from the leaves could act directly on existing primordia to alter their identity. Exposure to LD conditions caused irreversible commitment to flowering and the floral stimulus persisted even after the plants were returned to SD conditions.

In maize, experiments with excised apices showed that the presence of four to six leaves was required for the meristem to become committed to form flowers (Irish and Nelson, 1991; Irish and Jegla, 1997). In *Impatiens*, Poteau et al. (1997) observed that continued production of an inductive signal from the leaves was required to prevent reversion to the vegetative state.

Phytochrome A has been demonstrated to be a sensor of photoperiod for flowering response in *Arabidopsis* (Johnson et al., 1994). Other experiments showed that

PHYTOCHROME (PHY) A and *PHY B* could substitute for each other (Bagnall et al., 1995). A partial photoperiod response was retained even in *phy a/phy b* double mutants implying that additional phytochromes could also be involved in perceiving this signal (Reed et al., 1994). Jackson and Thomas (1997) suggested that while *PHY A* was an essential component of the daylength sensing mechanism in LD plants, there could be a potential interaction between *PHY C* and daylength sensitivity in SD plants.

After perception by phytochrome, the photoperiod signal must be transmitted to the meristem. The flowering time genes *CONSTANS (CO)* and *GIGANTEA (GI)* in *Arabidopsis* were implicated in the photoperiod-mediated floral induction pathway (Martinez-Zapater et al., 1994). *CO* mRNA was more abundant in long than in short days and the level of expression of *CO* affected flowering (Putterill et al., 1995).

Simon et al. (1996) observed that *CO* acted directly on transcript accumulation of floral meristem identity genes such as *LFY* and *AP1*. To test the possibility of *CO* playing a direct role in the promotion of flowering through the control of floral meristem identity genes, they designed a system to allow inducible expression of *CO* independent of day length. This system consisted of a transgene encoding a translational fusion of *CO* with the ligand binding domain of the rat glucocorticoid receptor (GR) placed under the control of the CaMV *35S* promoter. The promoter ensured high and constitutive transcription of the transgene but the GR domain prevented the fusion protein from entering the nucleus unless dexamethasone (DEX), a steroid ligand of the GR, was present. Upon addition of DEX, the fusion protein was translocated into the nucleus and *CO* activity was induced. The fusion protein (*CO-GR*) was able to activate expression of *LFY* under LD and SD conditions 24 hours after DEX treatment. This was as quickly as *LFY* expression was observed in wild-type plants after being shifted from SD to LD. Under LD, Simon et al. (1996) could detect *CO-GR* activated *AP1* expression 72 hours after DEX treatment, similar to the time course found for the wild type after a shift from SD to LD conditions. However, *CO-GR* activated *AP1* expression is delayed under SD (120 hours after DEX treatment), suggesting that *AP1* expression requires other factors that are present in LD grown plants but accumulate slowly under SD. The fact that *AP1* expression is eventually activated by *CO-GR* suggests

that *CO* might also activate the synthesis of other factors, such as *LFY*, required for *AP1* expression. However, *LFY* might not be the only factor as *LFY* is activated by *CO-GR* similarly under both LD and SD conditions. *AP1* expression is a point at which at least two pathways converge. One of the pathways seems to be mediated by *CO*, acting directly. Another might involve *CO* and *LFY*, and yet another could be *CO*-independent (Ma, 1998).

Lolium perenne requires a cold treatment of at least 12 weeks to induce flowering. During this period a decrease in *LpTFL1* message was detected in the ryegrass apex. However, upon subsequent induction with elevated temperatures and LD, *LpTFL1* message levels increased and reached a maximum when the ryegrass apex formed visible spikelets (Jensen et al., 2001) implicating the environmental control of flowering.

Hempel et al. (1997) reported that extension of red or far-red-enriched light induced *LFY* expression. Four hours of additional light was sufficient for floral determination in the far-red-enriched conditions while 12-16 hours of additional light was required in the red enriched conditions. *AP1* expression was first observed 16 hours after the start of photo-induction and since this time point was always after floral determination, *AP1* can be considered as an indicator of floral determination. Over-expression of *LFY* and *AP1* causes early flowering in *Arabidopsis* under both LD and SD conditions (Pineiro and Coupland, 1998).

1.2.2 Temperature and flowering

Genetic analysis has identified two classes of late-flowering *Arabidopsis* plants that respond to cold treatment. Naturally occurring late-flowering varieties collected from many locations across Europe carry the dominant alleles at the *FRIGIDA* (*FRI*) locus that delayed flowering, and these plants flowered early if given low temperature treatments (Burn et al., 1993; Lee et al., 1993; Clarke and Dean, 1994). A group of induced recessive mutations delaying flowering in early flowering *Arabidopsis* varieties have also been observed, and this phenotype could be corrected by low temperatures (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991). These mutations were assigned to the

autonomous flowering pathway, because they delayed flowering in all photoperiods. The fact that these mutants responded to low temperature treatment suggested that this could satisfy the requirement for the autonomous pathway.

FLOWERING LOCUS C (*FLC*) has been identified as playing a central role in the vernalisation response in *Arabidopsis* (Reeves and Coupland, 2000). It was observed that dominant *FRI* alleles or the *luminidepens* (*ld*) mutation that affects the autonomous pathway were shown not to delay flowering in the strain Landsberg erecta because it carried an inactive *FLC* allele. On the basis of these genetic interactions it was proposed that *FLC* inhibited flowering and that this inhibition was enhanced by *FRI* and repressed by *LD*. Analysis of *FLC* expression provided a means to test genetic models for the control of vernalisation. Late-flowering plants that could be induced to flower early if given a temperature treatment were shown to have high *FLC* mRNA levels, mainly in roots and at the shoot apex, and that these levels were reduced on vernalisation (Sheldon et al., 1999; Michaels and Amasino, 1999). Dominant alleles at the *FRI* locus, *ld* mutations and other mutations in *Arabidopsis* that affected the autonomous pathway increased *FLC* mRNA abundance. Thus it was found that genotypes with a vernalisation requirement had high *FLC* mRNA levels and vernalisation acted to reduce *FLC* mRNA abundance (Sheldon et al., 1999; Michaels and Amasino, 1999).

Michaels and Amasino (2000) observed that after cold treatment, as imbibed seeds or young seedlings, *FLC* transcript levels were downregulated and remained low for the remainder of the plant's life, but returned to high levels in the next generation. Plants containing a constitutively expressed *35S::FLC* construct remained late flowering after cold treatment, indicating that *FLC* levels must be downregulated for vernalisation to be effective. Thus the epigenetic downregulation of *FLC* appeared to be a major target of the vernalisation pathway and provides a molecular marker of the vernalised state. Michaels and Amasino (2001) found that the downregulation of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), also known as *AGL 20*, by *FRI* and autonomous pathway mutants was also mediated by *FLC*. Complete loss of *FLC* function, however, did not eliminate the effect of vernalisation. Thus, *FRI* and the autonomous

pathway were thought to act solely to regulate *FLC* expression, whereas vernalisation was able to promote flowering via *FLC*-dependent and *FLC*-independent mechanisms. Recently, Rouse et al. (2002) found that *SOC1* levels could be upregulated through the activities of other pathways, despite the repression by *FLC*.

Scortecci et al. (2000) described the identification and characterisation of a new MADS-box gene, *FLOWERING LOCUS M (FLM)*, in *Arabidopsis* which is involved in the transition from vegetative to reproductive development. *FLM* was similar in amino-acid sequence to *FLC*, another MADS-box gene involved in flowering time control. *flm* mutants were early flowering in both inductive and non-inductive photoperiods, and flowering time was sensitive to *FLM* dosage. *FLM* over-expression produced late flowering plants. Thus *FLM* acted as an inhibitor of flowering. *FLM* was expressed in areas of cell division such as root and shoot apical regions and leaf primordia.

1.2.3 Hormonal control of flowering

Bernier et al. (1993) pointed out that a number of different agents could control the transition to flowering, including carbohydrates, auxins, gibberellins, cytokinins and calcium. Bernier and Kinet (1986) concluded that endogenous growth substances or hormones were among the primary controlling agents of flowering and that several substances, either promoters or inhibitors, were usually implicated, each controlling particular events. Exceptions were certain rosette LD plants, e.g. *Rudbeckia bicolor*, and bromeliads, e.g. *Guzmania*, where a single limiting factor appeared to control the whole flowering process.

1.2.3.1 Cytokinins and flowering: The cytokinin content of root exudate and leaf exudate was determined for plants of *S. alba* given either a 22 h light period (LD) to induce them to flower or left vegetative (Lejeune et al., 1988). As early as 16 h after the start of the LD, there was an increase of cytokinin activity in both the root and leaf exudates of induced plants implicating the cytokinins as a component of the floral stimulus in this species.

Kinet et al. (1993) observed that, as in several species, root removal promoted flowering and high or low temperatures applied to the root system also influenced floral initiation in some plants. There was a clear implication that the root system possessed at least partial control of flowering. In various species, the inhibitory effect of the root could be mimicked by cytokinins which were known to be produced by roots. A few studies revealed, however, that the effect of exogenous cytokinins is strongly dependent on other factors, such as the applied concentration, the environmental conditions, and the time and site of application, and that promotion or inhibition could be observed (Kinet et al., 1993). These findings indicated that there was a permissive range of cytokinin concentrations and that the endogenous status of the plant affected cytokinin action, which was not comparable in all plant organs. Studies with the LD plant *S. alba* and the SD plant *Xanthium strumarium* (Kinet et al., 1993) suggested the existence of a shoot-to-root signal, which was under photoperiodic control and affected cytokinin synthesis in and/or release from the roots. As a result, cytokinin levels were altered in the plant: these changes, triggered by the inductive treatment, seemed to be transient and were not necessarily similar in different plant organs. Remarkably, in all species investigated, increases in cytokinin levels, most usually in buds or phloem sap, had been detected during the floral transition, suggesting that cytokinins could be required. A role as a mitotic stimulus was possible, as indicated by the work with *Sinapis* (Kinet et al., 1993).

Bonhomme et al. (2000) combined a physiological approach by testing the effects of three putative floral signals on *SaMADS A* expression in the SAM of *S. alba* plants with a transgenic approach using *A. thaliana* plants. A single application of a low dose of a cytokinin or a gibberellin to the apex of vegetative *S. alba* plants was capable of mimicking perfectly the initial effect of LD on *SaMADS A* transcription. A treatment combining the two hormones caused the same activation but seemed to enhance the level of *SaMADS A* expression. A sucrose application to the apex of vegetative plants was, in contrast, unable to activate *SaMADS A* expression. None of these chemicals, alone or combined, was capable of causing the floral shift at the SAM. The constitutive expression of *SaMADS A* led to precocious flowering in *A. thaliana* and antisense expression of a fragment of the *A. thaliana* homologue *AGL20* led to a delay in flowering time. These

results were consistent with *SaMADS A* activation being an intermediate event in a cytokinin- and/or gibberellin-triggered signal transduction pathway that was involved in the regulation of floral transition in *S. alba.*

1.2.3.2 Gibberellins and flowering:

Plants perceive daylength in the leaves, but reproductive changes occur in shoot apices, in response to the movement of a signal or signals throughout the plant. The signal(s) that move from leaves to the sites of reproductive development are not known, but there is good evidence that GAs may be important (Jackson and Thomas, 1997). Many studies have implicated GAs as causal or controlling factors in many aspects of higher plant growth and development, including shoot growth, bud development, bud break and flowering (Pharis and King, 1985; Takahashi et al., 1989).

In herbaceous, LD plants GAs can be potent tools for inducing flowering and studies by Pharis et al. (1987; 1989) and Evans et al. (1990; 1994) have shown distinct structure: function relations of the GA molecule for floral induction of the shoot apex versus elongation of the stem in *Lolium.*

GAs vary in their florigenic activity and for woody angiosperms applied GAs, especially GA_3 and GA_7, are generally inhibitory to flowering (Looney et al., 1985; Looney and Pharis, 1986; Oliveira and Browning, 1993). Van Staden and Dimalla (1980) have suggested that GAs may play an important role in maintaining *Bougainvillea* plants in a vegetative state. Moncur et al. (1994) have reported that *Eucalyptus nitens* grafts treated with paclobutrazol as a single collar application, reduced the endogenous GAs and enhanced umbel production. Moncur and Hasan (1994) observed that paclobutrazol application reduced the concentration of endogenous GAs in apical tissue and enhanced the reproductive activity of grafted trees of *Eucalyptus nitens* maintained outside over winter in Canberra, Australia. Grafts maintained in a warm greenhouse over winter did not produce flower buds, despite the paclobutrazol-induced reduction in GA concentration in the apical tissue. Addition of GA_3 to paclobutrazol-treated grafts reduced the effect of paclobutrazol on reproductive activity.

Clemens et al. (1995) studied the influence of GAs on bud break, vegetative shoot growth and flowering in *Metrosideros collina* 'Tahiti'. They observed reduced flowering in response to applied GAs which was explained as caused by the combined result of abortion of floral structures in quiescent buds, and a preferential inhibition of bud break of floral buds relative to vegetative buds. The response to GAs in woody species is thus quite obviously in contrast to the response of herbaceous plants.

Studies with mutants have shown that, in *Arabidopsis*, constitutive flowering promotion genes might operate on a pathway that converges with the GA activity pathway (Coupland, 1995). Double mutants with *co* and *ga* synthesis or perception mutants flower extremely late suggesting that *CO* and GA act on different pathways to mediate the transition to flowering (Putterill et al., 1995). Blasquez et al. (1997) observed that application of GA_3 enhanced *LFY::GUS* expression in SD and hastened flowering. To test whether these effects reflect a role for endogenous GAs in controlling *LFY* promoter activity, Blasquez et al. (1998) introduced a *LFY::GUS* transgene into *ga1-3* mutants, which have severely reduced endogenous GA levels. The failure of the plants to flower in SD was paralleled by the absence of *LFY* promoter induction. Flowering was delayed in LD compared with wild type, and was paralleled by the profile of *LFY::GUS* activity.

A constitutively expressed *LFY* transgene was able to restore flowering to *ga1-3* plants in SD. In contrast to SD, impairment of gibberellin biosynthesis caused merely a reduction of *LFY* expression when plants were grown in LD or with sucrose in the dark. These results suggest that GAs affect flowering through a pathway that controls *LFY* transcription, viz., GAs—>*LFY* promoter induction—>flowering. Blazquez and Weigel (2000) showed that GAs activated the *LFY* promoter through *cis* elements that were different from those that were sufficient for the daylength response, demonstrating that the *LFY* promoter integrated environmental and endogenous signals controlling flowering time.

The GA-deficient *Arabidopsis* mutant, *ga1*, flowered later when grown in LD conditions and did not flower in SD conditions, indicating that GA was required for the photoperiod-insensitive (autonomous) flowering pathway (Wilson et al., 1992). GA levels and response

to the hormone were sensitive to changes in photoperiod (Pharis et al., 1987; Zeevaart and Gage, 1993), suggesting a role for GA in photoperiodic induction of flowering. *Arabidopsis* mutants defective in phytochrome synthesis (Reed et al., 1994; Koornneef et al., 1995) or phytochrome function (Ahmad and Cashmore, 1996) flowered early, indicating that this light receptor was involved in repression of floral initiation.

At least some of the effects of phytochrome, may be mediated by GA as some studies (Reed et al., 1996; Jackson and Thomas, 1997) indicated. Okamuro et al. (1996) demonstrated that in *Arabidopsis*, a heterochronic switch from flower to shoot development or floral meristem reversion can be achieved by manipulating photoperiod in the mutant *agamous* (*ag*) and plants heterozygous for *lfy*. The transformation is suppressed by *hy1*, a mutation blocking phytochrome activity, by *spindly*, a mutation that activates basal gibberellin signal transduction in a hormone-independent manner, or by the exogenous application of GAs.

Okamuro et al. (1996) proposed that there are three possible pathways for the maintenance of flower meristem identity by a GA-mediated phytochrome signal transduction cascade in wild type plants under SD. First, GAs may promote the maintenance of flower meristem identity by a pathway independent of *LFY* or *AG*. Second, GAs may promote the activity of genes located genetically downstream of *LFY* and *AG* or genes that function together with *LFY* to promote determinate flower development such as *API*. Finally, GAs may repress floral meristem reversion by promoting *LFY* activity directly or indirectly.

Lee et al. (1998) from studies on sorghum, observed that the diurnal rhythm of GA levels play a role in floral initiation, and may be one way by which the absence of *PHY B* causes early flowering under most photoperiods. To learn how *PHYB* and GAs interact in the control of flowering, Blazquez and Weigel (1999) analysed the effect of a *phyB* mutation on flowering time in *Arabidopsis* and on the expression of the floral meristem identity gene *LFY*. They showed that the early flowering caused by *phyB* correlated with an increase in *LFY* expression, which complemented their previous finding that GAs were required for activation of *LFY* under noninductive photoperiods (Blasquez et al., 1998). Since *phyB* did not change the GA responsiveness of the *LFY* promoter and suppressed the lack of flowering of severe GA-deficient mutants under SD, these authors proposed that

PHYB modulated flowering time at least partially through a GA-independent pathway. The effects of *PHYB* on flowering did not seem to be mediated by transcriptional upregulation of genes such as *CO* and *FT*, which are known to mediate the effects of the photoperiod-dependent floral induction pathway.

Luciferase imaging screen by Meier et al. (2001) identified two types of recessive mutants with increased *LUC* activity and apparent GA-related growth phenotypes in *Arabidopsis*, a dwarf (*lue1*) and two late flowering mutants (*fpa1-3* and *fpa1-4*). Mapping and comple-mentation analyses showed that the late flowering mutants were allelic to *fpa1*. This provided genetic evidence for cross-talk between the autonomous and GA-dependent flowering pathways.

1.3 Floral and inflorescence meristem identity genes and flowering in herbaceous plants

In recent years, a significant understanding has been gained regarding the molecular mechanisms of floral determination and the differentiation of floral organs. The two plants in which the most progress has been made in this direction are *Arabidopsis* and *Antirrhinum majus*. In both these plants, flowering is a two-step process in which (1) the vegetative (V) meristems are transformed into inflorescence (I) meristems, and (2) the inflorescence meristems then produce floral (F) meristems in the axils of bracts (Fosket, 1994). The progression of floral development is thus of the V > I > F category. Coen & Meyerowitz (1991) have described two classes of homeotic mutation: those that affect meristem identity, and those that affect the fate of floral organ primordia. Mutations affecting regulatory steps earlier than those under the control of the organ homeotic genes might be expected to prevent or alter the formation of floral meristems. Mutants of this type, in which inflorescence meristems or structures intermediate between inflorescence meristems and floral meristems appear in the positions normally occupied by floral meristems, are *floricaula* (*flo*) and *squamosa* in *Antirrhinum* and *leafy* (*lfy*) and *apetala1* (*ap1*) in *Arabidopsis*. In *flo* mutants of *Antirrhinum*, vegetative growth and the transition to the inflorescence meristem are similar to wild type. Instead of floral meristems being

produced in the axils of bracts, the meristems in these positions were of the inflorescence type and indeterminate shoots were formed in the bract axils. Each of these shoots can in turn produce further shoots in the axils of their bracts. *Arabidopsis lfy* and *ap1* and *Antirrhinum squa* mutants have a similar phenotype, although they show only partial conversion of floral meristems to inflorescence meristems.

Flowering plants exhibit one of two types of inflorescence architecture: indeterminate, in which the inflorescence grows indefinitely, or determinate, in which a terminal flower is produced. The production of a terminal flower is thought to be an ancestral state from which the indeterminate condition evolved (Stebbins, 1974). In two mutants in distantly related species, *terminal flower1* in *Arabidopsis* and *centroradialis* in *Antirrhinum*, inflorescences that are normally indeterminate are converted to a determinate architecture. The *Arabidopsis* gene *TERMINAL FLOWER1* (*TFL1*) and the *Antirrhinum* gene *CENTRORADIALIS* (*CEN*) were shown to be homologous, which suggests that a common mechanism underlies indeterminacy in these plants (Bradley et al., 1997). However, unlike *CEN*, *TFL1* is also found to be expressed during the adult vegetative phase, where it delays the commitment to inflorescence development and thus affects the timing of the formation of the inflorescence meristem as well as its identity (Bradley et al., 1997).

The *TFL1* gene of *Arabidopsis* has an important role in regulating flowering time and in maintaining the fate of the inflorescence meristem (IM). The overall morphology of an *Arabidopsis* plant depends on the behaviour of its meristems. Meristems derived from the shoot apex can develop into either shoots or flowers. The distinction between these alternative fates requires separation between the function of floral meristem identity genes and the function of an antagonistic group of genes, which includes *TFLI*, required to maintain inflorescence meristem identity (Ratcliffe et al., 1999). A considerable body of knowledge has accumulated on the roles of *LFY*, *AP1* and *TFL1* in annuals and herbaceous plants, and any study of a woody perennial must be considered in this context.

1.3.1 *LEAFY* and *APETALA1*

LFY homologues have been isolated and studied from several species of dicotyledonous plants. They include homologues from cauliflower, tobacco, peas, pine, eucalyptus, poplar

and violet cress (Anthony et al., 1993; Kelly et al., 1995; Hofer et al., 1997; Pouteau et al., 1997; Mouradov et al., 1998; Southerton et al., 1998; Rottman et al., 2000; Shu et al., 2000). The first *LFY* homologue isolated was *FLO* from *Antirrhinum*. *FLO* transcripts are abundant in bracts and early floral meristems (Coen et al., 1990). In *Arabidopsis*, the highest expression of *LFY* was found in regions of the inflorescence meristem that formed floral meristems and in newly formed floral meristems. However, *LFY* was also expressed at low levels during vegetative development (Weigel et al., 1992). The *LFY* homologues in tobacco were found to be active not only in floral meristems, but also in indeterminate vegetative meristems (Kelly et al., 1995). The gene is expressed in both vegetative and floral meristems, in leaf primordia and leaves, and in the four floral organs. Blasquez et al. (1997) observed that *LFY* combines properties of flowering time and floral meristem identity genes. They analysed the expression of *LFY* during the plant life cycle and found that *LFY* is extensively expressed during the vegetative phase. Increasing the copy number of endogenous *LFY* reduces the number of leaves produced before the first flower is formed.

Characterisation of the tomato *falsiflora* mutant by Molinero-Rosales et al. (1999) showed that *fa* mutation mainly alters the development of the inflorescence resulting in the replacement of flowers by secondary shoots, but it also has a late flowering phenotype with an increased number of leaves below first and successive inflorescences. This pattern suggests that the *FALSIFLORA* (*FA*) locus regulates both floral meristem identity and flowering time in tomato in a similar way to the floral identity genes *FLO* of *Antirrhinum* and *LFY* of *Arabidopsis*.

The *LFY* gene stands out because its expression precedes that of other meristem identity genes with flower specific expression. Overexpression of *LFY* caused precocious flowering and the conversion of lateral inflorescence shoots into flowers in transgenic *Arabidopsis* (Weigel and Nilsson, 1995). It is also reported that *lfy* loss-of-function mutations have the strongest effect on meristem identity (Mandel et al., 1992; Weigel et al., 1992; Jofuku et al., 1994; Ingram et al., 1995; Kempin et al., 1995). Strong *lfy* mutants form axillary shoots subtended by leaves at the positions occupied by the first flowers of wild type plants, but

later positions are less severely affected and ultimately form flower-like structures. However, these structures do not form petals or stamens and show helical phyllotaxy rather than the typical arrangement of whorls (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992; Schultz and Haughn, 1993).

Mutations in *AP1* also have a stronger effect on flowers that develop at early positions on the shoot than on those that develop later. However, *ap1* mutant structures are less affected than shoots formed in *lfy* mutants. *SQUA* and *AP1* are perhaps not as critical as *FLO* and *LFY* in controlling floral meristem identity as evidenced by the phenotypic characteristics of their null mutants. The phenotypes of *lfy* and *ap1* single and double mutants indicate that these genes have partially redundant functions. *lfy/ap1* double mutants show a more severe phenotype than either single mutant, with flower-like structures seen only very rarely (Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993).

Redundancy is also evident between *LFY*, *AP1* and other floral meristem identity genes (Bowman et al., 1993) such as *CAULIFLOWER* (*CAL*) and *APETALA2* (*AP2*). These observations suggest that in wild type plants the four genes act collectively, mutually enhancing expression and acting additively on target genes to promote floral meristem identity. The reduced requirement for *LFY* and *AP1* in later flowers is probably a result of other floral meristem identity genes compensating for their loss of function (Pineiro and Coupland, 1998).

The redundancy and co-operation between floral meristem identity genes has made it difficult to study the roles of individual genes. However, despite the interrelationship between them, ectopic and high-level expression of a single meristem identity gene can be sufficient to specify floral development. In *Arabidopsis* plants ectopically expressing *LFY* or *AP1*, lateral meristems that would normally be shoots are converted into axillary flowers (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). In addition, the shoot apical meristem of *35S::LFY* and *35S::AP1* plants is determinate, forming a terminal flower similar to that of *terminal flower* mutants (*tfl*). These results demonstrate that both *LFY* and

AP1 are sufficient to convert shoot meristems into flowers. Introduction of *LFY* with the *35S* cauliflower mosaic virus promoter into *ap1* mutants and *35S::AP1* into *lfy* mutants suggests that *LFY* acts before *AP1* in conferring floral meristem identity (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995).

Use of steroid-inducible activation of *LFY* demonstrated that early expression of *AP1* is a result of transcriptional induction by *LFY* (Wagner et al., 1999). This *AP1* induction occurs specifically in the tissues at the developmental stage in which floral fate is assumed. Later expression of *AP1* appears to be only indirectly affected by *LFY*. Liljegren et al. (1999) present evidence that *AP1* expression in lateral meristems is activated by at least two independent pathways, one of which is regulated by *LFY*. In *lfy* mutants, the onset of *AP1* expression is delayed, indicating that *LFY*, which is upstream of *AP1*, is a positive regulator of *AP1*. These authors have also found that *AP1*, in turn, can positively regulate *LFY*, because *LFY* is expressed prematurely in the converted floral meristems of plants constitutively expressing *AP1*. Therefore, the normally sharp transition that occurs upon floral induction is promoted by positive feedback interactions between *LFY* and *AP1*.

Mandel and Yanofsky (1995) have shown that transgenic plants that constitutively express the *AP1* gene show transformations of apical and lateral shoots into flowers, and that these plants flower much earlier than wild type plants. These results indicate that *AP1* alone can convert inflorescence shoot meristems into floral meristems, and that ectopic *AP1* expression can dramatically reduce the time to flowering. However, converging lines of evidence suggest that the combined activities of *LFY* and *AP1* are more effective in conferring a floral fate on meristems than is either activity alone (Liljegren et al., 1999).

In *Arabidopsis*, the closely related *AP1* and *CAL* genes share overlapping roles in promoting flower meristem identity. In *ap1* mutants, sepals are transformed to leaf-like organs and the petals fail to develop. In the axils of these leaf-like organs, secondary flowers arise which repeat the same pattern as the primary ones. Although *cal* single mutants appear as wild type, *ap1/ cal1* double mutants displayed a massive proliferation of inflorescence-like meristems in positions that would normally be occupied by solitary

flowers. The functional redundancy shared by *AP1* and *CAL* could be explained by the fact that these two genes encode related members of the MADS-box family of regulatory proteins (Mandel et al., 1992; Bowman et al., 1993; Gustafson-Brown et al., 1994; Kempin et al., 1995). Later in flower development, the *AP1* gene is required for normal development of sepals and petals.

One of the roles of floral meristem identity genes is to activate the expression of organ identity genes that act later in flower development (Weigel and Meyerowitz, 1993). Analysis of a *LFY* responsive enhancer in the homeotic gene *AGAMOUS* indicated that direct interaction of *LFY* with the enhancer is required for its activity in plants (Busch et al., 1999). Thus *LFY* is a direct upstream regulator of floral homeotic genes.

Sector analysis and targeted expression in transgenic plants were used to study whether *LFY* and *AP1* could participate in cell-to-cell signaling between and within different layers of the floral meristem (Sessions et al., 2000). *LFY* signaled equally well from all layers and had substantial long-range action within layers. Non-autonomous action of *LFY* was accompanied by movement of the protein to adjacent cells, where it directly activated homeotic target genes. In contrast, *AP1* had only limited non-autonomous effects, apparently mediated by downstream genes because activation of early target genes by *AP1* was cell-autonomous. Proper development of petals and stamens in *Arabidopsis* flowers requires the activities of *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), whose transcripts can be detected in the petal and stamen primordia. Localised expression of *AP3* and *PI* requires the activities of at least three genes: *AP1*, *LFY*, and *UNUSUAL FLORAL ORGANS* (*UFO*). It has been proposed that *UFO* provides spatial cues and that *LFY* specifies competence for *AP3* and *PI* expression in the developing flower (Medard and Yanofsky, 2001). To understand the epistatic relationship among *AP1*, *LFY*, and *UFO* in regulating *AP3* and *PI* expression, Medard and Yanofsky (2001) generated two versions of *AP1* that had strong transcriptional activation potential. Genetic and molecular analyses of transgenic plants expressing these activated *AP1* proteins showed that the endogenous *AP1* protein acted largely as a transcriptional activator *in vivo* and that *AP1* specified petals by regulating the spatial domains of *AP3* and *PI* expression through *UFO*.

1.3.2. *TERMINAL FLOWER1*

The *TFL1* gene of *Arabidopsis* plays an important role in regulating flowering time and in maintaining the fate of the inflorescence meristem (IM). *TFL1* is a homologue of *CEN* from *Antirrhinum*, which is involved only in IM maintenance. Upon floral induction, the primary shoot meristem of an *Arabidopsis* plant begins to produce flower meristems rather than leaf primordia on its flanks. Shoot meristems maintain an identity distinct from that of flower meristems, in part through the action of genes such as *TFL1*, which bars *AP1* and *LFY* expression from the inflorescence shoot meristem (Shannon and Meeks-Wagner, 1993).

Liljegren et al. (1999) showed that this negative regulation could be mutual because *TFL1* expression was down-regulated in plants constitutively expressing *AP1*. Therefore, the normally sharp transition between the production of leaves with associated shoots and the formation of flowers, which occurs upon floral induction, was suggested to be promoted by positive feedback interactions between *LFY* and *AP1*, together with negative interactions of these two genes with *TFL1*.

Although the *Antirrhinum CEN* gene is the homologue of *TFL1* (Bradley et al., 1996, 1997), its interaction with *FLO* (the *LFY* homologue), is not the same as that between *TFL1* and *LFY*. The expression of *CEN* is slightly later than *FLO*, and is initially dependent on *FLO* for its initial expression (Bradley et al., 1996). Subsequently, *CEN* represses *FLO* expression at the centre of the inflorescence meristem, similar to the function of *TFL1*. Another difference is found with the tomato *CEN/TFL1* homologue *SELF PRUNING (SP)*. *SP* is expressed in inflorescence and floral meristems and co-expressed with the tomato *LFY* homologue (Pnueli et al., 1998). Therefore, in tomato, these two genes are not antagonistic with each other, unlike *TFL1* and *LFY*.

To investigate the regulation of meristem identity and the control of floral transition in perennial ryegrass (*Lolium perenne*), Jensen et al. (2001) isolated a ryegrass *TFL1*-like gene, *LpTFL1*, and characterised it for its function in ryegrass flower development. Analysis of the *LptTFL1* promoter fused to the *UidA* gene in *Arabidopsis* revealed that the

promoter is active in axillary meristems, but not the apical meristem. Therefore, they suggested that *LpTFL1* was a repressor of flowering and a controller of axillary meristem identity in ryegrass.

Ferrandiz et al. (2000) found that mutations in the *FRUITFUL* (*FUL*) MADS-box gene, when combined with mutations in *AP1* and *CAL*, lead to a dramatic non-flowering phenotype in which plants continuously elaborate leafy shoots in place of flowers. They demonstrated that this phenotype was caused both by the lack of *LFY* upregulation and by the ectopic expression of the *TFL1* gene. Their results suggested that the *FUL*, *AP1* and *CAL* genes acted redundantly to control inflorescence architecture by affecting the domains of *LFY* and *TFL1* expression as well as the relative levels of their activities.

FLOWERING LOCUS T (*FT*), which acts in parallel with the meristem identity gene *LFY* to induce flowering of *Arabidopsis*, was isolated by activation tagging (Kardailsky, 1999). Like *LFY*, *FT* was found to act partially downstream of *CO*, which promoted flowering in response to long days. Unlike many other floral regulators, the deduced sequence of the *FT* protein did not suggest that it directly controlled transcription or transcript processing. Instead, it was similar to the sequence of *TFL1*, an inhibitor of flowering that also shared sequence similarity with membrane-associated mammalian proteins. Loss of *FT* caused delay in flowering, whereas overexpression of *FT* resulted in precocious flowering independent of *CO* or photoperiod. Work conducted by Kobayashi et al. (1999) also showed that *FT* acted in part downstream of *CO* and mediated signals for flowering in an antagonistic manner with its homologous gene, *TFL1*.

Recent mutational studies and the genome project revealed that *TFL1* belongs to a small gene family in *Arabidopsis*, in which functional divergence may have occurred among the members (Mimida et al., 2001).

Table 1.1, adapted from Ma (1998), summarises the characteristics of *LFY*, *AP1* and *TFL1* from studies on *Arabidopsis* and *Antirrhinum*.

Table 1.1. *Arabidopsis* **and** *Antirrhinum* **genes controlling meristem identity**

Arabidopsis genes	*Antirrhinum* genes	Mutant phenotypes in meristem identity	Phenotypes of ectopic transgenics
LFY	*FLO*	Conversion of flower to shoot or shoot-like structures; floral reversion (*lfy/+*)	Conversion of lateral shoots to flower; terminal flower; early flowering
AP1	*SQUA*	Conversion of flower to shoot; branched flowers (*ap1*)	Conversion of lateral shoots to flower; terminal flower; early flowering
TFL1	*CEN*	Terminal flower; early flowering (*tfl1*)	No flowering; continuous production of leafy shoots in place of flowers

1.4 Expression patterns of *LEAFY* and *APETALA1* in woody perennials

Southerton et al. (1998) cloned two genes from *Eucalyptus globulus*, *ELF1* and *ELF2*, with sequence homology to the genes *LFY* in *Arabidopsis* and *FLO* from *Antirrhinum*. They reported that *ELF1* was expressed in the developing eucalypt floral organs in a pattern similar to *LFY*, while *ELF2* appeared to be a pseudogene. *ELF1* was expressed strongly in the early floral primordium and then successively in the primordia of sepals, petals, stamens and carpels. It was also reported to be expressed in the leaf primordia and young leaves of adult and juvenile trees. The authors observed that *ELF1* promoter coupled to a

GUS reporter gene directed expression in transgenic *Arabidopsis* in a temporal and tissue-specific pattern similar to an equivalent *Arabidopsis LFY* promoter construct. Strong expression was seen in young flower buds and then later in sepals and petals. No expression was seen in rosette leaves or roots of flowering plants or in any non-flowering plants grown under LD. Furthermore, ectopic expression of the *ELF1* gene in transgenic *Arabidopsis* caused the premature conversion of shoots into flowers, similar to a *35S::LFY* construct. Southerton et al. (1998) suggested that *ELF1* played a similar role to *LFY* in flower development and that the basic mechanisms involved in flower initiation and development in *Eucalyptus* were similar to those in *Arabidopsis*.

NEEDLY (*NLY*), the orthologue of *LFY* in *Pinus radiata*, has also been found to be expressed both in reproductive and vegetative meristems (Mouradov et al., 1998). Although the *NLY* protein shared extensive sequence similarity with its angiosperm counterparts, it lacked the proline-rich and acidic motifs thought to function as transcriptional activation domains. Mouradov et al. (1998) found that *NLY* was already expressed during vegetative development at least five years before the transition to the reproductive phase. Expression of *NLY* in transgenic *Arabidopsis* promoted floral fate, demonstrating that, despite its sequence divergence, *NLY* encoded a functional orthologue of the *FLO/LFY* genes of angiosperms.

PTLF, the *Populus trichocarpa* homologue of *LFY* and *FLO*, was cloned to assess its function in a dioecious tree species (Rottmann et al., 2000). *In situ* hybridisation studies showed that the gene was expressed most strongly in developing inflorescences. Expression was also seen in leaf primordia and very young leaves, most notably in apical vegetative buds near inflorescences, but also in seedlings. Although ectopic expression of the *PTLF* cDNA in *Arabidopsis* accelerated flowering, only one of the many tested transgenic lines of *Populus* flowered precociously. The majority of trees within a population of 3-year-old transgenic hybrid *Populus* lines with *PTLF* constitutively expressed showed few differences when compared to controls. Rottmann et al. (2000) concluded that *PTLF* activity appeared to be subject to regulation that did not affect heterologously expressed *LFY*, and was dependent upon tree maturation.

AP1 homologues have also been cloned from several woody species. Two apple (*Malus sylvestris* (L.) Mill. var. domestica (Borkh.) Mansf.) homologous fragments of *FLO/LFY* and *SQUA/AP1* (*AFL* and *MdAP1*, respectively) were analysed (Kotoda et al., 2000) to determine the relationship between floral bud formation and floral gene expression in 'Jonathan' apple. The *AFL* gene was expressed in reproductive and vegetative organs. By contrast, the *MdAP1* gene, identified as *MdMADS5*, which was classified into the *AP1* group, was expressed specifically in sepals concurrent with sepal formation. Based on these results, they suggested that *AFL* may be involved in floral induction to a greater degree than *MdAP1* since *AFL* transcription increased approximately two months earlier than *MdAP1*.

Elo et al. (1996) cloned three MADS box genes homologous to *SQUA* and *AP1* in silver birch (*Betula pendula*), whereas in *Eucalyptus*, Kyozuka et al. (1997) cloned two functional equivalents of *AP1* and named them *EAP1* and *EAP2*. Both the genes were predominantly expressed in flower buds. RNA blot analysis showed that while *EAP1* was expressed at all stages of floral morphogenesis, *EAP2* was expressed more predominantly in young flower buds. Neither gene was expressed at a significant level in leaves, stems or roots.

Sung et al. (1999), isolated a MADS-box gene, *MdMADS2* from 'Fuji' apple and its developmental expression pattern was studied during flower development. *MdMADS2* shared a high degree of amino acid sequence identity with the *SQUA* subfamily of genes. RNA blot analysis showed that *MdMADS2* was transcribed through all stages of flower development, and its transcription was seen in the four floral organs. RNA *in situ* hybridisation revealed that the *MdMADS2* mRNA was expressed both in the inflorescence meristem and in the floral meristem. The *MdMADS2* transcript was detected at all stages of flower development. Protein localisation analysis showed that MdMADS2 protein was excluded from the stamen and carpel primordia, in which a considerable *MdMADS2* mRNA signal was detected. This indicated that post-tanscriptional regulation may be involved in the *MdMADS2*-mediated control of flower development. Transgenic tobacco expressing the *MdMADS2* gene under the control of CaMV *35S* promoter showed early

flowering and shorter bolts, but did not show any homeotic changes in the floral organs. These results suggested that *MdMADS2* played an important role during early stages of flower development.

Partial orthologues of *LFY* and *AP1* have been isolated by Walton et al. (2001) from kiwifruit (*Actinidia deliciosa*) and have been named *ALF* and *AAP1*, respectively. Northern hybridisation analyses showed that *ALF* and *AAP1* had bimodal patterns of annual expression in developing first-order axillary buds and their subsequent shoots. This pattern of expression was consistent with the 2-year cycle of axillary bud, flower and fruit development observed in kiwifruit. The first period of expression was early in first-order bud development (late spring of the first growing season), when second-order meristems were initiated, and the second, approximately 10 months later when those meristems differentiated flowers (late spring of the second growing season). *In situ* hybridisation studies on axillary buds collected during late spring of the first growing season showed *ALF* expression throughout the developing first-order buds, and *AAP1* expression to be localised in developing second-order axillary meristems. During the spring of the second growing season, transcript accumulation for both *ALF* and *AAP1* was localised in differentiating flowers. Walton et al. (2001) suggested that important developmental events were occurring very early in kiwifruit first-order axillary bud development (spring of the first growing season) and it was likely that this included floral commitment.

No literature is available on the expression pattern of *TFL1*, as yet, in woody perennials. Gene bank data bases have also not listed any submissions on *TFL1* sequences from woody perennials other than *CiFT*, which the authors (Omura et al., 1999) have listed as a flowering time gene from *Citrus unshiu* with sequence homology to *TFL1*.

1.5 The interaction between flowering time and floral meristem identity genes

Flowering time mutants are reported to display their major effects on the duration of vegetative development, whereas mutations in floral meristem identity genes are found to

disrupt floral development. Therefore, flowering time genes are often assumed to act before floral meristem identity genes and to lead to their activation (Pineiro and Coupland, 1998).

The role of flowering time genes in the activation of floral meristem identity genes is being uncovered, but the greatest progress has been made in studying the interactions between flowering-time genes and *LFY*. It has generally been seen that the effects of *lfy* or *ap1* mutations are enhanced by mutations causing late flowering, indicating a close relationship between genes that promote flowering and the action of floral meristem identity genes (Putterill et al., 1995; Ruiz-Garcia et al., 1997).

The promotion of flowering by some treatments such as activation of *CO*, a flowering-time gene, was partially by causing an increase in the transcription of the *LFY* gene. Blasquez (1997) has observed that forced expression of *CO* promoted flowering through the transcriptional activation of *LFY*. However, the role of flowering-time genes could not simply be to activate *LFY* expression. Strong expression of *35S::LFY* was insufficient to cause flower development without the formation of several vegetative nodes, suggesting that the shoot apical meristem must also become competent to respond to *LFY* expression (Weigel and Nilsson, 1995).

In general, two classes of flowering time genes have been identified (Reeves and Coupland, 2000). The first class of genes appeared to be involved in the transcriptional upregulation of *LFY*. Mutations in genes from this class caused relatively weak upregulation of the *LFY* promoter activity during development and did not attenuate the early flowering of 35S::*LFY* plants. Genes from several genetically distinct flowering time pathways including *FCA/FVE* (autonomous pathway), *CO/G1* (photoperiod pathway), and *GIBBERELLIN RESPONSIVE1/GIBBERELLIN INSENSITIVE* (GA-dependent pathway) belonged to this group, indicating that *LFY* is a common target for these flowering pathways (Simon et al., 1996; Nilsson et al., 1998).

The second group consists mainly of a subgroup of genes in the photoperiod path-

way, namely *FT* and *FWA* (Reeves and Coupland, 2000). This class did not appear to be involved in the transcriptional upregulation of *LFY*, but was instead required for the response to *LFY* activity. Mutations in these genes had only a small effect on the *LFY* promoter and the late flowering phenotype of *Arabidopsis* mutants *ft* and *fwa* was epistatic to the early flowering of 35S::*LFY* plants (Nilsson et al., 1998). It was considered that one function of this second group was the activation of *AP1* (Ruiz-Garcia et al., 1997)

Genetic experiments also showed that some flowering time genes did not act through *LFY* but through other floral meristem identity genes. Two of the late flowering *Arabidopsis* mutants (*fwa* and *ft*) showed a more severe interaction with *lfy* than others (Ruiz-Garcia et al., 1997). The *fwa* and *ft* mutations also enhanced the *ap1* phenotype but the enhancement was not as strong as that of *lfy*. On the basis of these findings Ruiz-Garcia et al. (1997) proposed that *fwa* and *ft* did not act to promote flower development through *LFY* but through other floral meristem identity genes such as *AP1*. The severe phenotypes of *fwa/lfy* and *ft/lfy* plants could thus be explained as the impairment of partially redundant floral meristem identity functions, one which involved *LFY* and others that required *FWA* and *FT* to be activated (Ruiz-Garcia et al., 1997).

More detailed genetic analysis combining individual late flowering mutants of *Arabidopsis* with a range of meristem identity mutants also revealed that although some genes are involved in the transcriptional upregulation of *LFY*, this was unlikely to be their sole function (Aukerman et al., 1999; Melzer et al., 1999; Page et al., 1999). As an example, it was seen that double *fca/lfy* mutants showed an enhancement of the *lfy* phenotype, suggesting that *FCA* also acted to promote flowering and flower development in a pathway parallel to *LFY* (Page et al., 1999).

Studies conducted by Page et al. (1999) also highlighted an overlooked role that *AP1* had in the regulation of flowering. It had been observed that overexpression of *AP1* caused early flowering in *Arabidopsis* (Mandel and Yanofsky, 1995). This suggested that one function of *AP1* was to promote flowering, and the appearance of *AP1* gene expression in primordia is generally considered to be a good marker for the commitment to flowering

(Hempel et al., 1997). However, it was also seen that *ap1* mutants also flowered slightly earlier than wild type plants indicating that *AP1* gene may also repress flowering (Page et al., 1999). It was suggested that, as the late flowering of the *fca* mutant was epistatic to the early flowering of *ap1* plants, *AP1* could repress flowering by antagonising the action of the autonomous promotion pathway.

It has been suggested that exposure to ideal day lengths and the action of flowering time genes could activate meristem identity genes that act cooperatively with *LFY* to confer floral identity on meristems. Some flowering time genes could also act on meristems to facilitate the activity of floral meristem identity genes. Pineiro and Coupland (1998) observed that the homology of *FT* to *TFL* could suggest such a role for *FT*.

Flowering time genes were also considered to have a likely involvement in the increased expression of *TFL1* that occurred around the time of commitment to flowering, as the activation of *CO* led to increased expression of *TFL1* (Simon et al., 1996; Bradley et al., 1997). It has also been observed that the effects of *tfl* mutation in *Arabidopsis* were weakened by environmental conditions such as SD that delayed flowering (Shannon and Meeks-Wagner, 1991). It has also been shown that at least some mutations that caused late flowering also delayed the determinate phenotype of *tfl* mutants, so that the double mutants formed a terminal flower after producing more lateral flowers than that produced in *tfl* mutants (Ray et al., 1996; Ruiz-Garcia et al., 1997). The double mutants also flowered with a similar number of leaves as the late flowering parents, indicating that genes affected in the late flowering mutants were required for the early flowering seen in *tfl* mutants.

1.6 Herbaceous models in flowering

Several models have been developed through transgenic and mutant studies in annual, herbaceous plants such as *Arabidopsis* and *Antirrhinum* to account for how the various flowering time genes, the floral meristem identity genes and the organ identity genes interact in the process of flowering.

Schultz and Haughn (1993) have proposed a working model for transition from the vegetative to reproductive phase. They suggested that the activity level of a factor(s) changes with node production, and that at critical levels, morphological programs associated with a phase change are activated. They referred to the factor(s) as Controller(s) of Phase Switching (COPS). COPS activity decreases gradually throughout shoot development. At a critical COPS level the Floral Initiation Process (FLIP) genes (*LFY*, *AP1*, *AP2*) are activated and they together initiate floral initiation processes by repressing the inflorescence program, repressing Class C organ identity gene expression and activating perianth development. Late in flower development, a decrease in FLIP gene expression allows the expression of Class C organ identity genes, which, as well as activating reproductive organ development, continues maintenance of the floral program by repressing the inflorescence program.

Ma (1998) summarised a progressive specification of the floral fate as follows. During normal development under favourable conditions, the earliest floral meristem identity genes, such as *LFY* and *FLO*, are activated before the formation of the visible floral meristem. They are thus the first molecular signs of floral development and their expression marks the position of future meristems. Soon afterwards, *AP1* and *SQUA* are expressed in the newly formed floral meristems. In addition, *CAL* has a function that overlaps with but is not identical to that of *AP1*. *FLO/SQUA* and *LFY/AP1/CAL* promote the floral fate characterised by development of floral organs. *AP1* is not expressed at the centre when *AGAMOUS* (*AG*) begins to be expressed. At this time, *AG* is required to specify the floral meristem identity, to further commit it to a determinate program of development. The lack of *AG* function or the reduction of *LFY* function allows the lateral meristem to revert to an inflorescence meristem. *CEN/TFL1* are needed to prevent the expression of *FLO/LFY*, *AP1* and probably *SQUA* from spreading into the centre of the inflorescence meristem, thereby maintaining the inflorescence meristem identity. In *Arabidopsis*, the flowering time gene *CO* can activate *LFY* expression and genetic experiments suggest that both *CO* and *FCA* interact positively with *AG*. Owing to the sequential action of all *FLO/LFY*, then *SQUA/AP1* genes and finally *AG/PLENA* (*PLE*), cells in the lateral meristem become increasingly committed to reproductive development.

Environmental controls, mediated by *CO* and other genes, can affect the expression and/or function at each of these steps. When the floral meristem identity genes are expressed constitutively in transgenic plants, upstream events are bypassed and flowering occurs precociously.

Among the models for the control of floral organ identity, the ABC model (Haughn and Somerville, 1988; Schwarz-Sommer et al., 1990; Bowman et al., 1991; Coen and Meyerowitz, 1991) is the most widely known. The basic tenets of the model are that, firstly, three gene classes (A, B and C) with overlapping fields of expression control whorl identity. In *Arabidopsis*, the A-function genes are *AP1* and *APETALA2* (*AP2*); B-function genes are *APETALA3* (*AP3*), *PISTALLATA* (*PI*); and the C-function gene is *AGAMOUS* (*AG*). Class A genes act in whorls one and two, Class B genes act in whorls two and three, and Class C genes act in whorls three and four. Secondly, the model states that the expression of Class A and Class C genes are mutually exclusive, such that each gene class restricts the expression of the other. Class A activity is also found to influence Class B expression, since carpels could be found in the second and third whorls of strong *AP2* alleles (Kunst et al., 1989; Haughn et al., 1993) and *AP3* transcript levels were reduced in some *AP2* alleles (Jack et al., 1992).

Recent studies have shown that a trio of closely related genes, *SEPALLATA1/2/3* (*SEP1/2/3*), are required for petal, stamen and carpel identity, and are thus necessary for the function of B and C class genes (Pelaz et al., 2001). Studies by Pelaz et al. (2001) also revealed that in *Arabidopsis*, *AP1* and *SEP3* interacted to promote flower development. Mutations in the *SEP3* gene, as well as *SEP3* antisense plants that had a reduction in *SEP3* RNA, displayed phenotypes that closely resembled intermediate alleles of *AP1*. Furthermore, the early flowering phenotype of plants constitutively expressing *AP1* was significantly enhanced by constitutive *SEP3* expression.

More and more genes are being discovered in different pathways of floral induction and flowering and the complexity of the interactions are increasing. A model referred to in this

thesis as the *Arabidopsis* model or herbaceous model is presented below in Figure 1.2 which is an adaptation from Zhao et al. (2002).
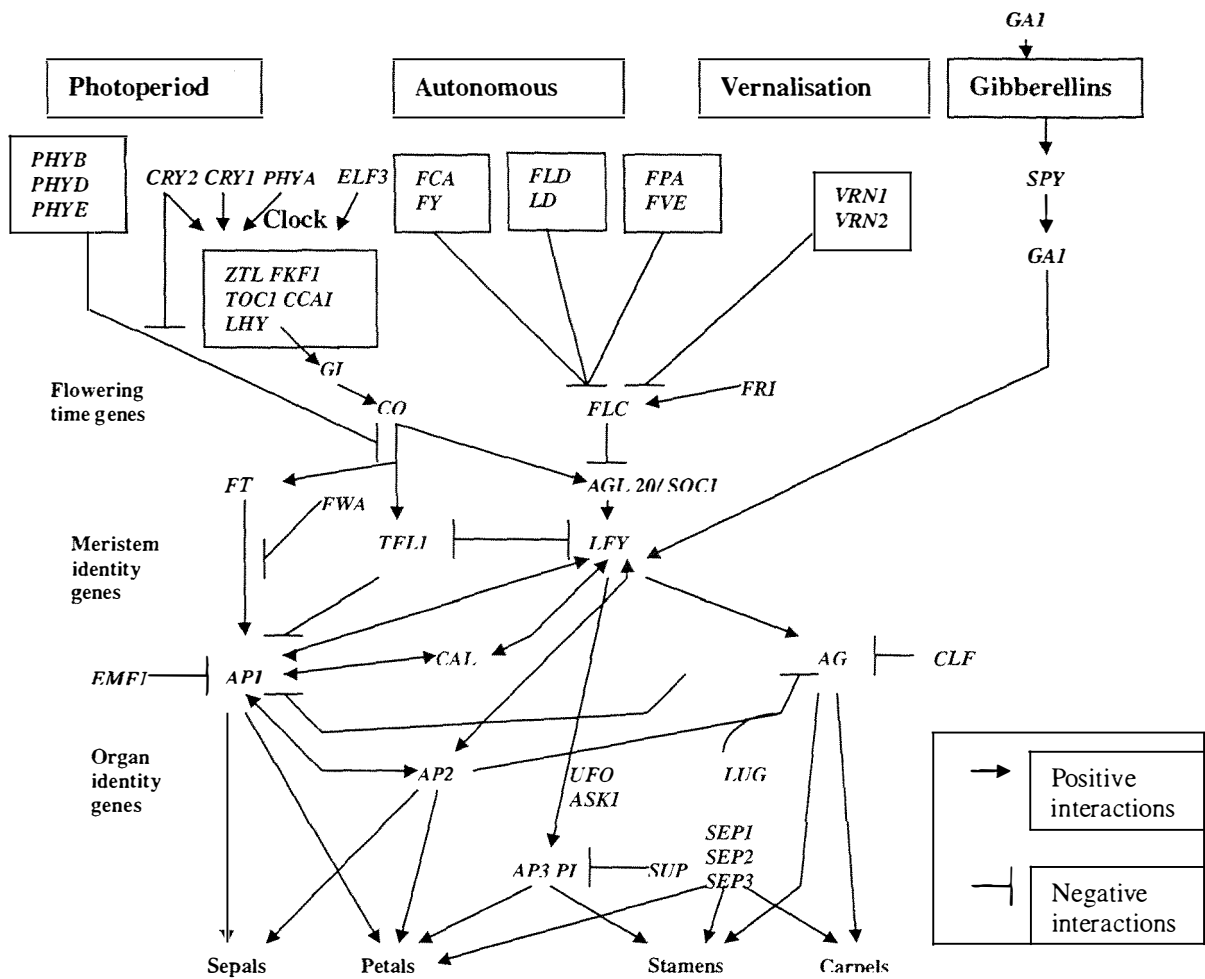
Figure 1.2 Genetic interactions between flowering time, meristem identity and floral organ identity genes; the *Arabidopsis* model.

1.7 Flowering in *Metrosideros* and related species

Dawson (1968a) concluded that the genus *Metrosideros* comprises over 45 described species distributed throughout the Pacific, with outlying representatives in the Borin Islands and South Africa. Eleven species are endemic to New Zealand, of which *M. excelsa* has special cultural and ornamental value. Dawson (1968b) observed that apices of vegetative shoots aborted during the growing season and pairs of over-wintering buds developed in the axils of the adjacent leaf pairs. These buds form the vegetative shoots or inflorescences of the next summer. The trees flower in December and the inflorescence is composed of a primary axis with up to 10 pairs of cymules arranged in an opposite, decussate pattern. Each cymule comprises one terminal flower and two lateral flowers. The primary axis is indeterminate or "open" (Dawson, 1968b), terminating in a vegetative bud that usually remains quiescent between floral bud break in spring and anthesis in midsummer. Vegetative growth can resume from this inflorescence meristem, or the whole floral shoot may abscise following maturation of the fruits and shedding of seed in autumn.

Orlovich (1996) studied floral development in the *Metrosideros* group (Myrtaceae) with special emphasis on the androecium. The basic pattern of flower development was reported to be essentially the same for all the 11 taxa studied which did not include *M. excelsa* or *M. collina*. The flower apical meristem is reported to be convex only until the first couple of sepal primordia are initiated, after which it becomes prominently invaginated, and the subsequent perianth primordia form on the steeply inclined wall of the developing floral tube. The first two sepals are positioned decussate to the floral prophylls but spiral phyllotaxy is quickly attained and continued into the corolla.

Orlovich (1996) reported in the same study that initiation of the floral organs does not follow a strict centripetal order in many of the flowers examined; sepals and petals are formed in order, but the first signs of gynoecium are apparent before the inception of stamen primordia. Stamen primordia are inserted in the expanding hypanthial region between the developing corolla and gynoecium. Orlovich considered the inclined walls of the floral apex equivalent to the flank of the apical dome of a normal, convex,

floral meristem.

Floral biology studies by Schmidt-Adam et al. (1999) revealed that trees flowered over a peak period of two weeks, and compound inflorescences contained an average of 14.3 hermaphrodite, red brush flowers that remained open for seven days. A brief female flower stage (mean duration 1.3 d) was followed by the main hermaphrodite phase, which lasted for four days.

In phenology studies conducted by Schmidt-Adam and Gould (2000), the development of compound inflorescences in *M. excelsa* over a 10-week period was described. They identified six key developmental stages from dormancy to bud break according to morphological characteristics and presented a time course of inflorescence development. Vegetative and compound inflorescence buds were initially morphologically identical, with several bud scales and a spheroidal shape (stage I). Allometric changes resulted in ellipsoidal buds for approximately eight days (stage II). Deciduous bracts subtending the secondary inflorescence axes partly covered cymose inflorescence buds at stage III. These bracts abscised by stage IV. A pair of bracts and two pairs of bracteoles, subtending lateral flower buds in each cymose inflorescence, became visible at stage V. After all bracts and bracteoles have abscised, individual flower buds appeared clearly separated and petals became visible (stage VI).

Concurrent with the present study, Henriod (2001) was investigating environmental factors influencing flowering in *M. excelsa*. Henriod (2001) found that *M. excelsa* 'Vibrance' was highly responsive to photoperiod, but that a significant number of 'Scarlet Pimpernel' plants flowered under both SD and LD. However, few or no 'Vibrance' plants flowered under LD. The intensity of flowering was reduced by exposure to LD in both the cultivars. Hence, *M. excelsa* is considered to be a facultative short day plant.

Henriod et al. (2000) showed that flowering in *M. excelsa* could be manipulated by applying or withholding cool temperatures and SD and by advancing flowering time using warm, LD conditions after inductive conditions had been applied. Henriod et al. (2000)

observed that *M. excelsa* plants transferred to a forcing greenhouse at 24/17°C after 15 weeks at a temperature treatment of 17/14°C and 10 h photoperiod had the highest proportion of flowering plants with significantly more inflorescences per plant, as compared to plants that had been at 12/9°C and 16 h photoperiod. No flowering was observed in plants transferred after 0, 5 or 10 weeks exposure to inductive treatments indicating that a minimum period of cool temperatures combined with SD was optimal for flowering.

While the floral morphology and floral development studies in the *Metrosideros* group by Dawson, Orlovich and Schmidt-Adam each provide key information, none of these studies tracked the progression of flowering from floral initiation to floral organ differentiation and development. These studies on flowering in *Metrosideros* also did not correlate the events happening during floral development, with genetic, environmental or hormonal controls of flowering.

1.8 Summary

A multitude of signals are involved in the induction, evocation and initiation of flowering. Molecular and genetic studies are identifying and characterising a growing number of genes involved in this process. Current knowledge points to a complex regulatory mechanism that is in operation, and that this system mediates the transmission of environmental and developmental cues to the shoot apex where they programme the vegetative meristem to undergo the transition to flowering. Through selected manipulation of this signaling network it may be possible to promote or inhibit flowering. It is important to relate the expression of these genes to actual morphological development in plants and to track the progress of floral development in parallel with the expression patterns of these genes. However, flowering can be completed in plants such as *Arabidopsis* in a very brief span of time during which it would be difficult to clearly assess how these genes are temporally and spatially regulated. It is important to test whether these models apply to woody perennials. Furthermore, woody perennials present the opportunity to assess temporal and spatial expression patterns of flowering genes over a longer period as

the whole process of flowering can be protracted in these species.

Only Walton et al. (2001), whose research proceeded parallel with the research work reported in this thesis, has attempted to track the expression of *LFY* and *AP1* in a woody perennial. Walton and co-workers showed that these genes have a bimodal pattern of expression in *A. deliciosa*, but this study did not monitor the expression pattern of these genes through to floral organ development. The floral development of *A. deliciosa* is also quite different from *M. excelsa* which belongs to a different family. It is known that *TFL1* has a major role to play in flowering in herbaceous plants such as *Arabidopsis* and that it interacts with *LFY* and *AP1* to determine floral fate. The equivalent of this very important gene had not yet been studied in a woody perennial.

GAs have been found to promote flowering in several herbaceous and long day plants. However, they have been found to be inhibitory to florigenesis in several angiosperms. Thus, a brief study of the influence of GAs on expression of floral meristem identity genes in *M. excelsa* (which is a facultative short day plant) was included in the study. It is in this background, that the present project, "Molecular studies on flowering in *M. excelsa*" was conducted to test the herbaceous model with the objectives outlined below.

1.9 Aims of the study

The aim of the project reported in this thesis was to assess the applicability of the herbaceous model to flowering in a woody perennial tree, with a particular focus on floral and inflorescence meristem identity genes. The tree chosen was *M. excelsa* as it was a native tree with significant ornamental and cultural value in New Zealand. Partial funding for this project came from the Public Good Science Fund Native Ornamental Plants Programme.

The objectives were to:
- Develop microscopy techniques for viewing meristems of *M. excelsa*.

- Develop a calendar of floral development in *M. excelsa*, through microscopy studies, to track the process of floral development from floral initiation to floral organ development.

- Study how floral meristems develop compared to vegetative meristems.

- Isolate, sequence and analyse the partial orthologues of the floral meristem identity genes *LFY* and *AP1*, and the *TFL1-* equivalent from *M. excelsa.*

- Study the temporal pattern of expression of floral meristem identity genes, *LFY* and *AP1*, and *TFL1* in meristems of *M. excelsa* through RT-PCR analysis

- Study the spatial pattern of expression of floral meristem identity genes, *LFY* and *AP1*, and *TFL1* in meristems of *M. excelsa* using *in situ* hybridisation techniques.

- Investigate the influence of GAs on *LFY* and *AP1* expression in juvenile plants of *M. excelsa.*

The term 'partial orthologue' is used throughout this thesis to refer to a fragment of the gene with sequence similarity and *presumed* functional similarity to the genes under consideration from the herbaceous plants. The term 'homologous' implies common ancestry and sequence similarity of gene sequences while the term 'orthologous' implies 'homology' combined with similarity in function.

Chapter 2. Microscopy studies and calendar of floral development in *Metrosideros excelsa*

2.1 Introduction

Previous studies of *M. excelsa* did not track the progression of flowering from floral initiation to floral organ differentiation and development. Tracking the development of these meristems through microscopy enabled a calendar of floral development to be produced which has been published (Sreekantan et al., 2001).

Meristems of *M. excelsa* are covered by at least six to eight pairs of very waxy bud scales that prevent easy penetration of clearing and staining fluids. The scales are covered with hairs that make sectioning very difficult. Different methods were tested before the meristems could be successfully viewed through microscopy. Investigations were also carried out to assess whether buds and meristems of difficult specimens such as *M. excelsa* and soft tissues such as maize meristems could be stored at −80°C and later sectioned successfully.

2.2 Microscopy techniques for viewing meristems of *M. excelsa*

2.2.1 Plant material and study site

Five 10-15 year old seedling pohutukawa trees located at the Palmerston North campus of Massey University were used for the study. The trees were of similar habit and proportions. All trees had flowered profusely for at least six years before the study commenced. The distal axillary buds on either side of the aborted apices of elongating shoots were collected over a period from March 1998 to November 1998 for use as specimens. Buds were placed in the fixatives immediately after excision.

2.2.2 Techniques

Seven procedures were investigated. These involved different fixing, staining, and sectioning methods and confocal (Leica DMRBE) as well as light microscopy (Leica MZ12 or Zeiss Axiophot). The composition of fixatives and stains used are given in Table 2.1.

Procedure 1: Whole buds were kept in a clearing fluid (two parts by weight of 85% lactic acid, chloral hydrate, phenol, clove oil and 1 part xylene) for 1, 2, 3 or 4 d. They were then washed in 95% ethanol, and further cleared in 95% ethanol for 2 d, and stained with either:

  a) fast green 0.5%

  b) safranin 1%

  c) methylene blue 1.5% or

  d) safranin for 24 h and counter stained with fast green.

Staining whole buds and then hand sectioning was tested as well as post-section staining. Similarly, clearing under vacuum for the initial 2 d was also tested as well as staining under vacuum. Buds were hand sectioned and viewed under confocal and light microscopes.

Procedure 2: Whole buds and buds with 2-3 outer scales removed were immersed in Histoclear (National Diagnostics, Georgia, USA) for 2-4 d and then hand sectioned and stained using either fast green or safranin, under vacuum for 2 h. The sections were viewed both under confocal and light microscopes.

Procedure 3: Whole buds were fixed using Formalin-Aceto-Alcohol (FAA) (Johansen, 1940) for 24 h. Thereafter the buds were washed three times with 70% ethanol at 2-hourly intervals, cleared using the clearing solution in Procedure 1 for 24 h, rinsed in 95% ethanol 3-4 times, and stained with either fast green or safranin. Specimens were kept immersed in stain for 24 h, with the initial 2 h under vacuum. The buds were then hand sectioned and viewed under the confocal and light microscopes.

Procedure 4: Buds were fixed overnight under vacuum in Formalin-Propiono-Alcohol

Table 2.1 Composition of fixatives and stains

Fixative / Stain	Composition
FAA	70 % ethyl alcohol----------------90ml
	glacial acetic acid-----------------5ml
	37% formalin -----------------5ml
FPA	70% ethyl alcohol-----------------90ml
	propionic acid ------------------5ml
	37% formalin ------------------5ml
Carnoy's Fluid	100% ethyl alcohol--------------60ml
	glacial acetic acid-----------------10ml
	chloroform -----------------30ml
Safranin	1% safranin in 70% ethyl alcohol
Picro- alcohol	0.5% picric acid in 95% ethyl alcohol
Fast green	clove oil --------------------15ml
	methyl cellosolve------------------15ml
	95 % ethyl alcohol----------------90ml
	glacial acetic acid-----------------30ml
	fast green ------------------0.3 g
Clove oil/Absolute alcohol/ Histoclear	------------------50:25:25
Histoclear/Absolute alcohol	------------------50:50
Methylene blue	-------- 1.5% in 96% alcohol

(FPA) (Sass, 1958). The fixing solution was replaced with 70% ethanol and the samples were taken through an ethanol series (85, 95, 100%) with one hour between the changes. The tissues were left in 100% ethanol overnight to complete the fixation process and to eliminate chlorophyll. The samples were then brought through a decreasing ethanol series (95, 85, 70, 50, 30, 15%, to distilled water) with >30 min for each change. Tissues were next stained with propidium iodide, a nuclear stain, rinsed and cleared as per Running et al. (1995) but substituting the xylene with Histoclear. The buds were viewed under the confocal and light microscopes after sectioning by hand.

Procedure 5: Two fixatives were assessed: FAA and Carnoy's fluid (Johansen, 1940). Whole buds were collected directly in fixatives taken to the plants in the field. The outermost 2-3 pairs of scales were removed with the buds still in the fixing solution in the case of large buds (>4 mm in width) and the buds fixed under vacuum overnight. The buds were left in the fixatives for 4 d. After fixing, they were washed with 70% ethanol at least three times at 2-hourly intervals. After the buds were taken through serial dehydration solutions of tertiary butyl alcohol (Figure 2.1) they were embedded in paraplast using an embedding machine (Leica EG1160). The embedded blocks were hardened on the chill tray of the embedder.

The next day the blocks of embedded tissue were microtomed to serial sections of 10 μm thickness on a Leica RM2145 machine. Ribbon pieces were floated on water at 42°C in a Leica HI 1210 water bath for 1 min to take the compression out of the tissue. Superfrost-plus treated microscope slides (Biolab Scientific) were positioned in the water under the ribbon pieces and the ribbons were scooped on to them. Water was drained off the slides and they were incubated on a slide warmer set to 45°C for 24-48 h. Slides were then taken through a staining process (Figure 2.2) that was a modification of the schedule of Johansen (1940). Sections were then mounted in DPX mountant (BDH, Prod 360294H). After drying in the fume hood for at least 2-3 h, sections were viewed under the light microscope.

Procedure 6: Buds of *M. excelsa* and longitudinal pieces of maize shoots (1-2 cm in length) containing meristems were harvested into liquid nitrogen and were taken to the

Washing fixed tissue with 70% ethanol x 3 (at 2 h intervals. Tissue left in 70% ethanol between washes)

▼

tertiary butyl alcohol/95% ethanol/ distilled water   20:50:30 (2 h)

▼

tertiary butyl alcohol/95% ethanol/distilled water   35:50:15 (2 h)

▼

tertiary butyl alcohol/95% ethanol   55:45 (2 h)

▼

tertiary butyl alcohol/absolute alcohol   75:25 and a pinch of erythrosine dye (2 h)

▼

100% tertiary butyl alcohol x 3 (2 h between changes)

▼

Overnight in 100% tertiary butyl alcohol*

▼

tertiary butyl alcohol/liquid paraffin*   50:50 (2 h)

▼

Transfer tissue with tertiary butyl alcohol/liquid paraffin mix into universal bottles half filled with paraplast chips and put in oven at 60°C

▼

Decant away the solution and put in pure molten Paraplast x 5 (3 h between changes)
▼
Embedding

*Temperature to be kept around 30-35°C in a water bath or using a fan heater to prevent solutions from solidifying at cooler temperatures.

Figure 2.1 Flow chart of tissue dehydration and wax infiltration for paraffin embedding of buds of *M. excelsa*
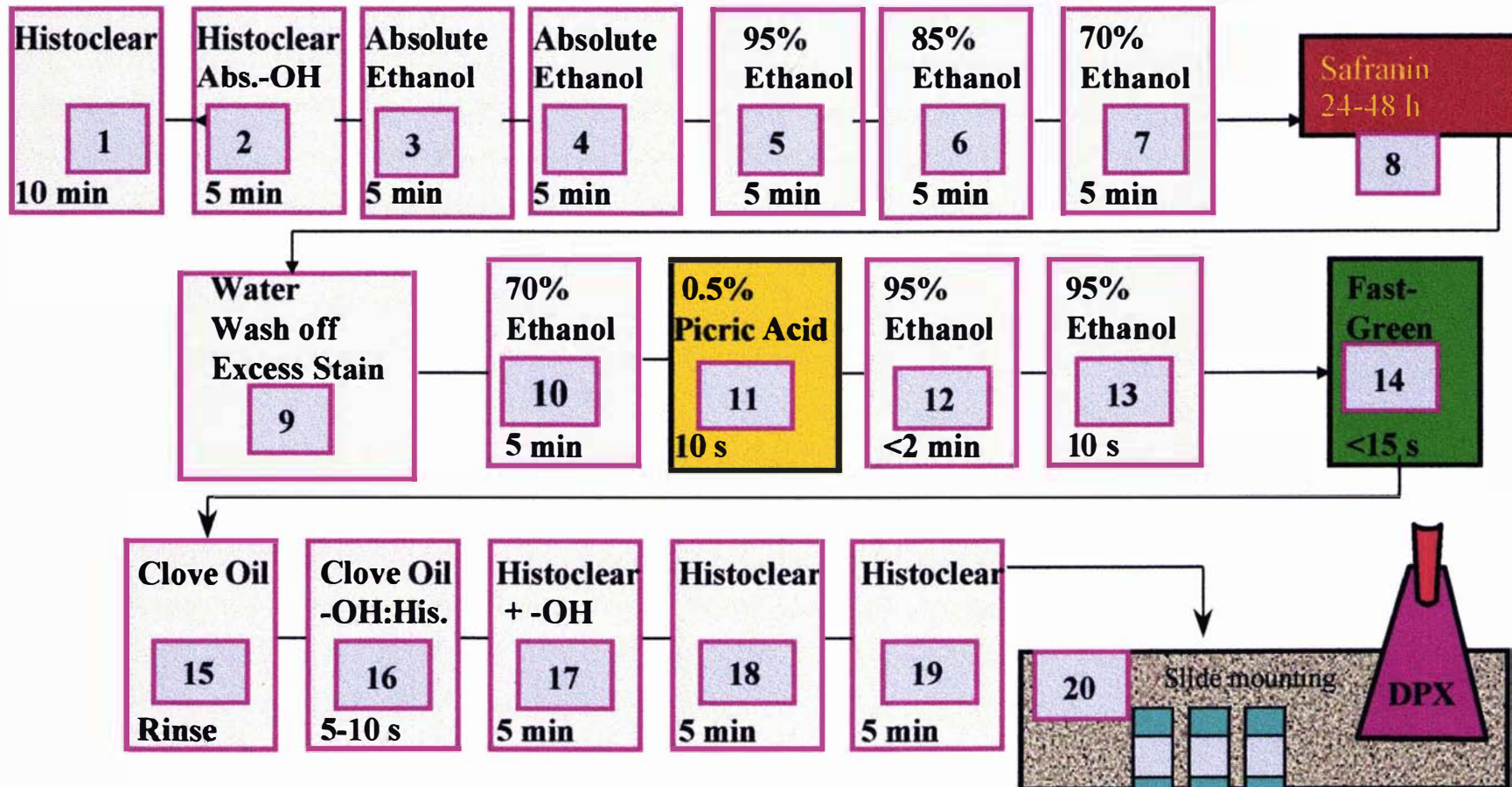
Figure 2.2 Staining schedule with safranin and fast green
(Adapted from Nickless, L.)

laboratory and stored at $-80°C$ for more than a week. These tissues were transferred to fixatives (FAA and Carnoy's fluid) that had been stored at $-20°C$ for temperature stabilisation overnight. The tissue then went through one of the following treatments: either immediate vacuum fixation overnight at room temperature and left in fixative at room temperature for 4 d, or left in the fixative at $-20°C$ for 1-2 d, transferred to $4°C$ for 1-2 d followed by vacuum fixation overnight at room temperature and left in fixative at room temperature for 4 d. The fixed tissue was taken through the embedding and staining procedure outlined in Procedure 5.

A shorter fixation of *M. excelsa* buds transferred directly from the freezer into fixatives was also tried with vacuum fixation for 30 min. The buds were then left in the fixative overnight (compared with 4 d as in Procedure 5) and were embedded and sectioned as in Procedure 5.

Procedure 7: Some buds embedded as per Procedure 5 were microtomed to the depth where the meristem was exposed and were dewaxed in Histoclear for 5 d with the Histoclear solution changed every day. These sections were then put in 70% ethanol for 3-4 d, again changing the solution every day. Afterwards, these sections were viewed under the confocal microscope using either auto-fluorescence or after staining with fastgreen for 24 h.

Some large flower buds that were fixed as per Procedure 5, were also hand sectioned without embedding and were viewed under the confocal microscope using either auto-fluorescence or after staining with fast green for 24 h.

2.2.3 Results

Buds treated as per the first two procedures did not give consistently good sections as the meristems shattered on sectioning. Only one out of 12 buds in the first procedure gave a reasonable image under the confocal microscope. However, even with this section, little could be seen beyond the surface. The third procedure, which used FAA as a fixative,

yielded images for thin sections (Figure 2.3 A) but was not good for thicker sections under the confocal microscope. The fact that the meristem was shattering had pointed to the necessity for using fixatives. However with the use of fixative, all the numerous hairs on the bud scales also became fixed and this was the major problem with Procedure 3. The numerous hairs on the scales were very prominent and obstructed the clear viewing of the meristem. The buds again did not appear to be adequately fixed. This was the difficulty again with the fourth procedure.

Procedure 5 consistently gave the best results and it was possible to track the development of floral and vegetative buds of *M. excelsa* under the light microscope (Figure 2.3 B).

Buds that were embedded and microtomed until the meristem was visible, and then dewaxed with histoclear (Procedure 7), also gave fairly good images (Figure 2.3 C) under the confocal microscope as did floral buds that were just fixed, hand sectioned and viewed through the confocal microscope (Figure 2.3 D).

Buds of *M. excelsa* and maize meristems kept at −80°C (Procedure 6) could also be successfully sectioned. In this case, vacuum fixing the tissues straight from the freezer overnight was as good as, or even slightly better than, taking them through a temperature series and then applying vacuum. The shorter fixation time tested with *M. excelsa* buds taken from −80°C did not give good sections. In the case of a soft tissue, such as the maize meristem, the sections obtained after −80°C storage were as good (Figure 2.4) as those fixed and embedded using Procedure 5. With *M. excelsa*, however, buds taken through Procedure 5 without going through the −80°C treatment were easier to section. In all cases, maize meristems were considerably easier to section and stain than *M. excelsa* buds.

A comparison of the effectiveness of Carnoy's fluid and FAA as fixatives clearly showed that, although marked differences could not be seen between the two in fixing maize meristems, Carnoy's fluid was definitely superior to FAA in the case of *M. excelsa* buds (Figure 2.5). There was no browning of sections obtained with the use of Carnoy's fluid where as there was considerable browning with FAA.
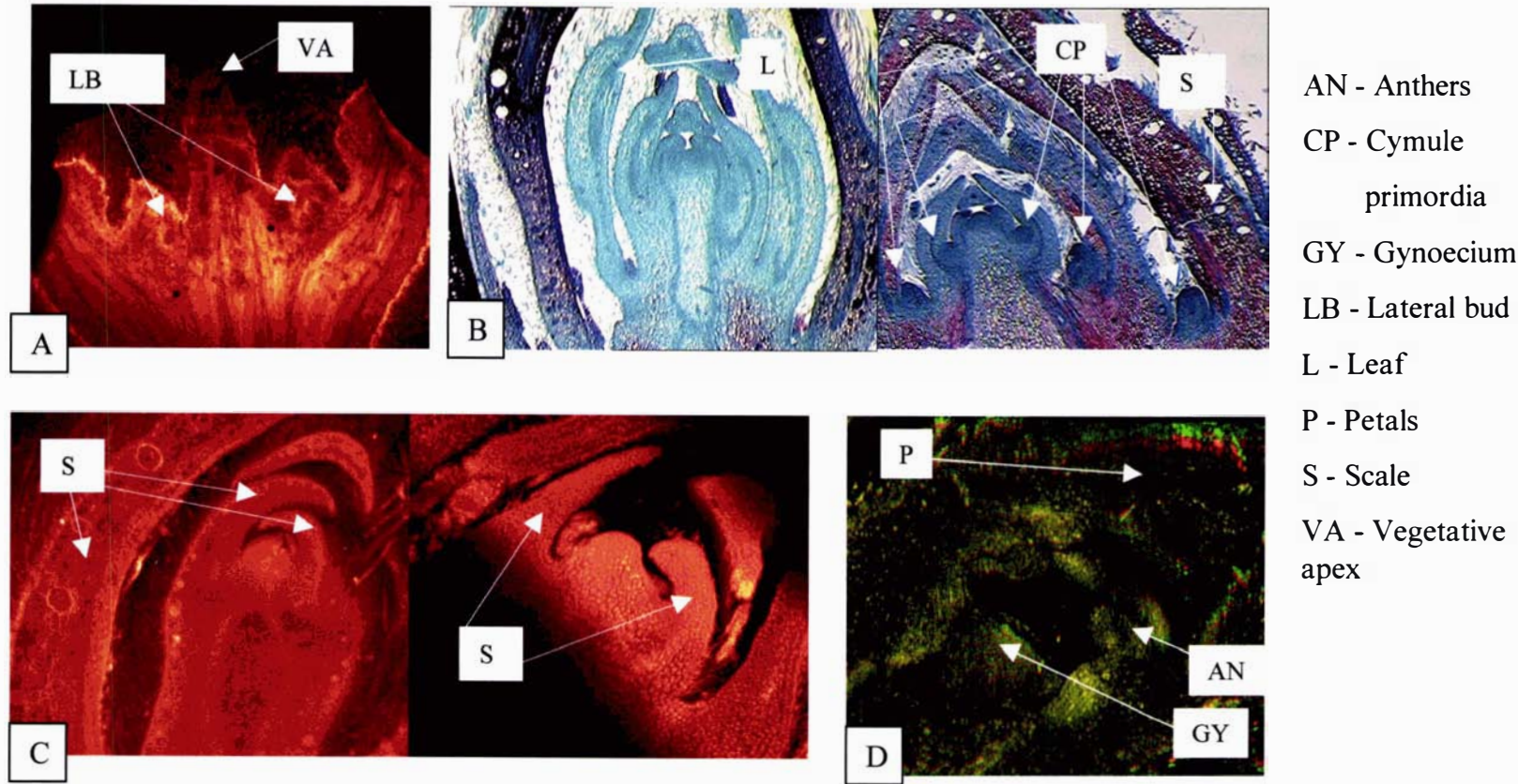
Figure 2.3 Images of meristems of *M. excelsa* generated through different microscopy techniques. (A) Confocal image after fixing with FAA, clearing, safranin staining and hand sectioning. (B) Vegetative (left) and floral (right) buds fixed with Carnoy's fluid, embedded, microtomed and stained with safranin and fast green. (C) Fixed, embedded, microtomed and dewaxed meristems viewed under confocal microscope through autofluorescence. (D) Fixed and hand sectioned, 3 dimensional image of a floral bud viewed under confocal microscope through autofluorescence.
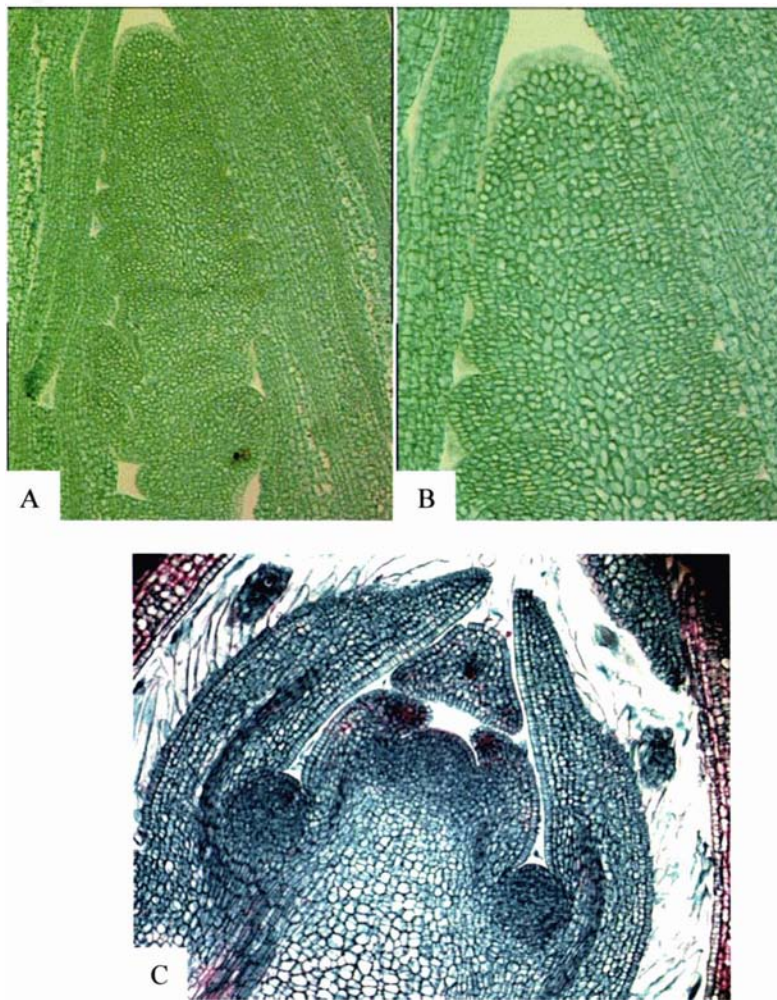
Figure 2.4 Comparison of fixation protocol for softer tissues and that of *M. excelsa* (A) Maize meristem fixed, embedded, microtomed and stained with safranin and fast green. (B) Maize meristem fixed, embedded, microtomed and stained with safranin and fast green from -80°C. (C) *M. excelsa* floral meristem fixed, embedded, microtomed and stained with safranin and fast green from -80°C.

Figure 2.5 Comparison of browning caused by different fixatives
(A) *M. excelsa* bud fixed with FAA, embedded and microtomed.
(B) *M. excelsa* bud fixed with Carnoy's fluid, embedded and microtomed.

2.2.4 Discussion

The reason why the first two procedures were not successful could be because that the clearing solution and stains were not effectively reaching the meristem in these procedures. Only a superficial image could be obtained in Procedure 3 probably because the bud had been stained with safranin and then sectioned and safranin did not fluoresce well enough for the laser of the confocal microscope to pick up the signals. Good images could not be obtained for Procedure 3 and Procedure 4 probably because the fixing times were too short as well.

The success of the Procedure 5 could be attributed to the longer vacuum fixation whereby fixatives could penetrate through the waxy and pubescent scales to the meristems protected within, and prevented them from shattering on sectioning. FAA can be used on almost any plant material intended for anatomical or morphological studies. Material may be left in it almost indefinitely without appreciable damage. However, Carnoy's fluid has greater penetrating power and is frequently used for difficult specimens (Johansen, 1940). Browning during fixation with FAA, notably in oil glands, epidermal cells and stigmatic cells of mature flowers, has been reported in *Eucalyptus* by Southerton et al. (1998) even with a much shorter fixing time. This is in agreement with the observation in the present study that FAA caused much browning of tissues in *M. excelsa* (Figure 2.5). Carnoy's fluid was superior in the case of *M. excelsa* tissues compared to maize where the difference between the two fixatives was not marked probably because the former has high amounts of phenolics.

The serial sections of 10 μm thickness, obtained through Procedure 5, could effectively be stained by safranin and fast green and viewed under the light microscope. Safranin stains cutinised and lignified cell walls and nuclear material. Fast green stains cellulose and cytoplasm. Picric acid-alcohol solution helps to differentiate safranin (Johansen, 1940).

With confocal microscopy, visualisation was possible utilising the high auto-fluorescence of the tissue. Kohler et al. (1997) observed that some plant tissues such as leaves have high

auto-fluorescence. In all sections of *M. excelsa*, the glands that could be producing oils or wax were very prominent. These could be the substances imparting the auto-fluorescence to the specimens. Some reactions occurring while fixing may also be responsible for imparting the auto-fluorescence to the specimens. Auto-fluorescence could only be observed in fixed buds. Many lasers in laser scanning confocal microscopes emit wavelengths that overlap with auto-fluorescence from chlorophyll and other plant molecules. Auto-fluorescence could interfere with the rhodamine signal (Taylor et al., 1996) that is used for fast green. This could be one of the reasons that stained sections did not give good images on the confocal microscope.

It can be concluded that the procedure that was successful for *M. excelsa* can safely be adopted for both difficult specimens and soft tissues. For soft tissues, a shorter fixing time after removal from –80°C should be tested but this was beyond the scope of this study.

For *in situ* hybridisation techniques as described by Drews et al. (1991), the fact that tissues, even soft ones such as maize meristems, could be stored at –80°C and then fixed and sectioned is very important. The mRNAs would remain preserved at –80°C and fixing and sectioning can be done conveniently at a later date. However, suitable fixatives and procedures had to be tried to get good signals from the *in situ* hybridised sections as harsh fixatives such as Carnoy's fluid could reduce signals. The details of these procedures are presented in Chapter 5.

In summary, meristems of *M. excelsa* were particularly difficult specimens and several procedures were tried before anatomical details could be successfully viewed through microscopy. Overnight vacuum fixation and leaving the specimens in the fixatives (FAA or Carnoy's fluid) for 4 d was required for successful viewing of anatomical details under both light microscopy and confocal microscopy. Taking the fixed whole buds through a dehydration series of tertiary butyl alcohol and paraffin embedding and microtoming gave very good sections. Buds fixed in this way gave the best image in confocal microscopy with autofluorescence. It was also possible to obtain good sections with this procedure for *M. excelsa*, and the much softer maize meristems, that had been stored at –80°C for several

weeks. The sections when stained with safranin and fast green gave good visualisation under light microscope. Hence this procedure was chosen as the ideal microscopy technique for viewing meristems of *M. excelsa* over a period of nine months to track their progression and form a calendar of floral development (Sreekantan et al., 2001).

2.3 Calendar of floral development in *Metrosideros excelsa*

2.3.1 Materials and methods

The distal axillary buds from the shoots of five 12-15 year old seedling trees growing at the Palmerston North campus of Massey University were excised randomly between March 1998 and November 1998. Buds were labeled as small (<0.8 mm diameter), medium (0.8 mm-1.5 mm), and large (>1.5 mm) in early March 1998. Ten buds from each size group, were collected at intervals of four weeks until August and thereafter at fortnightly intervals. The buds were immediately placed in FAA or Carnoy's fluid. The buds were fixed overnight under vacuum, and left in the fixatives for at least 4 d. Thereafter, they were washed with 70% ethanol. Samples of 10-15 buds were then drawn out and embedded in paraffin using an embedding machine (Leica EG1160). Subsequently, they were sectioned using a microtome (Leica RM2145) to serial sections of 10 µm thickness and were stained with safranin and fast green with differentiation in picric acid (Johansen, 1940). The details of the embedding and staining procedures are provided above under Procedure 5. The buds were then viewed under a light microscope to study the pattern of bud development.

2.3.2. Results

Microscopy studies revealed that the meristem of each distal axillary bud was protected by six to eight pairs of scales in decussate pattern. The scales were very pubescent and waxy with wax glands clearly visible as large open circles (Figure 2.6 A). Prior to May, no activity other than the formation of the full complement of scales could be distinguished in the buds (Table 2.2, Figure 2. 6 A). In the first week of May, slight protrusions and accelerated cell division could be observed in the axils of the scales of two out of 15 buds

Table 2.2 Timing and progress of floral development in distal axillary buds of

M. excelsa

Date of sampling	Number of buds examined	Number of buds in which floral development was evident	Comments
12 March '98	12	0	Bud scales were forming. No other activity could be observed
9 April '98	12	0	Bud scales were forming. No other activity could be observed
7 May '98	15	2	Small bulges in the axils of scales with actively dividing cells
4 June '98	12	5	Small bulges in the axils of scales with actively dividing cells in floral buds; apex dividing in opposite decussate pattern forming leaf primordia in vegetative buds
20 June '98	15	9	In 4 out of the 9 floral buds the bulges had clearly grown to large floral primordia while the rest showed small bulges in the axils of scales
4 August '98	15	10	The bracts subtending the three flowers could be seen to develop in some floral buds. Vegetative buds still not much elongated
18 August '98	15	9	,, ,, ,,
1 September '98	15	9	Cymules continued to develop in floral buds while vegetative buds started to elongate
16 September '98	5	5	Floral organs could be seen developing in the individual flowers. Stopped tracking development of vegetative buds
30 September '98	5	5	All floral organs were fully differentiated in individual flowers
14 October '98	5	5	Floral organs fully differentiated and growing
12 November '98	5	5	Organs fully differentiated and developing. Ovules and pollen visible.

examined in that period (Figure 2.6 B). By the first week of June such axillary activity could be seen in five out of 12 buds examined. The ratio of floral buds to total buds examined also did not increase much after June. The percentage of floral buds remained at 60 - 65% in buds examined from late June to September (Table 2.2). The axillary protrusions had grown to distinguishable cymule primordia by the end of June (Figure 2.6 C and D). Cymules continued to develop from these primordia during the period from August to September (Figure 2.6 E). The three bracts subtending each flower in a cymule were the structures to form first (Figure 2.7A). Organogenesis progressed inwards with the formation of sepals, petals, the androecium and the gynoecium (Figure 2.7 B). The anthers grew inwards as protrusions from the sides of the inner walls of the operculum, and later the filaments elongated. The gynoecium grew from the basal inner wall of the operculum (Figure 2.7 B), with the apical dome clearly outlined during early September (Figure 2.3D), and later elongated to form a style. By the end of September, all the floral organs had clearly differentiated in the three flowers in each cymule (Figure 2.7C). Sections at this stage revealed numerous tightly curled filaments and a very long convoluted style within the young flower buds. Pollen grains and ovules could be distinguished in bud sections by mid-November. The trees were in full bloom by the second week of December.

The buds that developed into vegetative shoots developed in a very different pattern compared to floral buds. No axillary activity could be seen in the axils of these buds until August (Figure 2.8 A), and the shoot apical meristem (SAM) continued to produce leaves in an opposite decussate pattern even after the formation of the full complement of scales (Figure 2.8 B). The internode length started to increase by September (Figure 2.8 C) at which stage some axillary buds could sometimes be seen in some sections. Vegetative buds were not tracked beyond September as bud break had started to occur.
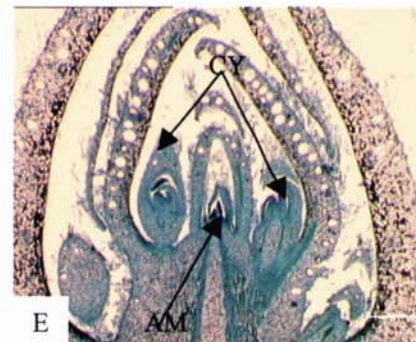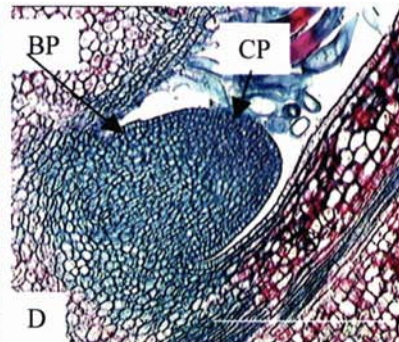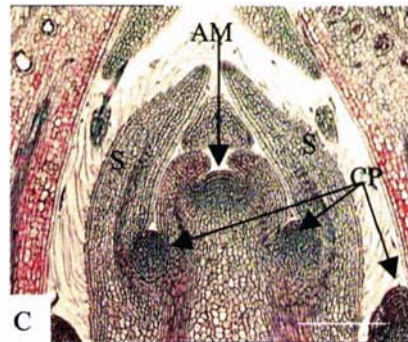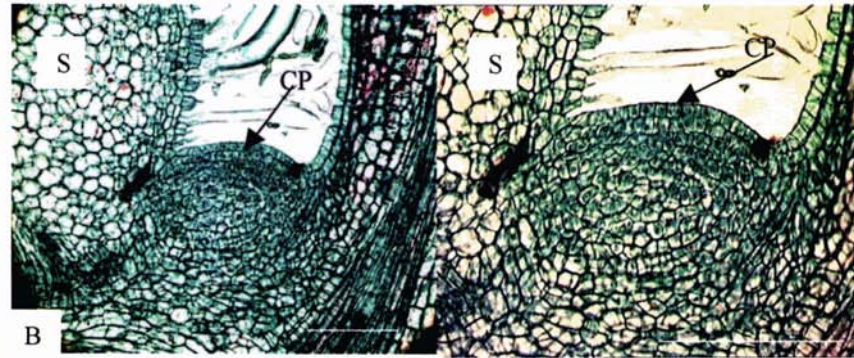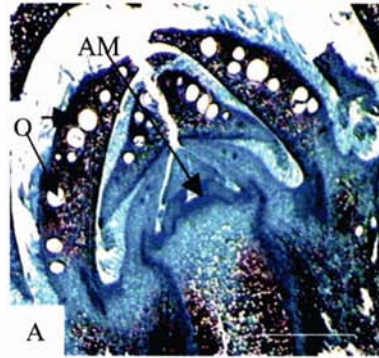
AM = Apical meristem
BP = Bract primordium
CP = Cymule primordia
CY = Cymules
O = Oil gland
S = Scales

Figure 2.6 Development of floral buds of *M. excelsa* from March to August. (A) Distal lateral bud in March with the scales protecting the meristem. Bar = 500 μm. (B) Initiation of floral primordia in scale axils in May. Bar = 100 μm (left and right). (C) Cymule primordia in scale axils in June. Bar = 100 μm. (D) Cymule primordia in late June. Bar = 100 μm. (E) Inflorescence in August. Bar = 500 μm.
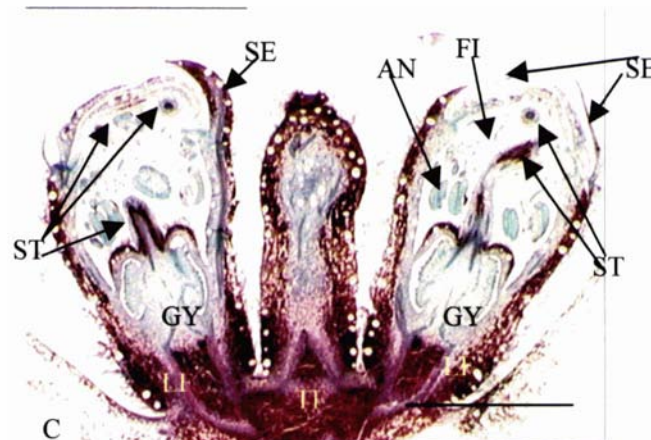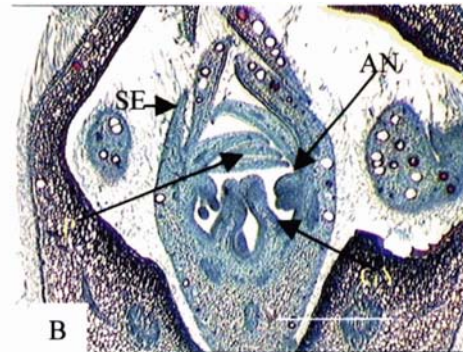
AN = Anthers
BE = Bracteole
BR = Bract
FI = Filament
GY = Gynoecium
LF = Lateral flower
P = Petals
SE = Sepals
ST = Style
TF = Terminal flower

Figure 2.7 Development of floral buds of *M. excelsa* from August to October (A) Development of bracts and bracteoles and three flowers in a cymule in August. Bar = 100 µm.(B) Formation of sepals, petals, androecium and gynoecium in mid-September. Bar = 500 µm. (C) Differentiation of all floral organs in late September. Bar = 1mm

AB = Axillary bud
AM = Apical meristem
L = Leaf
LP = Leaf primordium
S = Scale
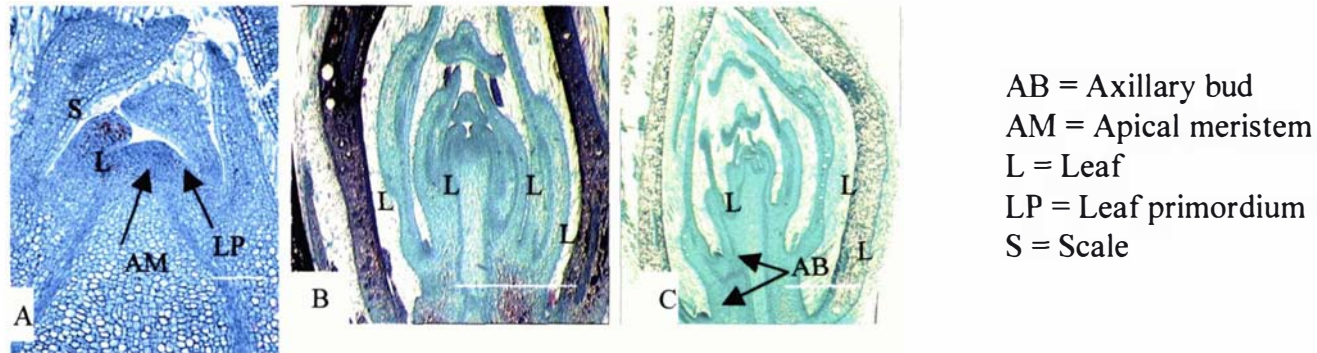
Figure 2.8 Development of vegetative buds of *M. excelsa* from June to September (A) Formation of scales with no axillary protrusions in June. Bar = 100 μm. (B) Apical meristem continuing to form decussate pairs of leaves in August. Bar = 500 μm. (C) Elongation of internodes in September with continued production of leaves. Bar = 500 μm.
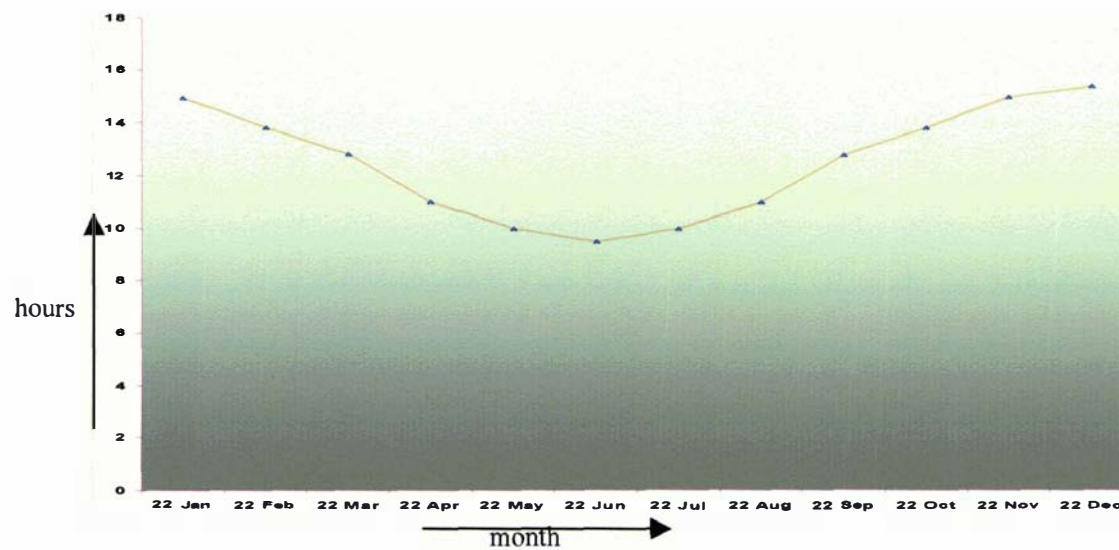
Figure 2.9 Photoperiod greater than 107.6 lux (hours) in Palmerston North, New Zealand (after Francis, 1972).

2.3.4. Discussion

The steady decline in photoperiod could be the inductive signal for floral initiation rather than the shortest days *per se*. It was observed that floral primordia started to form even before the shortest days which occurred in June (Figure 2.9). The same could also be said about temperature as floral initiation started earlier than the coldest days in winter. Floral induction may be occurring over a certain period and need not be just a once on-off signal as buds at different stages of development were observed at any one time. In December fully opened inflorescences could be seen on the same tree with flower buds that needed at least another two weeks for anthesis. However, activity in the axils of the scales that started later than June would probably not become flowers as all the floral buds had progressed well into formation of cymules by then. This could account for some of the aborted axillary structures seen in vegetative buds in August-September.

Observations also revealed that all the 6-8 pairs of scales in each bud need not necessarily bear cymules in their axils. The first formed outermost pairs of scales often lacked cymules in their axils. It is possible that this was due to a decline in floral initiation signals from the apex downwards. Factors other than the environment may also play a role in floral induction as buds exposed to the same conditions often had different developmental fates (Sreekantan et al., 2001). This was particularly evident in cases where even the two buds in a pair of adjacent axillary buds sometimes developed in two different patterns: one forming into a vegetative shoot and the other an inflorescence.

In the study by Orlovich (1996) on floral development in the *Metrosideros* group with special emphasis on the androecium, the basic pattern of flower development was reported to be essentially the same for all the 11 taxa studied which did not include *M.excelsa* or *M. collina*. The flower apical meristem is reported to be convex only until the first couple of sepal primordia are initiated, after which it becomes prominently invaginated, and the subsequent perianth primordia form on the steeply inclined wall of the developing floral tube. Sections from *M. excelsa* showed similar invagination when flower development begins within the cymule primordia (Figure 2.7A).

Orlovich (1996) also reported that the first two sepals are positioned decussate to the floral prophylls but spiral phyllotaxy is quickly attained and continued into the corolla, Stamen primordia are inserted in the expanding hypanthial region between the developing corolla and gynoecium. Orlovich (1996) considered the inclined walls of the floral apex to be equivalent to the flank of the apical dome of a normal, convex, floral meristem. Sections from *M. excelsa* in the present study also revealed that stamen primordia arise from the hypanthial region between the developing corolla and the gynoecium (Figure 2.7 B)

In the model species *Arabidopsis* and *Antirrhinum*, it is the apical meristem that switches from vegetative to floral development as the plants enter the reproductive phase (Coen and Meyerowitz, 1991; Hempel and Feldman, 1994). In contrast, in *Eucalyptus*, which is a close relative of *Metrosideros*, the apical meristem generally remains vegetative. Lateral meristems, arising in the axils, may give rise to a leafy shoot or to an inflorescence in response to inductive environmental conditions, such as daylength and temperature. Both of these structures are enveloped by a pair of bracts that protect the primordia. The inflorescence in *Eucalyptus* is determinate and converts directly to a floral meristem(s). The flowers are generally arranged in a dichasium, which terminates in a single flower, the subtending, deciduous bracts of which enclose all other flowers in the developing inflorescence (Southerton et al., 1998a).

While *M. excelsa* and *Eucalyptus* inflorescences are similar in many ways there are some differences in their formation. In *M. excelsa* also, inflorescences are typically axillary as in *Eucalyptus*. The apices of elongating shoots abort sometime in spring (September to November) and then the lateral meristems overwinter and give rise to leafy shoots or inflorescences. However, unlike Eucalyptus, the inflorescence axis is indeterminate. The primary axis of the inflorescence remains "open" terminating in a vegetative bud. This vegetative bud is inactive during flowering but may later develop into a leafy branch (Dawson, 1968a; Sreekantan et al., 2001).

Southerton et al. (1998) had reported that the pattern and timing of organ development in *Eucalyptus* is similar to that of *Arabidopsis* and *Antirrhinum* even though their flowers

were structurally very different. The flowers are comprised of four whorls as in *Arabidopsis* and *Antirrhinum* with the sepals forming first, followed by the petals, then the androecium and finally the innermost whorl of gynoecium. Southerton et al. (1998) then concluded that these common developmental features suggested that the key floral regulatory genes could be the same in all these species.

However, Orlovich (1996) observed that the initiation of the floral organs does not follow a strict centripetal order in many of the *Metrosideros* species examined: sepals and petals are formed in order, but the first signs of gynoecium are apparent before the inception of stamen primordia. In sections of *M. excelsa* buds also, the gynoecium was evident at all stages when the anthers were first visible (Figures 2.3 D and 2.7 B) indicating that the initiation of floral organs was not in a strictly centripetal pattern. Hence it is important to investigate whether the key regulatory genes in flowering function the same way in *M. excelsa* as in the model plants, *Arabidopsis* and *Antirrhinum.*

Once the calendar of floral development for *M. excelsa* had been established, the next step was to isolate the floral and inflorescence meristem identity genes (*LFY*, *AP1* and *TFL1* equivalents) in this woody species (Chapter 3). The calendar of floral development then provided the basis for comparison of actual events occurring in the floral meristem versus the temporal expression patterns of the meristem identity genes (Chapter 4). The microscopy procedures also provided the basis for the in situ localisation of the meristem identity genes (Chapter 5). Once the temporal and spatial expression patterns of these genes have been revealed, the validity of applying the herbaceous model of flowering to a woody perennial could then be assessed.

Chapter 3. Isolation, cloning and Southern analysis of floral and inflorescence meristem identity genes from *Metrosideros* species

3.1 Introduction

The floral meristem identity genes, *LEAFY* (*LFY*), *APETALA1* (*AP1*) and the inflorescence meristem identity gene *TERMINAL FLOWER1* (*TFL1*), play key roles in the process of flowering as has been observed in studies with *Arabidopsis* and several other plants. *LFY* and *AP1* promote flowering and *TFL1* acts like a flowering time gene, functioning as a repressor antagonistic to *LFY*. While giving the *Arabidopsis* model its due importance, it is still necessary to test this model in other plant systems, especially woody perennials, such as *M. excelsa* which is a facultative short day plant (Henriod et al., 2000) in contrast to the long day annual *Arabidopsis*. Even though structurally very different to flowers of *Arabidopsis* or *Antirrhinum*, flowers of *M. excelsa* also are comprised of four whorls, the sepals, the petals, the androecium and finally the innermost whorl of gynoecium. It was therefore hypothesised that there would be equivalents of the important floral meristem identity genes, *LFY*, *AP1* and the inflorescence meristem identity gene *TFL1*, in *M.excelsa* and that they would be expressed in the distal axillary buds that formed the inflorescences. The equivalents of *LFY* had been isolated from several plant species, including perennials, such as *Eucalyptus* (Southerton et al., 1998). *MEL*, the partial orthologue of *LFY* in *M. excelsa*, had also been isolated by McKenzie et al. (1997). *AP1* equivalents had also been isolated from several plant species including *EAP1* and *EAP2* from *Eucalyptus* (Kyozuka et al., 1997). However, *TFL1* had not so far been isolated from a perennial. In the present study, the aim was to assess whether similar genes existed in *M. excelsa* and to characterise their pattern of expression.

MEL, the partial orthologue of *LFY* was first isolated from genomic DNA by McKenzie et al. (1997) through polymerase chain reaction (PCR) using degenerate primers. In the current project, its isolation was attempted both through PCR from genomic DNA and through RT-PCR from total mRNA from *M. excelsa* buds. The strategy used for isolation of *MESAP1* and *MTAP1*, the partial orthologues of *AP1* from *M. excelsa* and *M. collina*

'Tahiti' respectively, was RT-PCR with primers specific for *AP1* in *Brassica oleraceae*. The method used for isolation of *METFL1* and *MTTFL1*, the partial orthologues of *TFL1* from *M. excelsa* and *M. collina* 'Tahiti' respectively, was RT-PCR with degenerate primers on the outside followed by a nested reaction using primers designed to a monocot (rice) and a dicot sequence (Jensen et al. 2001). The work was carried out with the approval from Environmental Risk Management Authority, New Zealand and the Gene Technology Committee, Massey University (GMO 00/ MU/19 and GTC 98/08).

Genes such as *AP1* and *TFL1* have been found to belong to multi-gene families. At least two *LFY* genes have been isolated from some plant species such as *Nicotiana tabacum* (Kelly et al., 1995) and *Brassica* species (Brunel et al., 1998). In *Eucalyptus*, two functional equivalents of *AP1* and two *LFY* genes, (one of which was described as a pseudo gene) have been isolated (Kyozuka et al., 1997; Southerton et al., 1998). Hence, a Southern analysis was also performed on *M. excelsa* DNA using the isolated fragments as probes to determine the number of copies of these genes in the plant. The current Chapter details the strategies adopted to isolate partial orthologues of these genes, the analysis of the sequential similarities of these genes from other plants and the Southern analysis.

3.2 Materials and Methods

Common recipes for buffers and procedures such as gel electrophoresis and DNA plasmid preparations are detailed in Appendix I.

3.2.1 Extraction and purification of DNA

Genomic DNA was extracted from very young leaves of *M. excelsa* harvested from the same five trees that were used for the microscopy study in Chapter 2, using the cetyl trimethyl ammonium bromide (CTAB) procedure as described by Reichardt and Rogers (1994).

Leaf tissue (10 g) was collected and immersed immediately in liquid nitrogen. It was then stored in the -80°C freezer until the time of DNA extraction. From this pool, 3 g was used

in two sets of 1.5 g each for isolation of DNA. On the day of the extraction, 2-mercapto-ethanol (2-ME) was added to the required amount of CTAB extraction solution to give a final concentration of 2% (v/v). This solution, and the CTAB/NaCl solution were heated to 65°C in a water bath. 2-ME /CTAB extraction solution (4 ml) and CTAB/NaCl solution (0.5 ml) were used per gram of leaf tissue. The leaf tissue was chilled and pulverised to a fine powder in liquid nitrogen using a sterile mortar and pestle and transferred to sterile oakridge tubes. Warm 2-ME/CTAB extraction solution was added to the pulverised tissue and mixed thoroughly along with 1% (v/v) polyvinylpyrrolidone. The samples were then incubated for 60 min at 65°C with occasional mixing.

The homogenate was then extracted with an equal volume of 24:1(v/v) chloroform/isoamyl alcohol. After mixing well by inversion, the homogenate was centrifuged at $7500g$ using a SS34 rotor in a Sorvall centrifuge for 5 min and the upper aqueous phase was recovered. 1/10 volume of 65°C CTAB/NaCl solution was added to the recovered aqueous phase and mixed well by inversion. The extraction was repeated with an equal volume of chloroform/isoamyl alcohol, by mixing, centrifuging and recovering as described above. The nucleic acids were then precipitated by adding exactly 1 volume CTAB precipitation solution and mixing well by inversion. The mixture was centrifuged for 5 min at $500g$ at 4°C. The supernatant was removed and the pellet was resuspended in high-salt TE buffer (1 ml g^{-1} of starting material). The nucleic acids were then precipitated by adding 0.6 volume isopropanol, mixing well and centrifuging for 15 min at $7500g$ at 4°C.

The DNA pellet was resuspended in 1 M NaCl (500 µl). This step removed additional polysaccharides and was found to be essential in *M. excelsa*. The polysaccharides were pelleted by centrifuging at $14000g$ for 10 min, and the supernatant, containing the DNA, recovered. The nucleic acids were then precipitated with 1 volume of ice cold isopropanol. The pellet was washed with 80% ethanol and re-suspended in 1 ml of TE (Appendix I) and an aliquot (15 µl) electrophoresed on a 0.8% gel to check recovery.

CTAB extraction solution:

> 2% (w/v) CTAB
>
> 100 mM Tris.Cl, pH 8.0
>
> 20 mM EDTA, pH 8.0

CTAB / NaCl solution:

> 10% (w/v) CTAB
>
> 0.7 M NaCl

CTAB pecipitation solution:

> 1% (w/v) CTAB
>
> 50 mM Tris.Cl, pH 8.0
>
> 10 mm EDTA, pH 8.0

3.2.2 Extraction of RNA

One to 5 g of distal axillary buds from the shoots of the five 12-15 year-old seedling trees growing at the Palmerston North campus of Massey University were excised randomly between March and November. At the time of harvest they were instantly put in liquid nitrogen. On taking them to the laboratory, they were stored at -80°C until the time of RNA extraction. The procedure used for total RNA isolation was the hot-borate extraction method as described by Wilkins and Smart (1996).

DTT, PVP and NP-40 were added to hot-borate extraction (XT) buffer and warmed to 85°C in an oak ridge tube. Frozen tissue (1 g) of *M. excelsa* buds was ground to a fine powder in the pre-chilled mortar and pestle using liquid nitrogen as needed. XT buffer at 2.5 ml g^{-1} tissue was dispensed into a room temperature mortar and pestle and the ground tissue was transferred into it. After blending well for 1 min the homogenate was quickly decanted into an oak ridge tube with 105 μl of the proteinase K (20 mg ml^{-1} in water) solution. The mortar was rinsed with 1 ml of XT buffer and that was also transferred to the proteinase K homogenate in the oak ridge tube. The tube was then incubated at 42°C for 1.5 h with gentle swirling and mixing every 10 min. After that, 280 μl of 2 M KCl was added to a final concentration of 160 mM. The mix was gently swirled and then incubated on ice for 1 h. It was then centrifuged at 12000g for 20 min at 4°C to remove the debris. The supernatant was transferred carefully to another clean oak ridge tube, $^1/_3$ volume of 8 M LiCl was added (to a final concentration of 2 M LiCl) and the mixture incubated

overnight at 4°C.

Precipitated RNA was then pelleted by centrifugation at 12000g for 20 min at 4°C. The supernatant was decanted and discarded. The pellet was washed in 5 ml of ice-cold 2 M LiCl four times, each time centrifuging at 12000g for 10 min at 4°C and discarding the supernatant. After the fourth wash, the pellet was suspended in 2 ml of 10 mM Tris-HCl (pH 7.5) by gentle vortexing. The sample was warmed to room temperature to dissolve. The insoluble material was pelleted by centrifugation at 12000g for 10 min at 4°C. The supernatant was transferred to another oak ridge tube and 1/10 volume of 2 M potassium acetate (pH 5.5) was added and the mix incubated on ice for 15 min. This was followed by centrifugation at 12000g at 4°C for 10 min to remove the polysaccharides and insoluble material. The supernatant was transferred to a further clean oak ridge tube and RNA was precipitated with the addition of 2.5 x volume of 100% ethanol and left overnight at -20°C.

The next morning, the RNA was pelleted by centrifugation at 9800g for 30 min at 4°C. The ethanol was decanted and discarded. The RNA pellet was washed gently with 2 ml of ice-cold 70% ethanol and centrifuged at 9800g for 5 min at 4°C. The ethanol was decanted and discarded and the residual ethanol was removed under vacuum. The pellet was re-suspended in 200 µl RNase-free autoclaved milliQ water and transferred to a 1.5 ml Eppendorf tube. The oak ridge tube was rinsed with another ml of water and that too was transferred to the Eppendorf. For further purification, the RNA was precipitated with $^1/_{10}$ volume of 3 M sodium acetate, pH 6.0, and 2.5 x volume of ice cold 100% ethanol at 20°C for 2 h. RNA was pelleted and collected by centrifugation (16000g) at 4°C for 20 min in a microcentrifuge. The ethanol was aspirated and the pellet washed with cold 70% ethanol and centrifuged for another 5 min. Residual ethanol was removed and the pellet dried under vacuum. The RNA was re-suspended in sterile RNase-free water and an aliquot run on a gel.

The quantity and the quality of RNA were estimated based on the A_{230}, A_{260}, A_{280}, and A_{320} values in a spectrophotometer (Hitachi U-1100). Subtracting the A_{320} values from the A_{260} readings gave the RNA concentrations corrected for polysaccharides and other

contaminants. RNA quantification was carried out using the formula 1 A_{260} = 40 μg RNA. A_{230}/A_{260} / A_{280} ratio of 1:2:1 indicated a preparation of high quality.

Hot-borate extraction (XT) buffer:

0.2 M Na borate decahydrate (Borax)

30 mM EDTA

2% (w/v) SDS

10 mM dithiotrietol (DTT)*

1% Nonidet P-40* (NP-40)

2% (w/v) polyvinylpyrrolidone* (PVP)

To dissolve, components were added to pre-warmed RNase free milliQ water, pH adjusted to 9 with NaOH and stock solution was autoclaved. *DTT, PVP and Nonidet P-40 were added only immediately before the extraction.

3.2.3 Polymerase chain reaction (PCR)

The partial equivalent of *LFY* was originally isolated from the genomic DNA of *M. excelsa* and cloned into pUC19 by McKenzie et al. (1997) and was repeated for this thesis. The primer sequences are given below:

LFY Left primer: 5' GCGAATTCACIAAYCARGTITTYMGIYAYGC 3'

LFY Right primer: 5' CGGATCCGTGICKIARIYKIGTIGGIACRTA 3'

The composition of the PCR mix is given below.

10 x buffer	: 5 μl
MgCl$_2$ (25mM)	: 5 μl
dNTPs (2.5 mM)	: 5 μl
Platinum Taq DNA Polymerase (5 Uμl^{-1})	: 0.2 μl
Water	: 32.8 μl

Forward and Reverse primers were added to make a final concentration of 50 pmol each. DNA (1/1000 dilution) was added to give a final concentration of 10 ng μl^{-1} in a total of 50 μl of PCR mix.

The PCR protocol was:

94°C - 4 min

94°C - 30 s

45°C - 30 s   29 cycles

72°C - 1min

72°C - 3 min

4°C

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

The partial equivalents of *AP1* and *TFL1* were first isolated through RT-PCR. A partial orthologue of *LFY* was isolated also through RT-PCR. The reverse transcriptase reaction was performed as follows.

One µg RNA, 1 µl of oligo dT primer and RNase-free sterile water to make a final volume of 10 µl was warmed at 65°C for 10 min in an Eppendorf tube in a water bath. The reaction mix was put on ice to cool and then 4 µl of 5 x buffer, 2 µl of 100 mM DTT, 2 µl of 10 mM dNTP mix, 1 µl of RNase inhibitor (40 U µl^{-1}) (Roche) and 1 µl of Expand Reverse Transcriptase (Roche) were added to make a final volume of 20 µl. The reaction mix was incubated at 42°C for 60 min. It was then cooled on ice and the synthesised cDNA stored at -20°C until the PCR reaction.

The PCR reaction mix was essentially as described in Section 3.2.3 except that 3 µl of cDNA was used in the 50 µl reaction mix instead of DNA.

3.2.5 Isolation of the partial orthologue of *LEAFY* (*MEL*) from *M. excelsa*

The protocol followed for isolation of *MEL* from genomic DNA is described in Section 3.2.3. *MEL* was also isolated from RNA through RT-PCR using the same primers as mentioned earlier and *LFY* specific primers designed based on the original sequence (Appendix VII). The sequences of the *LFY* specific primers were as follows:

LFY Specific Forward Primer: 5' GCGAATTCCAAGCCGAAGATGAGGCACTAC 3'

LFY Specific Reverse Primer: 5' GCATTGAAGATAGCGTCGATG 3'

Twelve μl of the RT-PCR product was run on a 1.5% gel and visualised after ethidium bromide (EtBr) staining. The PCR reaction was repeated with three samples and the total quantities run on a gel. The appropriate sized bands were cut out and the DNA purified using Concert™Rapid Gel Extraction system (Life Technologies) and quantified using a mass ladder (High DNA Mass™ Ladder, Life Technologies). The purified fragment was cloned into the pGEM-T Easy vector (Promega) as outlined in Section 3.2.8.

3.2.6 Isolation of the partial orthologues of *APETALA1* from *M. excelsa* (*MESAP1*) and *M. collina* 'Tahiti' (*MTAP1*)

cDNA prepared from buds harvested from *M. excelsa* on 14 October 1998 and cDNA prepared from flower buds of *Metrosideros collina* var. Tahiti were used to isolate the partial *AP1* equivalents. The primers used were based on the *AP1* sequence in *Brassica oleraceae* (Appendix VII) and were as follows:

AP1 Forward Primer: 5' CAAGCTTGAAGAGGATAGAG 3'

AP1 Reverse Primer: 5' GCTTAAGAGCAGTATCAAGCTG 3'

The RT-PCR reaction was as described earlier in Section 3.2.4. Three μl of the cDNA was used in the PCR reaction mix. Twelve μl of the PCR products were run on a 1% agarose gel and visualised after ethidium bromide (EtBr) staining. The PCR reaction was repeated for three samples of *M. excelsa* and *M. collina* and entire quantities run on 1% agarose gel, the appropriate sized bands cut out and the DNA purified using Concert™ Rapid Gel Extraction system (Life Technologies) and quantified using High DNA Mass™ Ladder (Life Technologies). The purified fragments from *M. excelsa* and *M. collina* were then cloned into pGEM-T and pGEM-T Easy vectors (Promega) respectively as outlined in Section 3.2.8. Vector maps are provided in Appendix II.

3.2.7 Isolation of the partial orthologue of *TERMINAL FLOWER1* from *M. excelsa* (*METFL1*) and *M. collina* 'Tahiti' (*MTTFL1*)

Partial equivalents of *TFL1* were isolated using RT-PCR from samples of buds harvested from March to August in *M. excelsa* and from closed buds of *M. collina*. Initial attempts at isolating the fragments with one round of PCR did not produce bands that could be

visualised on a gel. Hence one round of PCR was done using degenerate primers at 48°C annealing temperature. A dilution ($^1/_{10}$) of the resultant PCR product was used to do a second round of PCR with an annealing temperature of 51°C with a set of nested primers designed from an alignment of *TFL1* equivalents between dicots and rice (Jensen et al., 2001). Mutiple alignment of sequences for designing the primers and the primer regions are presented in AppendixVII. The primer sequences were:

TFL1 outer forward primer: 5' ARSAARSNNGTYWNYAATGGNCRTG 3'

TFL1 outer reverse primer: 5' SMRTTRAAGWARACNGCNGCNACNGG 3'

TFL1 nested forward primer: 5' GGTTATGACAGACCCAGATGT 3'

TFL1 nested reverse primer: 5' CGAACCTGTGGATACCAATG 3'

Twelve μl of this final PCR product was run on a 2% agarose gel and visualised after EtBr staining. The entire remaining second round products were then run on a 2% agarose gel, the appropriate sized bands cut out and the DNA purified using the Concert™ Rapid Gel Extraction system (Life Technologies) and quantified using High DNA Mass™ Ladder (Life Technologies). The fragment from *M. excelsa* was sequenced from this purified PCR product as it could not be cloned because necessary approval for the same could not be obtained from Environmental and Risk Management Authority, New Zealand as *M. excelsa* is a native species. The fragment from *M. collina* was cloned into pGEM-T Easy vector (Promega) and sequenced.

3.2.8 Cloning of the gene fragments

The fragments were cloned by TA cloning into pGEM-T and pGEM-T easy vector systems (Promega).

Ligation : The ligation reactions were set up in 0.5 ml tubes as given below.

2 x Rapid ligation buffer, T4 DNA ligase   : 5 μl

pGEM -T or pGEM-T Easy vector (25 ng μl⁻¹): 2 μl

PCR product (20-50 ng μl⁻¹)   : 1-2 μl

T4 DNA Ligase (3 Weiss units μl⁻¹)   : 1 μl

Sterile milliQ water to a final volume of 10 μl

The reactions were mixed by pipetting and were then incubated overnight at 4°C.

Transformation: The next morning, the tubes containing the ligation reactions were pulse centrifuged to collect the contents at the base of the tube. Four µl of each ligation reaction was pipetted into a sterile 1.5 ml microcentrifuge tube, on ice. Competent DH5 α *E. coli* cells prepared earlier (Appendix III) were taken from -80°C storage and placed in an ice bath until just thawed. Thereafter, 100 µl of cells was transferred into the tubes with the 4 µl ligation product. The contents were gently mixed by flicking the tubes and the tubes were placed on ice for 20 min. The cells were heat-shocked for 50 s in a water bath at 42°C and immediately returned to ice for 2 min. Next, 950 µl of room temperature LB medium (Appendix I) was added to the tubes with the transformed cells and they were incubated for 1.5 h at 37°C in a shaking incubator (150 rpm). One hundred µl of each transformant culture were plated onto LB plates with 25 µl of 100 mg ml^{-1} ampicillin, 80 µl of 10 mg ml^{-1} isopropyl-ß-D-thiogalactopyranoside (IPTG) and 15 µl of 50 mg ml^{-1} 5-bromo-4-chloro-3-indoyl-ß-D-galactoside (X-Gal) in dimethyl formamide spread over the surface of each 25 ml plate. The plates were incubated overnight at 37°C and then transferred to a refrigerator at 4°C.

3.2.9 DNA plasmid preparation

Single white colonies were picked and each grown overnight in 10 ml LB medium with ampicillin (10 µl of 100 mg ml^{-1} stock) at 37°C in a shaking incubator at 225 rpm. Plasmids with *AP1* fragments as inserts were isolated and purified as per the protocol of Sambrook et al. (1989). Details of plasmid preparation and purification are given in Appendix IV. The plasmids with *LFY* and *TFL1* equivalents as inserts were isolated and purified using the QIAprep spin miniprep plasmid preparation kit from Qiagen. The plasmids were run on 1% agarose gels to assess their quantity and quality.

3.2.10 Sequencing

All the sequencing was carried out at Massey University DNA Analysis Service (MUSeq), Palmerston North, New Zealand. *LFY* and *AP1* fragments from *M. excelsa* and *TFL1* fragment from *M. collina* were sequenced from the plasmids provided at a concentration of 200 ng µl^{-1}. *TFL1* from *M. excelsa* was sequenced from purified PCR product provided at a concentration of 20 ng µl^{-1}. Primers were used at a concentration of 0.8 pmol µl^{-1}. T7 and

SP6 promoter primers were used to do the forward and reverse sequencing from the plasmids. *TFL1* nested forward primer and *TFL1* nested reverse primer (sequences given earlier) were used for sequencing from the PCR product.

3.2.11 Southern Analysis

The procedure was based upon the method of Southern (1975). Southern analysis was performed on the genomic DNA from *M. excelsa* using radio-labeled probes prepared from the cloned fragments of *MEL*, *MESAP1* and *MTTFL1* to assess the fidelity of these fragments as probes for expression studies and to identify the number of homologous sequences in the genome.

Electrophoresis of DNA digests and blotting: *M. excelsa* genomic DNA (30 µg) was digested with the restriction enzyme *BamH1* for analysing each of the three gene fragments isolated. The digested DNA samples were electrophoresed on a 1% gel. The gel was transferred from the gel tray to a plastic container and soaked in 0.2 M HCl with gentle shaking for 30 min for depurination. The HCl was poured off, the gel rinsed once in distilled water and then sufficient denaturation buffer (1.5 M NaCl, 0.5 M NaOH) was added to cover the gel. The gel was soaked in this buffer with gentle shaking for 30 min. The denaturation buffer was poured off, the gel rinsed once with distilled water and sufficient neutralisation buffer (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001 M EDTA) was added to cover the gel. The gel was soaked in this buffer with shaking for 15 min. This was repeated with fresh neutralisation buffer for another 15 min. The neutralisation buffer was poured off and the gel rinsed with distilled water. The DNA was transferred overnight onto Hybond^{TM}-N+; positively charged nylon membrane from Amersham Life Science with 20 x SSC (3 M NaCl, 0.3 M Na$_3$ citrate). The next day, the membrane was rinsed in 2 x SSC and the DNA fixed onto the membrane by UV crosslinking.

Probe synthesis: PCR was used to produce DNA from the cloned inserts of *MEL*, *MESAP1* and *MTTFL1* in the pGEM-T vectors. *MEL* and the primers used were designed based on the original *M. excelsa LFY* and *AP1* sequences. The sequences of the *LFY*-specific primers are given in Section 3.2.5. The *AP1* specific primer sequences are

given below.

AP1 specific Forward Primer: 5' GCGAATTCGAGATGTGGGCTGCTGAAGAAG 3'

AP1 specific Reverse Primer: 5' GCGGGATCCCAAGCTGGTGCTCCAAATTCTG 3'

TFL1 nested primers as described in Section 3.2.7 were used to amplify *MTTFL1*. The PCR reactions were carried out as outlined in Section 3.2.3 but at annealing temperatures of 52°C. The reactions were then run on a 1.5% agarose gel, the correct sized bands sliced out and the DNA purified using the Concert™ Rapid Gel Extraction system (Life Technologies). DNA was quantified by running 4 μl samples on a gel along with High DNA mass ladder.

Labeling of the probe was done using the ^{T7}QuickPrime^R kit from Amersham Pharmacia Biotech. DNA fragments (50 ng) in 36.5 μl sterile water were denatured at 100°C for 5 min, chilled immediately on ice and 10 μl of labeling mix, 2.5μl of $\propto^{32}P$-(dCTP), and 1 μl of T7 DNA polymerase added. The reactions were mixed gently and incubated at 37°C for 30 min. The labeled probes were then purified using ProbeQuant™ spin columns (Amersham Pharmacia Biotech). The purified probes were denatured in boiling water for 5 min and cooled on ice before hybridisation.

Hybridisation and detection of radioactive probes: The membranes were placed in hybridisation tubes and pre-hybridisation solution [50% formamide, 50 mM Tris pH 7.5, 1 mM EDTA, 3 x SSC, 1 x Denhardt's solution (Sigma), 0.5% w/v SDS, 100 mg ml^{-1} *E. coli* tRNA (Roche Molecular Biochemicals)] was added. Denatured sonicated salmon sperm DNA (Sigma), to a concentration of 100 μg ml^{-1}, was also added to the pre-hybridisation solution and the membranes were pre-hybridised at 42°C for 6 h. Following incubation, the pre-hybridisation solution was removed and hybridisation solution was added along with the denatured radioactive labelled probe. The hybridisation solution varied from the pre-hybridisation solution by the addition of dextran sulfate (Sigma) to a final concentration of 10%. The membranes were hybridised overnight at 42°C. They were then briefly washed in 2 x SSC, 0.1% SDS. Subsequently, they were washed three times for 10 min each with 1 x SSC, 0.1% SDS soloution at 65°C, followed by two washes for 10

min each in 0.1 x SSC, 0.1% SDS at 65°C. The membranes were then dried and exposed to films (Kodak Scientific Imaging Film) in casettes at -70°C for 1-14 days depending on the strength of the signal.

3.3 Results

Bands, of the expected 188 bp in size could be seen (Figure 3.1 A) after RT-PCR using *LFY* specific primers. A bright band of 357 bp in size could be seen (Figure 3.1 B) after RT-PCR for the *AP1* equivalent in *M. excelsa*. Twelve µl products of the second round of PCR for *TFL1* equivalent in *M. excelsa* after reverse transcription when run on a 2% agarose gel and visualised after EtBr staining showed a band of the expected size of 175 bp (Figure 3.1 C).

3.3.1 Sequences of *MEL*

The nucleotide sequence of the fragment isolated by McKenzie et al. (1997) and the putative amino acid sequence are given below.

Nucleotide sequence: GAAGAAGGCGGGAGCAAGCTACATAAACAAGCCGAAGA
TGAGGCACTACGTCCACTGCTACGCCCTGCACTGCATGGACGAGCACGCCTCC
AACGCCCTCCGCAAGAGCTTCAAGGAGCGCGGGGAGAACGTCGGCGCCTGGA
GGCAAGCCTGCTACCACCCCCTGGTTACCATCGCCGGCCGCAGGGCCGGCTGG
GACATCGACGCTATCTTCAATGCCCACCCCCGCCTCTGCATCTGG

Amino acid sequence: KKAGASYINKPKMRHYVHCYALHCMDEHASNALR
KSFKERG ENVGAWRQACYHPLVTIAGRRAGWDIDAIFNAHPRLCIW
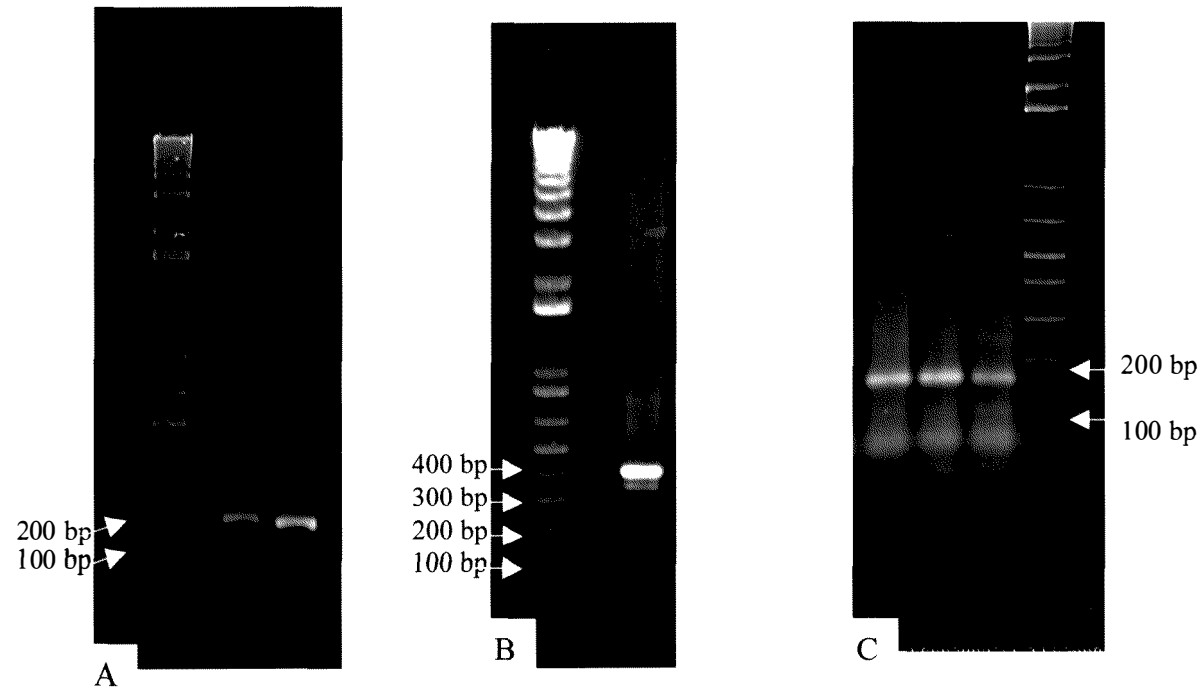
Figure 3.1 Isolation of *MEL*, *MESAP1* and *METFL1* by RT-PCR. (A) Bands approximately 188 bp in size that sequenced to give *MEL*. (B) Band approximately 357 bp in size that sequenced to give *MESAP1*. (C) Bands approximately 175 bp in size that sequenced to give *METFL1*.

The nucleotide and the deduced amino acid sequences of the fragment cloned by RT-PCR with *LFY* specific primers are given below.

Nucleotide sequence: CAAGCCGAAGATGAGGCACTACGTCCACTGCTACGCCCT GCACTGCATGGACGAGCACGCCTCCAACGCCCTCCGCAAGAGCTTCAAGGAGC GCGGGGAGAACGTCGGCGCCTGGAGGCAAGCCTGCTACCACCCCCTGGTTACC ATCGCCGGCCGCAGGGCCGGCTGGGACATCGACGCTATCTTCAATGC

Amino acid sequence: KPKMRHYVHCYALHCMDEHASNALRKSFKERGENVGAW RQACYHPLVTIAGRRAGWDIDAIFN

Both the isolated fragments were partial orthologues of a *LFY*-like gene from *M. excelsa*. The fragment isolated by RT-PCR was nine amino acids shorter at the 5' end and eight aminoacids shorter at the 3' end of the fragment cloned from genomic DNA.

3.3.2 Alignment of the amino acid sequence of *MEL* with other *LFY*-like amino acid sequences

A comparison of the deduced amino acid sequence of *MEL* with selected *LFY* genes in the GenBank data base of the National Centre for Biotechnology Information (NCBI) is given below. The amino acids differing from *MEL* are written in bold and underscored.

```
MEL
    1 KKAGASYINKPKMRHYVHCYALHCMDEHASNALRKSFKERGENVGAWRQACYHPLVTIAG 60

Eucalyptus gobulus (ELF1) Accession No. 7227892 Identities = 79/80 (98%), Positives =
80/80 (99%)

  240 KKAGASYINKPKMRHYVHCYALHCLDEHASNALRKSFKERGENVGAWRQACYHPLVTIAG 299

Arabidopsis thaliana Accession No. 285245 Identities = 67/80 (83%), Positives = 73/80 (90%)

  286 KKSGASYINKPKMRHYVHCYALHCLDEEASNALRRAFKERGENVGSWRQACYKPLVNIAC 345

Nicotiana tabacum LFY1 Accession No. 7227893 Identities = 69/80 (86%), Positives = 73/80
(91%)

  299 KKAGASYINKPKMRHYVHCYALHCLDEEASNALRRAFKERGENVGAWRQACYKPLVAIAA 358
```

Nicotiana tabacum LFY2 **Accession No. 7227894 Identities = 69/80 (86%), Positives = 73/80 (91%)**

299 KKAGASYINKPKMRHYVHCYALHC<u>L</u>DE<u>E</u>ASNALRR<u>RA</u>FKERGENVGAWRQACY<u>K</u>PLV<u>A</u>IA<u>A</u> 358

Antirrhinum majus **Accession No. 123096 Identities = 68/80 (85%), Positives = 73/80 (91%)**

282 KKAGA<u>N</u>YINKPKMRHYVHCYALHC<u>L</u>DE<u>A</u>ASNALRR<u>RA</u>FKERGENVGAWRQACY<u>K</u>PLV<u>A</u>IA<u>A</u> 341

Brassica oleraceae **Accession No. 584851 Identities = 65/80 (81%), Positives = 71/80 (88%)**

295 KK<u>S</u>GA<u>N</u>YINKPKMRHYVHCYALHC<u>L</u>DE<u>E</u>ASNALR<u>SA</u>FK<u>V</u>RGENVG<u>S</u>WRQACY<u>K</u>PLV<u>D</u>IA<u>C</u> 354

Pinus radiata **Accession No. 7227903 Identities = 55/80 (68%), Positives = 66/80 (81%)**

307 K<u>HN</u>GA<u>V</u>YINKPKMRHYVHCYALHC<u>L</u>D<u>SEQ</u>SN<u>H</u>LR<u>RLY</u>KERGENVGAWRQACY<u>Y</u>PLV<u>A</u>IA- 365

Populus balsimifera **subspp.** *trichocarpa* **Accession No.7227895 Identities = 69/80(86%), Positives=74/80 (92%)**

267 KKAGASYINKPKMRHYVHCYALHC<u>L</u>DE<u>D</u>ASNALRR<u>RA</u>FKERGENVGAWRQACY<u>K</u>PLV<u>A</u>IA<u>S</u> 326

Actinidia deliciosa **Accession No.6010659 Identities = 68/80(85%), Positives = 74/80 (92%)**

9   KKAGASYINKPKMRHYVHCYALHC<u>L</u>DE<u>E</u>ASNALRR<u>RA</u>FKERGENVGAWRQACY<u>K</u>PLV<u>A</u>IA<u>A</u> 68

MEL	61	RRAGWDIDAIFNAHPRLCIW 80
E. globulus	300	RRAGWDIDAIFNAHPRLCIW 319
A. thaliana	346	R<u>H</u>-GWDIDA<u>V</u>FNAHPRL<u>S</u>IW 364
N. tabacum LFY1	359	R<u>Q</u>-GWDID<u>T</u>IFNAHPRL<u>A</u>IW 377
N. tabacum LFY2	359	R<u>Q</u>-GWDID<u>T</u>IFNAHPRL<u>A</u>IW 377
A. majus	342	R<u>Q</u>-GWDID<u>T</u>IFNAHPRL<u>S</u>IW 360
B. oleraceae	355	R<u>H</u>-GWDIDA<u>V</u>FNAHPRL<u>S</u>IW 373
P. radiata	366	R<u>ENN</u>WDI<u>EGI</u>FN<u>R</u>NEK<u>L</u>KIW 385
P. balsamifera	327	R<u>Q</u>-GWDID<u>S</u>IFNAHPRL<u>A</u>IW 345
A. deliciosa	69	R<u>Q</u>-GWDID<u>S</u>IFNAHPRL<u>A</u>VW 87

It is apparent from the above data that at the amino acid level, the isolated fragment had more than 80% homology to selected *LFY* homologues, except for the *Pinus radiata LFY* protein. Even with *P. radiata*, the homology was as high as 68%. *MEL* had the greatest similarity (98%) to *ELF1*, the *LFY* homologue from *Eucalyptus globulus*. Alignment of the

fragment with other genes also showed that it is very close to the 3' end of the gene with only about 120 nucleotides left to be sequenced to reach the end of the gene.

3.3.3 Sequences of *MESAP1* and *MTAP1*

The nucleotide sequences of the fragments isolated from *M. excelsa* and *M. collina* 'Tahiti' through RT-PCR were named *MESAP1* and *MTAP1* respectively. They were found to be partial orthologues of the *AP1* equivalents in *Metrosideros*. The nucleotide sequences and amino acid sequences are given below.

Nucleotide sequences

MESAP1: AACAAGATCAATAGGCAAGTTACGTTCTCGAAGCGGAGATGTGGG CTGCTGAAGAAGGCCCACGAGATCTCCGTCCTCTGCGACGCGGAGGTCGCCCT CATCGTCTTCTCCACCAAGGGCAAGCTCTTCGAGTACGCCACCGATGCTTGCAT GGAGAAGATCCTTGAGCGCTATGAAAGATATTCATATGCAGAGAGACAGATTC TCACAAACAACGCCGAAACCAATGGGAACTGGACTTTGGAACATGCAAAACT CAAGGCCAGGATGGAGATTTTGCAGAAAAATCAAAAGAATCTGATGGGAGAA GAACTTGATTCCTTAAGCCTCAAAGAGCTTCAGAATTTGGAGCACCAG

MTAP1: AACAAGATCAATAGGCAAGTTACGTTCTCGAAGCGGAGATGTGGGC TGCTGAAGAAGGCCCACGAGATCTCCGTCCTCTGCGACGCGGAGGTCGCCCTC ATCGTCTTCTCCACCAAGGGCAAGCTCTTCGAGTACGCCACCGATGGTTGCAT GGAGAAGATCCTTGAGCGCTATGAAAGATATTCATATGCAGAGAGACAGATTC TCACAAACAACGCCGAAACCAATGGGAACTGGACTTTGGAACATGCAAAACT CAAGGCCAGGATGGAGATTTTGCAGAAAAATCAAAAGAATCTGATGGGAGAA GAACTTGATTCCTTAAGCCTCAAAGAGCTTCAGAATTTGGAGCACCAG

A comparison of the nucleotide sequences of *MESAP1* and *MTAP1*, given below, shows that there was 99% homology. Identities = 356/357 (99%)

```
MESAP1:1   AACAAGATCAATAGGCAAGTTACGTTCTCGAAGCGGAGATGTGGGCTGCTGAAGAAGGCC
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MTAP1:1    AACAAGATCAATAGGCAAGTTACGTTCTCGAAGCGGAGATGTGGGCTGCTGAAGAAGGCC


MESAP1:61  CACGAGATCTCCGTCCTCTGCGACGCGGAGGTCGCCCTCATCGTCTTCTCCACCAAGGGC
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MTAP1:61   CACGAGATCTCCGTCCTCTGCGACGCGGAGGTCGCCCTCATCGTCTTCTCCACCAAGGGC


MESAP1:121 AAGCTCTTCGAGTACGCCACCGATGCTTGCATGGAGAAGATCCTTGAGCGCTATGAAAGA
           ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
MTAP1:121  AAGCTCTTCGAGTACGCCACCGATGGTTGCATGGAGAAGATCCTTGAGCGCTATGAAAGA


MESAP1:181 TATTCATATGCAGAGAGACAGATTCTCACAAACAACGCCGAAACCAATGGGAACTGGACT
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MTAP1:181  TATTCATATGCAGAGAGACAGATTCTCACAAACAACGCCGAAACCAATGGGAACTGGACT


MESAP1:241 TTGGAACATGCAAAACTCAAGGCCAGGATGGAGATTTTGCAGAAAAATCAAAAGAATCTG
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MTAP1:241  TTGGAACATGCAAAACTCAAGGCCAGGATGGAGATTTTGCAGAAAAATCAAAAGAATCTG


MESAP1:301 ATGGGAGAAGAACTTGATTCCTTAAGCCTCAAAGAGCTTCAGAATTTGGAGCACCAG 357
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MTAP1:301  ATGGGAGAAGAACTTGATTCCTTAAGCCTCAAAGAGCTTCAGAATTTGGAGCACCAG 357
```

Amino acid sequences

MESAP1 : **NKINRQVTFSKRRCGLLKKAHEISVLCDAEVALIVFSTKGKLFEY
AT**DACMEKILERYERYSYAERQILTNNAETNGNWTLEHAKLKARMEILQKNQK
NLMGEELDSLSLKELQNLEHQ

A BLAST search on the GenBank data base revealed that the isolated fragment spanned
part of the MADS box region and a DNA-binding and dimerisation domain or the Serum
Response Function type Transcription Factor (SRF-TF), and part of the K-box region. The
MADS Domain covering residues1-48 is bold underlined in bold. SRF-type transcription
factor encompassing residues 1-47 is written in bold. The K-box region is underlined with
dots and covers the residues 61-119.

MTAP1 : **NKINRQVTFSKRRCGLLKKAHEISVLCDAEVALIVFSTKGKLFEYAT**
DGCMEKILERYERYSYAERQILTNNAETNGNWTLEHAKLKARMEILQKNQKNLM
GEELDSLSLKELQNLEHQ

There was one amino acid difference in between *MESAP1* and *MTAP1* caused by the C-G difference at position 146 of the nucleotide sequences. Thus the GCT sequence (position 145-147) in *MESAP1* that codified for Alanine was substituted by GGT (position 145-147) in *MTAP1* that codified for Glycine. This change fell immediately outside the MADS box region.

3.3.4 Alignment of the deduced amino acid sequences of *MESAP1* and *MTAP1* with other *AP1*-like amino acid sequences

The deduced amino acid sequences of *MESAP1* and *MTAP1* were aligned with other *AP1*-like amino acid sequences. Selected alignments are presented below.

```
MESAP1     NKINRQVTFSKRRCGLLKKAHEISVLCDAEVALIVFSTKGKLFEYATDACMEKILERYER 60

MTAP1 Identities = 118/119 (99%)
           NKINRQVTFSKRRCGLLKKAHEISVLCDAEVALIVFSTKGKLFEYATDGCMEKILERYER 60

Eucalyptus globulus EAP2L Accession No.1120557 Identities = 114/119(95%),Positives =
117/119 (97%)
        13 NKINRQVTFSKRRCGLLKKAHEISVLCDADVALIVFSTKGKLFEYATDCCMERILERYER 72

Eucalyptus globulus EAP2S Accession No 11037010 Identities = 114/119 (95%), Positives =
117/119 (97%)
        13 NKINRQVTFSKRRCGLLKKAHEISVLCDADVALIVFSTKGKLFEYATDCCMERILERYER 72

Betula pendula MADS5 Accession No 1483232 Identities = 100/119 (84%), Positives = 113/119
(94%)
        13 NKINRQVTFSKRRSGLLKKAHEISVLCDAEVALIVFSTKGKLFEYSTDSCMERILERYER 72

Eucalyptus globulus EAP1 Accession No 10946429 Identities = 93/119 (78%), Positives =
108/119 (90%)
        13 NKINRQVTFSKRRAGLLKKAHEISVLCDAEVALIVFSAKGKLFEYSTDSCMERILERYER 72

Malus domestica Accession No 3947985 Identities = 95/119 (79%), Positives = 110/119 (91%)
        13 NKINRQVTFSKRRSGLMKKAHEISVLCDAEVALIIFSTKGKLFEYSNDSCMERILERYER 72

Nicotiana tabacum NAP1-1 Accession No 4102111 Identities = 95/119 (79%), Positives =
107/119 (89%)
        13 NKINRQVTFSKRRSGLLKKAHEISVLCDAEVGLIVFSTKGKLFEYSTDSCMERILERYER 72

Populus tremuloides female Apetala1 Accession No 6465897 Identities = 87/119 (73%),
Positives = 106/119 (88%)
         2 NKINRQVTFSKRRAGLLKKAHEISVLCDAEVALIVFCHKGKLFEYSTNACMEKILERHER 61

Populus tremuloides male Apetala1 Accession No 6465895 Identities = 85/119 (71%), Positives
= 107/119 (89%)
         2 NKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEYSTDSCMEKILERYER 61

Brassica oleracea BOAP1. Accession No. 887392.Identities = 84/119 (70%), Positives =
105/119 (87%)
        13 NKINRQVTFSKRRAGLFKKAHEISVLCDAEVALVVFSHKGKLFEYSTDPCMEKILERYER 72

Antirrhinum majus SQUAMOSA Accession No 82313 Identities = 87/119 (73%), Positives =
110/119 (92%)
        13 NKINRQVTFSKRRGGLLKKAHELSVLCDAEVALIVFSNKGKLFEYSTDSCMDRILEKYER 72
```

Arabidopsis lyrata Apetala1 **Accession No. 6707086 Identities = 85/119 (71%), Positives =** 108/119 (90%)

13 NKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEYSTDSCMEKILERYER 72

Sinapis alba Saap1 **Accession No 3913047 Identities = 85/119 (71%), Positives = 107/119** (89%)

13 NKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEYSTDSCMEKILERYER 72

Arabidopsis thaliana AP1 **Accession No. 543813 Identities = 85/119 (71%), Positives =** 107/119 (89%)

13 NKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEYSTDSCMEKILERYER 72

MESAP1

61 YSYAERQILTNNAETNGNWTLEHAKLKARMEILQKNQKNLMGEELDSLSLKELQNLEHQ 119

MTAP1

61 YSYAERQILTNNAETNGNWTLEHAKLKARMEILQKNQKNLMGEELDSLSLKELQNLEHQ 119

Eucalyptus globulus EAP2L

73 YSYAESQVLTNNAETNGNWTLEHAKLKARMEILQKNQKNLMGEELDSLSLKELQNLEHQ 131

Eucalyptus globulus EAP2S

73 YSYAESQVLTNNAETNGNWTLEHAKLKARMEILQKNQKNLMGEELDSLSLKELQNLEHQ 131

Betula pendula MADS5

73 YSYADRQLLANDLEQNGSWTLEHAKLKARIEVLQRNQKHFVGEDLDSLSLKELQNLEQQ 131

Eucalyptus globulus EAP1

73 YSYAEHQVLASETESIGSWTLEHAKLKARLEVLHRNYRHFMGEDLDSLSLRDLQNLEQQ 131

Malus domestica

73 YSYTERQLLANDNESTGSWTLEHAKLKARVEVLQRNQRHYMGEDLQSLSLKELQNLEQQ 131

Nicotiana tabacum NAP1-1

73 YSYAERQLTATDHETPGSWTLEHAKLKGRLEVLQRNQGHYAGEDLDSLCMKELQNLEHQ 131

Populus tremuloides **female** *Apetala1*

62 YPYAERQLVATDLDSQGNWTLEYNRLKEKVELLQRNHRHYLGEDLDSVSLKELQNLEKQ 120

Populus tremuloides **male** *Apetala1*

62 YSYAERQLIAPESDVNTNWSMEYNRLKAKIELLKRNQRHYLGEDLQAMSPKELQNLEQQ 120

Brassica oleracea BOAP1

73 YSYAERQLIAPESDVNTNWSMEYNRLKAKIELLERNQRHYLGEDLQAMSPKELQNLEQQ 131

Antirrhinum majus SQUAMOSA

73 YSFAERQLVSNEPQSPANWTLEYSKLKARIELLQRNHRHYMGEDLDSMSLKEIQSLEQQ 131

Arabidopsis lyrata Apetala1

73 YSYAERQLIAPESDVNTNWSMEYNRLKAKIELLERNQRHYLGEDLNAMSPKELQNLEQQ 131

Sinapis alba Saap1

73 YSYAERQLIAPESDVNTNWSMEYNRLKAKIELLERNQRHYLGEDLQAMSSKELQNLEQQ 131

Arabidopsis thaliana AP1

73 YSYAERQLIAPESDVNTNWSMEYNRLKAKIELLERNQRHYLGEDLQAMSPKELQNLEQQ 131

MESAP1 and *MTAP1* had the highest homology of 95% at the amino acid level with *EAP2*, one of the two *AP1* equivalents isolated from *Eucalyptus* (Kyozuka et al., 1997). A homology search for the SRF region of *MESAP1* revealed that the isolated fragment had the highest homology (91%) to the SRF region of the *AP1* gene than the other MADS box genes with which it had only 80% or less identities.

```
MESAP1
1 ---------            NKINRQVTFSKRRCGLLKKAHEISVLCDAEVALIVFSTKGKLFEYAT 47
AP1 SRF Arabidopsis Accession No 543813
1  MGRGRVQLKRIENKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEYST 59
AGL2 Accession No 113512
1  MGRGRVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCS 59
AGL1 Accession No. 113511
16 LGRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALVIFSTRGRLYEYAN 74
FBP Floral binding protein Petunia hybrida Accession No. 729464
1  MGRGKIEIKRIENSSNRQVTYSKRRNGILKKAKEISVLCDARVSVIIFASSGKMHEFSS 59
Floral homeotic protein DEFICIENS Antirrhinum majus Accession No. 118426
1  MARGKIQIKRIENQTNRQVTYSKRRNGLFKKAHELSVLCDAKVSIIMISSTQKLHEYIS 59
Consensus
1  MGRGKIEIKRIENKTNRQVTFSKRKNGLLKKAYELSVLCDAEVALIIFSSTGKLYEYSS 59
```

BLAST search using the nucleotide sequence of *MESAP1* for identifying homologous sequences of the MADS Domain produced the following alignment. The greatest homology again was to the *AP1* amino acid sequence (*ZAP1*).

```
MESAP1
1-----------        NKINRQVTFSKRRCGLLKKAHEISVLCDAEVALIVFSTKGKLFEYATD 48

Zea mays AP1 ZAP1 Accession No. 939785
1    MGRGKVQLKRIENKINRQVTFSKRRNGLLKKAHEISVLCDAEVAVIVFSPKGKLYEYATD 60

Floral homeotic protein AGL15 Accession No. 3831486
1    MGRGKIEIKRIENANSRQVTFSKRRAGLLKKAHELSVLCDAEVAVIVFSKSGKLFEFSST 60

Cucumis sativus Agamous like putative transcription factor CAG1 Accession No. 4103342
1    MGRGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRLYEYSNN 60

Consensus
1    MGRRKIKIKRIENKTNRQVTFSKRRNGLFKKAHELSVLCDAEVALIVFSPTGKLYEFSSP 60
```

A comparison of the MADS domains of other genes with that of *MESAP1* is given below

```
MESAP1          NKINRQVTFSKRRCGLLKKAHEISVLCDAEVALIVFSTKGKLFEYATD 48
Eucalyptus EAP2 NKINRQVTFSKRRCGLLKKAHEISVLCDADVALIVFSTKGKLFEYATD
EAP1            NKINRQVTFSKRRAGLLKKAHEISVLCDAEVALIVFSAKGKLFEYSTD
SQUAMOSA        NKINRQVTFSKRRGGLLKKAHELSVLCDAEVALIVFSNKGKLFEYSTD
AP1            NKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEYSTD
AG             NTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRLYEYSNN
AGL1           NTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALVIFSTRGRLYEYANN
AGL2           NKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCSS
AGL3           NKINRQVTFAKRRNGLLKKAYELSVLCDAEIALLIFSNRGKLYEFCSS
AGL4           NKINRQVTFAKRRNGLLKKAYELSVLCDAEVSLIVFSNRGKLYEFCST
AGL5           NTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALVIFSTRGRLYEYANN
AGL6           NKINRQVTFSKRRNGLLKKAYELSVLCDAEVALIIFSSRGKLYEFGSV
AP3            NQTNRQVTYSKRRNGLFKKAHELTVLCDARVSIIMFSSSNKLHEYISP
PI             NANNRVVTFSKRRNGLVKKAKEITVLCDAKVALIIFASNGKMIDYCCP
```

It is evident that the MADS domain of *MESAP1* has greater homology with *AP1* and its equivalents (written in bold) rather than with other MADS box genes. The dissimilarities were more pronounced towards the 3' end of the domain.

3.3.5 Sequences of *METFL1* and *MTTFL1*

The fragments of *TFL1* equivalents isolated from *M. excelsa* and *M. collina* 'Tahiti' were named *METFL1* and *MTTFL1* respectively. Homology search showed them to be partial orthologues of *TFL1* equivalents. The sequences of the two fragments are given below.

Nucleotide sequences

METFL1: ACTAGTGATTGGTTATGACAGACCCAGATGTGCCTGGTCCTAGTGA TCCATATCTAAGGGAGCACTTGCACTGGATGGTGACGGACATCCCGGGCACAA CAGATGCCACATTTGGAAAGGAGGTGGTGGAGTACGAGATGCCGAGGCCCAA CATTGGTATCCACAGGTTCGAATC

MTTFL1: ACTAGTGATTGGTTATGACAGACCCAGATGTGCCTGGTCCTAGTGA TCCATATCTAAGGGAGCACTTGCACTGGATGGTGACGGACATCCCGGGCACAA CAGATGCCACATTTGGAAAGAAGGTGGTGGAGTACGAGATGCCGAGGCCCAA CATTGGTATCCACAGGTTCGAATC

The alignment of the nucleotide sequences of *METFL1* and *MTTFL1* is presented below.

```
METFL1  ACTAGTGATTGGTTATGACAGACCCAGATGTGCCTGGTCCTAGTGATCCATATCTAAGGG
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MTTFL1  ACTAGTGATTGGTTATGACAGACCCAGATGTGCCTGGTCCTAGTGATCCATATCTAAGGG


METFL1  AGCACTTGCACTGGATGGTGACGGACATCCCGGGCACAACAGATGCCACATTTGGAAAGG
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MTTFL1  AGCACTTGCACTGGATGGTGACGGACATCCCGGGCACAACAGATGCCACATTTGGAAAGA


METFL1  AGGTGGTGGAGTACGAGATGCCGAGGCCCAACATTGGTATCCACAGGTTCGAATC
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MTTFL1  AGGTGGTGGAGTACGAGATGCCGAGGCCCAACATTGGTATCCACAGGTTCGAATC
```

There was 99% homology between the nucleotides of the two fragments (Identities = 174/175).

Amino acid sequences

The deduced amino acid sequences of *METFL1* and *MTTFL1* are presented below.

METFL1: LVMTDPDVPGPSDPYLREHLHWMVTDIPGTTDATFGKEVVEYEMPRPNIGIHRFE

MTTFL1: LVMTDPDVPGPSDPYLREHLHWMVTDIPGTTDATFGRKVVEYEMPRPNIGIHRFE

3.3.6 Alignment of the deduced amino acid sequences of *METFL1* and *MTTFL1* with other *TFL1*-like proteins

The alignment of the deduced amino acid sequences of *METFL1* and *MTTFL1* with other selected *TFL1*-like amino acid sequences in the GenBank data base of NCBI is given below.

```
METFL1
      LVMTDPDVPGPSDPYLREHLHWMVTDIPGTTDATFGKEVVEYEMPRPNIGIHRFE 55

MTTFL1 Identities = 53/54 (97%)
      LVMTDPDVPGPSDPYLREHLHWMVTDIPGTTDATFGRKVVEYEMPRPNIGIHRFE 55

Antirrhinum majus Centroradialis Accession No. 12195101 Identities = 49/54 (90%),
 Positives = 53/54 (97%)
72    LIMTDPDVPGPSDPYLREHLHWIVTDIPGTTDSSFGKEVVSYEMPRPNIGIHRF 125

Lycopersicon esculentum Self-pruning protein Accession No 17367328 Identities = 47/54
(87%), Positives = 51/54 (94%)
68    LIMIDPDVPGPSDPYLREHLHWIVTDIPGTTDCSFGREVVGYEMPRPNIGIHRF 121

Oryza sativa Cen-like protein FDR1 Accession No. 5360180 Identities = 45/54 (83%),
Positives = 52/54 (95%)
67    LVMTDPDVPGPSDPYLREHLHWIVTDIPGTTDASFGREIISYESPKPSIGIHRF 120
```

Arabidopsis thaliana Terminal flower1 **Accession No. 15237535 Identities = 46/54 (85%), Positives = 52/54 (96%)**
70   LVMIDPDVPGPSDPFLKEHLHWIVTNIPGTTDATFGKEVVSYELPRPSIGIHRF 123

Lolium perenne terminal flower 1-like protein **Accession No. 11139708 Identities = 45/54 (83%), Positives = 51/54 (94%)**
67   LVMTDPDVPGPSDPYLREHLHWIVSNIPGTTDASFGGEVMSYESPKPNIGIHRF 120

Nicotiana tabacum CEN-like protein 1 **Accession No. 17367234 Identities = 42/54 (77%), Positives = 51/54 (93%)**
68   LIMTDPDVPGPSDPYLREHLHWIVTDIPGSTDSSFGREIVSYESPKPVIGIHRY 121

Brassica napus BNTFL1-1 **Accession No. 3650419 Identities = 43/54 (79%), Positives = 50/54 (91%)**
70   LVMTDPDVPNPSDPFLKERLHWLVMNIPGTTDATFGKEVVSYELPKPNIGIHRY 123

Brassica rapa BRTFL1-1 **Accession No 3650425 Identities = 43/54 (79%), Positives = 50/54 (91%)**
70   LVMTDPDVPNPSDPFLKERLHWLVMNIPGTTDATFGKEVVSYELPKPNIGIHRY 123
Brassica oleracea BOTFL1-1 **Accession No. 3650429 Identities = 40/53 (75%), Positives = 47/53 (88%)**
70   LVMTDPDVPNPSDPFLKERLHRLVMNIPGTTDATLGKEVVSYELPKPNIGIHRY 122

Citrus unshiu **Flowering Time gene (CiFT).Accession No. 4903139 Identities = 39/54 (72%), Positives = 45/54 (83%)**
67   LVMVDPDAPSPSDPSLREYLHWLVTDIPATTGASFGQEIVNYESPRPTMGIHRF 120

Oryza sativa **FT-like protein Accession No. 17221650 Identities = 39/53 (73%), Positives = 45/53 (84%)**
68   LVMVDPDAPSPSNPNLREYLHWLVTDIPGTTGATFGQEVMCYESPRPTMGIHR 120

Arabidopsis thaliana **flowering locus T (FT) Accession No. 15218709 Identities = 37/53 (69%), Positives = 42/53 (78%)**
67   LVMVDPDVPSPSNPHLREYLHWLVTDIPATTGTTFGNEIVCYENPSPTAGIHR 119

Arabidopsis thaliana TWIN SISTER OF FT (TSF) TFL1 like protein **Accession No. 15237061 Identities = 37/53 (69%), Positives = 41/53 (76%)**
67   LVMVDPDVPSPSNPHQREYLHWLVTDIPATTGNAFGNEVVCYESPRPPSGIHR 119

The BLAST search again showed that *METFL1* and *MTTFL1* could be fragments of the *TFL1* gene in *M. excelsa* and *M. collina* 'Tahiti' respectively as their amino acid sequences had more than 70% homology with most of the *TFL1* genes in the data base. The highest homology (90%) was with *CENTRORADIALIS*, the *TFL1* homologue in *Antirrhinum*. *METFL1* and *MTTFL1* also had 85% homology with *TFL1* from *Arabidopsis*. The isolated fragments also showed high homology (69-73%) to flowering time genes such as *FT* from *Arabidopsis*.

METFL1 and *MTTFL1* also included a phosphatidylethanolamine-binding domain as found in *TFL*, indicating that they are also phosphatidylethanolamine-binding proteins (PEBP).

They also had high homology (82%) to mammalian PEBP-like proteins from *Homo sapiens.*

An alignment of *METFL1* with the conserved PEBP domain in the database is presented below.

METFL1:1 LVMTDPDVPGPSDPYLREHLHWMVTDIPGTTDATFGKEVVEYE--MPRPNIGIHRF 54

PEBP: 65 LVMTDPDAPSRSNPKYREWLHWLVVNIPGTTDISKGNTISEYEGPGPPKNTGLHRY 120

3.3.7 Southern analysis

After exposure for seven days, Southern analysis showed a single band when probed with the *MEL* fragment (Figure 3.2 A). One dark band indicating strong hybridisation and one lighter band indicating weaker hybridisation could be seen when hybridised with the *MESAP1* fragment (Figure 3.2 B). A single band could also be seen when hybridised with the *MTTFL1* fragment (Figure 3.2 C).

3.4 Discussion

Sequencing and homology searches revealed that *MEL* was a partial orthologue of *LFY*. Similarly, *MESAP1* and *MTAP1* were confirmed to be partial equivalents of *AP1* and *METFL1* and *MTTFL1*, the partial orthologues of *TFL1*. Thus the hypothesis that there would be expression of the equivalents of the herbaceous floral and inflorescence meristem specification genes in the distal axillary buds of *M. excelsa* was proved true. The amino acid sequence of *MEL* had a very high homology of 98% with its counterpart in *Eucalyptus*. This is explained by the fact that *Eucalyptus* belongs to the family *Myrtaceae* and is a close relative of *Metrosideros*. Among other woody perennials, *MEL* amino acid sequence had a homology of 86% with *Populus balsamifera* ssp. *trichocarpa*, and 85% with *A. deliciosa*. It had more than 80% homology at the amino acids level to *LFY* equivalents in *Arabidopsis*, *Antirrhinum*, *Brassica oleraceae* and *Nicotiana tabacum*. It is
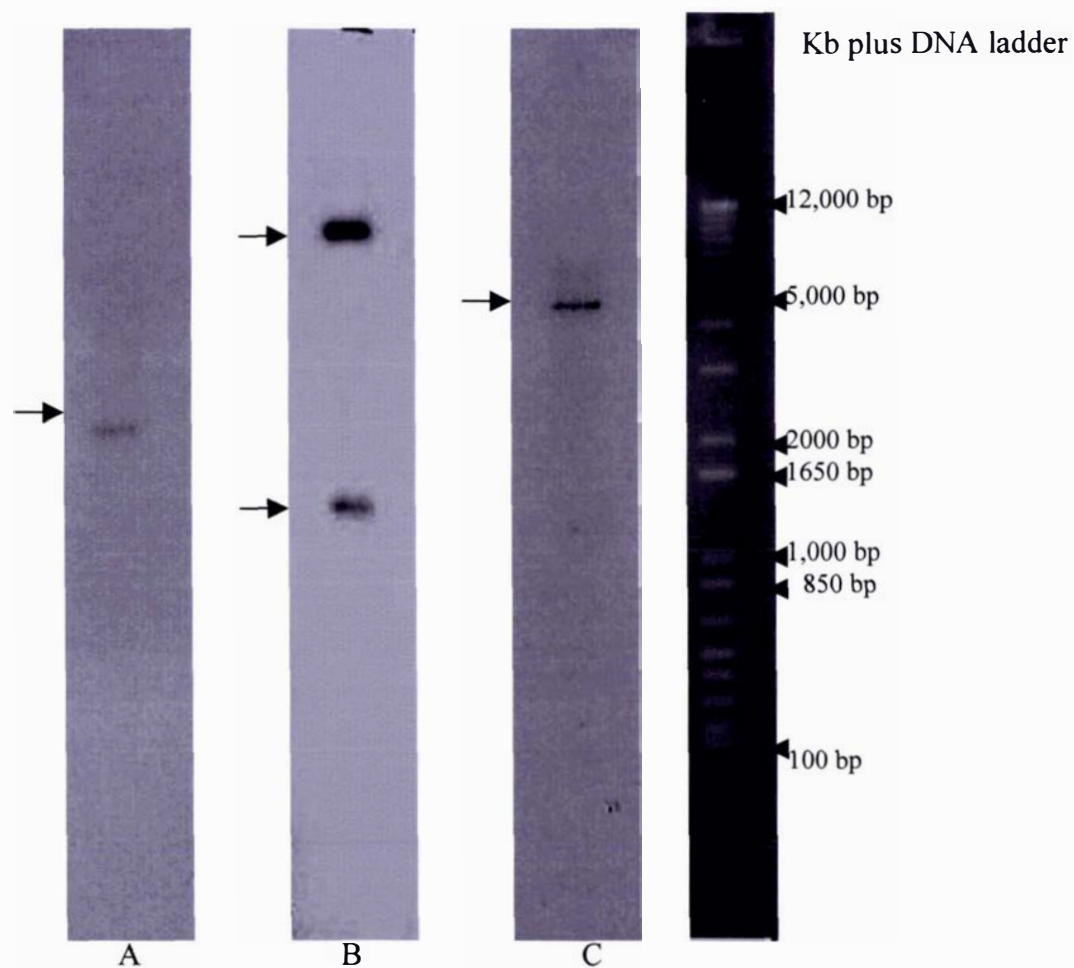
Figure 3.2 Southern analysis on *M. excelsa* genomic DNA after restriction digestion with *BamH1*.
(A) Hybridisation of the probe *MEL*. (B) Hybridisation of the probe *MESAP1*. (C) Hybridisation of the probe *MTTFL1*

thus evident that the PCR fragment isolated was derived from a *Metrosideros excelsa* orthologue of the *LFY* gene.

However, in *Eucalyptus*, Southerton et al. (1998) identified two *LFY* equivalents from the genomic DNA, although only one was functional. In the Southern analysis conducted in the present study, *MEL* hybridised to only one gene. A second *LFY* equivalent in *Metrosideros* was not thus revealed.

The fact that *MESAP1* and *MTAP1* had 70% or more identities with other *AP1* genes in the data bank confirms that they are indeed fragments of the *AP1* gene in *Metrosideros*. Two functional *AP1* equivalents, *EAP1* and *EAP2*, have been isolated by Kyozuka et al. (1997) from *Eucalyptus*. Both these genes are reported to be expressed predominantly in flower buds with slightly different expression levels during flower development. *EAP1* is expressed at all stages of floral morphogenesis whereas *EAP2* is expressed more predominantly in young flower buds.

It is evident that *MESAP1* and *MTAP1* had the highest homology (95%) at the amino acids level with *EAP2L* and *EAP2S*. *EAP2L* and *EAP2S* are reported as two mRNAs formed by alternative splicing of *EAP2* (Kyozuka et al.,1997). *EAP2* produces two different polypeptides arising from differential splicing at an intron, the shorter *EAP2* protein diverging from the longer sequence after amino acid 197 and having a translation stop after residue 206. This truncated protein includes both MADS- and K-box amino acid sequences. *MESAP1* had the next highest homology (84%) with *MADS5*, the *AP1* homologue in *Betula pendula*, followed by *EAP1* (78%), the other *AP1* equivalent in *Eucalyptus*.

The Southern analysis for *MESAP1* (Figure 3.2 B) showed hybridisation to one strong band and one weak band. As mentioned above, a homology search revealed that *MESAP1* had a homology of 95% with *EAP2*, but only 78% with *EAP1*. It is possible that the second, weaker, band found in the Southern analysis could be the equivalent of *EAP1* in *M. excelsa*.

The isolated fragments of *MESAP1* and *MTAP1* showed putative conserved sequences such as the MADS domain with the Serum Response Factor type transcriptional activator and the K-box region. Members of the MADS-box family of transcription factors are found in eukaryotes ranging from yeast to humans. In plants, MADS-box proteins regulate several developmental processes including flower, fruit and root development. Most known floral homeotic genes belong to the MADS box family and their products act in combination to specify floral organ identity by an unknown mechanism.

The MADS domain proteins *AP1*, *AP3*, *PI*, and *AG* specify the identity of *Arabidopsis* floral organs. *AP1* and *AG* homocomplexes and *AP3-PI* heterocomplexes bind to CArG-box sequences (Riechmann et al., 1996). The DNA-binding properties of these complexes were investigated by Riechmann et al. (1996) and they found that *AP1*, *AG* and *AP3-PI* were all capable of recognising the same DNA-binding sites, although with somewhat different affinities. In addition, the three complexes induced similar conformational changes on a CArG-box sequence. The molecular dissection of *AP1*, *AP3*, *PI* and *AG* indicated that the boundaries of the dimerisation domains of these proteins varied. For *AP1* and *AG* only a part of the L region was needed to form a DNA-binding complex in addition to the MADS box.

It has been suggested that the K-box is a coiled-coil structure and has a possible role in multimer formation and is a domain not found in non-plant MADS box factors, indicating that the plant MADS box factors may have different criteria for interaction (Davies et al., 1996). In *Antirrhinum majus*, West et al. (1998) observed that *SQUA* differed from the characterised mammalian and yeast MADS-box proteins as it could efficiently bind two different classes of DNA-binding sites. *SQUA* induced bending of these binding sites and the sequence of the site played a role in determining the magnitude of these bends. They demonstrated that the MADS-box and I-domains are sufficient for homodimer formation by *SQUA*. However, the K-box in *SQUA* could also act as an oligomerisation motif and in the full-length protein, the K-box played a different role in mediating dimerisation in the context of *SQUA* homodimers or heterodimers with *PLENA*.

Serum response factor is a MADS box transcription factor that binds to consensus sequences, CC(A/T)(6)GG, found in the promoter region of several serum-inducible genes (Meshi and Iwabuchi, 1995). Shore and Sharrocks (1995) observed that several MADS-box proteins specifically recruit other transcription factors into multi-component regulatory complexes. Such interactions with other proteins appear to be a common theme within this family and play a pivotal role in the regulation of target genes.

From all the assessments conducted through homology analyses, it could be safely concluded that *MESAP1* was a partial orthologue of *AP1* or *SQUA* from *M. excelsa*. The presence of MADS domain with SRF-type transcription factor and part of the K-box region in the isolated fragment establishes the important role *MESAP1* could play in floral development. Wagner et al. (1999) had concluded that *LFY* acts as a transcriptional activator and that it exerts its meristem identity activity in part by direct transcriptional activation of *AP1*. The presence of these domains in *MESAP1* indicates that it could thus be activated by *MEL* and, in turn, activate selectively targeted genes to complete the process of floral organogenesis and floral development in *M. excelsa*.

METFL1 and *MTTFL1* revealed a phosphatidylethanolamine binding (PEBP) domain in their sequences. Proteins belonging to the PEBP family are highly conserved throughout nature and have no significant sequence homology with other proteins of known structure or function. A variety of biological roles have previously been described for members of this family, including lipid binding, roles as odorant effector molecules or opioids, interaction with the cell-to-cell signalling machinery, regulation of flowering, plant stem architecture, and a function as a precursor protein of a bioactive brain neuropeptide. The crystal structure of *Antirrhinum CEN* to 1.9 $\overset{\circ}{A}$ resolution was determined by Banfield and Brady (2000). This structure confirmed the *CEN* proteins as a subset of the family of phosphatidylethanolamine-binding proteins (PEBP), as predicted from sequence homology. Mammalian forms of PEBP have been found to act as inhibitors of MAP kinase signalling, a central signalling cascade regulating cell differentiation. The structure of *Antirrhinum CEN* suggested a role as a kinase regulator. Studies by Banfield et al. (1998) ascribed hPEBP, the human PEBP protein, with a primary biological role as a transducer

of signals from the interior membrane surface. The high homology that *METFL1* and *MTTFL1* had with human PEBP domain (82%) could also be important in ascribing roles to this gene in *Metrosideros*.

Homology searches for *METFL1* and *MTTFL1* revealed that they were the first *TFL1* orthologues to be isolated from woody perennials. Compared to *MEL* and *MESAP1*, the *TFL1* orthologues in *Metrosideros* seemed to be relatively lowly expressed in the flower buds as a second round of nested PCR was necessary to visualise these products on the gel after staining. The sequences of *METFL1* and *MTTFL1* had high homology to genes such as *FLOWERING LOCUS T* (*FT*), which acts in parallel with the meristem-identity gene *LFY* to induce flowering of *Arabidopsis* and the floral inducer *CiFT* from *Citrus unshiu*. *FT* acts in part downstream of *CO* and mediates signals for flowering in an antagonistic manner with its homologous gene, *TFL1* (Kobayashi et al., 1999). In *Arabidopsis*, the deduced sequence of the *FT* protein did not suggest that it directly controlled transcription or transcript processing. Instead, it was similar to the sequence of *TFL1*, an inhibitor of flowering that also shared sequence similarity with membrane-associated mammalian proteins (Kardailsky, 1999). While *FT* is known to promote flowering, *TFL1* is known as a repressor of flowering. The fact that these two genes share high homology prompts one to speculate whether they regulate flowering by competing for the same factors and binding sites.

Southern analysis was carried out not only to identify the number of homologues in the genome, but also to validate the use of the fragments as probes for analysis of gene expression. A heterologous probe had to be used for *METFL1* as permission to clone the *TFL1* equivalent from *M. excelsa* could not easily be obtained because of the regulations imposed by the Environmental and Risk Management Authority of New Zealand on the cloning of genes from native plant species. Although the probe used for the Southern analysis to detect the *TFL1* equivalent in *M.excelsa* DNA was *MTTFL1*, its counterpart in *M. collina*, there was one clear band that developed indicating that this sequence could safely be used in expression studies in *M. excelsa* as well. It was also significant that only

one band was formed indicating that there was little chance of the probe hybridising to other genes in this multi-gene family and thereby confirming its fidelity.

The isolated fragments of *MEL*, *MESAP1* and *MTTFL1* were considered suitable for use as probes for studying expression patterns of *LFY*, *AP1* and *TFL1* equivalents in *M. excelsa*. It was necessary to study both the temporal and spatial expression patterns of the isolated orthologues to confirm whether they behaved and played the same roles as that of their equivalents in *Arabidopsis* and other plants. These studies were subsequently conducted and are presented in the following Chapters.

Chapter 4. Temporal expression patterns of floral and inflorescence meristem identity genes in *M. excelsa*

4.1 Introduction

Although a considerable knowledge has accumulated on the roles of *LFY*, *AP1* and *TFL1* in annuals and herbaceous plants, their roles in woody perennials is scanty. The expression of these floral genes in woody perennials could be quite different from herbaceous annuals such as *Arabidopsis* and *Antirrhinum*. In kiwifruit (*Actinidia deliciosa*) Walton et al. (2001) observed bimodal patterns of expression of *LFY* and *AP1* equivalents over the two seasons that kiwifruit flowers developed. In *Pinus radiata*, *NDLY*, the *LFY/FLO* equivalent, shared extensive homology with its angiosperm counterparts, but was already expressed during vegetative development at least five years before the transition to the reproductive phase (Mouradov et al., 1998). However, Southerton et al. (1998) observed that *ELF1*, the *Eucalyptus globulus LFY*, was strongly expressed in young flower buds and then later in sepals and petals. The two *AP1* equivalents in *Eucalyptus* (*EAP1* and *EAP2*) were found to be predominantly expressed in flower buds (Kyozuka et al., 1997).

In annuals, such as *Arabidopsis* and *Antirrhinum*, the process of flowering is completed within relatively very short time periods. In *Arabidopsis*, especially, events are happening in such a short span of time that subtle changes may not have been recorded. With *M. excelsa*, the flowering process is spread over a long period. Using such a plant may provide a clearer picture of the temporal expression patterns of these genes. It is also important to assess whether the *Arabidopsis* models of expression of the floral genes hold in plants such as *M. excelsa* since *M. excelsa* is a facultative short day plant (Henriod et al., 2000) and *Arabidopsis* is a facultative long day plant. The present Chapter describes how the expressions of *MEL*, *MESAP1* and *METFL1* were tracked in *M. excelsa* buds from March to November by which time the floral organs had all differentiated and developed. It was hypothesised that the temporal expression patterns of *MEL*, *MESAP1* and *METFL1*, being considered the equivalents of *LFY*, *AP1* and *TFL1* respectively in *M. excelsa* would be

similar to floral and inflorescence meristem identity genes in other species. As cymule primordia initiation began in May in *M. excelsa* (Figure 2.6 B), it was hypothesised that *MEL* and *MESAP1* expression would be detected in distal axillary buds before May if *MEL* functioned as a developmental switch that induced flowering, and *MESAP1* was an indicator for floral commitment similar to *AP1* (Hempel et al., 1997). It was also hypothesised that, the expression pattern of floral and inflorescence meristem identity genes in woody perennials would be influenced by seasonal changes in temperature and / or photoperiod, these differences being easily observed because of the longer period of expression that could be studied. It was anticipated that expression of floral meristem identity genes would be downregulated in winter as expression of *LFY* equivalent was not detected in over-wintering flower buds of *Eucalyptus* by Southerton et al. (1998) and a downregulation of expression of *LFY* and *AP1* equivalents in winter was also noted in *Actinidia deliciosa* by Walton et al. (2001). In the case of *METFL1*, it was anticipated that it would be expressed throughout the period of floral development as was characteristic of inflorescence meristem identity genes.

The technique of Northern analysis was initially attempted, and then RT-PCR analyses were carried out to monitor gene expression. Expression Southerns were also performed on RT-PCR products to validate results. As the Southern analysis in Chapter 3 had shown that the probe for *MESAP1* hybridised to two bands, although the hybridisation was weak in the second instance, a restriction enzyme analysis was also carried out to ensure that the PCR products in the band seen after RT-PCR for *MESAP1* had not been amplified from any other genes.

4.2 Materials and Methods

4.2.1 Sampling of buds and RNA extraction

Distal axillary buds (1-5 g) from the shoots of the five 12-15 year old seedling trees growing at the Turitea campus of Massey University were excised randomly between March and November 1998. The dates of harvests were 12 March, 9 April, 7 May, 4 June, 20 June, 4 August, 18 August, 1 September, 16 September, 30 September, 14 October and

12 November. These were the dates when buds were sampled for microscopy for the determination of the calendar of floral development as described in Chapter 2. Vegetative buds (1-5 g) as evidenced by the emergence of young leaves after bud break, were also collected separately on 14 October. At the time of harvest all tissues were instantly put in liquid nitrogen. On taking them to the laboratory, the buds were stored at -80°C until the time of RNA extraction. The procedure used for total RNA isolation was the hot-borate extraction method described in Section 3.2.2. The RNA extracted was quantified using a spectrophotometer as described in Section 3.2.2.

4.2.2 Northern analysis

Initially, Northern analysis was attempted twice on RNA samples for the period from 9 March to 30 September to detect *MEL* expression. The two Northerns differed only in the post-hybridisation washes.

4.2.2.1 RNA electrophoresis and blotting: Appropriate volumes of RNA

providing 10 µg of total RNA were ethanol precipitated and re-suspended in 6 µl milliQ and added to 5 µl formaldehyde, 12 µl formamide and 2.5 µl buffer/ethidium bromide mix. This mixture was incubated at 65°C for 10 min and cooled on ice. Loading buffer (2.5 µl) was added and the samples loaded on a 1.5% gel containing 6.6% formaldehyde and 1x RNA running buffer. The gel was electrophoresed at 80V for 4.5 h and the RNA was blotted overnight onto a Hybond^{TM}-N+, positively charged nylon membrane (Amersham Life Science) with 20 x SSC. The next day, the membranes were rinsed in 2 x SSC, air-dried at room temperature, and UV cross-linked.

5x buffer/ethidium bromide mix: 100 µl 50 x RNA running buffer

          25 µl 10 mg ml^{-1}ethidium bromide

          875 µl milliQ

50x RNA running buffer: 1 M 4-morpholinepropanesulfonic acid (MOPS)

          400 mM sodium acetate pH 7

          50 mM EDTA

Loading buffer: 10.0 g sucrose

          0.02 g bromophenol blue

          20 ml milliQ

20 x SSC : Described in Section 3.3.2

4.2.2.2 Probe synthesis: PCR was used to produce DNA complementary to *MEL* mRNA. The PCR reactions were carried out as outlined in Section 3.2.3 using *MEL* cloned in pUC19 as the template for amplification, and increasing the annealing temperature to 50°C. Purification, quantification and labeling of the probe was done as described for Southern analysis in Section 3.2.11.

4.2.2.3 Hybridisation and detection of radioactive probe: The membrane was placed in a hybridisation tube and pre-hybridisation solution (65°C) added. Bubbles were removed and the membrane incubated in the pre-hybridisation solution at 65°C with gentle agitation for 2 h. Following incubation, labelled probe (Section 4.2.2.2) was added, all bubbles removed and the membrane was incubated overnight at 65°C. Subsequently, for the first trial, the membrane was rinsed briefly in 2 x SSC and then washed three times (15 min each) in 2 x SSC/0.1% SDS, followed by a 1 h wash in 0.1 x SSC/0.1% SDS at 65°C. For the second attempt, the membrane was washed at a lower stringency. Washing temperature was 65°C but the last wash was with 1 x SSC/1% SDS for 1 h. After the washes the membrane was dried and exposed to Kodak Scientific Imaging Film in a cassette at -70°C for 1-14 d and then the films were developed.

4.2.3 RT-PCR analysis

4.2.3.1 Isolation and sequencing of a fragment of actin for loading control: RT-PCR was carried out on pooled RNA samples to isolate a fragment of the constitutively expressed gene, actin, to be used as loading control. Five reactions were performed. The protocol followed was the same as described in Section 3.2.1.4. Degenerate primers were designed for the PCR:

Actin forward primer: 5' GCGAATTCTTCACCACYACHGCYGARCG 3'
Actin reverse primer: 5' GCGGATCCCCRATCCARACACTGTAYTTC C 3'

Aliquots (12 µl) of the reactions were run on a 1% agarose gel along with 0.8 µl of 1 Kb plus DNA ladder™ (Life Technologies) and visualised after EtBr staining.

The remaining quantities of the PCR reactions were then run on a 1% agarose gel, stained with EtBr and visualised. The correct sized (approximately 450 bp) bands were cut out. The DNA was purified using Concert^{TM} Rapid Gel Extraction System (Life Technologies) and quantified by using High DNA Mass^{TM} Ladder (Life Technologies). Sequencing was carried out at the Massey University Seqencing facility using purified PCR product at a concentration of 20 ng μl^{-1} as a template and the actin forward and reverse primers.

4.2.3.2 RT-PCR of *MEL, MESAP1, METFL1* and actin: RT-PCR was

performed on RNA prepared from the twelve sampling periods (Section 4.2.1) for *MEL*, *MESAP1* and *METFL1*. The protocol for the reverse transcriptase reaction was the same as described in Section 3.2.1.4. For PCR, a higher annealing temperature of 50°C was used for *MEL* and *MESAP1*. The *LFY*-specific primers were used for *MEL* (Section 3.2.5). In the case of *MESAP1*, *AP1* specific primers based on the sequence of the earlier isolated fragment, were used. The primer sequences were:

AP1 specific forward primer: 5' GCGAATTCGAGATGTGGGCTGCTGAAGAAG 3'
AP1 reverse primer: 5' GCGGGATCCCAAGCTGGTGCTCCAAATTCTG 3'

For *METFL1*, two rounds of PCR were carried out as described in Section 3.2.7 using the primers described there.

cDNA (1 μl) from each date of sampling was used for PCR, using primers for actin as a loading control at the same time, when PCR was carried out for *MEL, MESAP1*, and during the first round PCR in the case of *METFL1*. The primers used for actin were as given in the preceding section.

The PCR products (12 μl) were run on a 2% agarose gel in the case of *MEL* and *METFL1* and a 1.5% gel in the case of *MESAP1* and actin, along with 0.8 μl of 1 Kb plus DNA ladder^{TM} (Life Technologies) and visualised after EtBr staining.

4.2.3.3 Polymerase chain reaction using RNA: PCR was carried out on RNA

prepared from the 12 sampling periods (Section 4.2.1) without reverse transcription to

check for possible DNA contamination. The procedure was as described for the PCR steps in the preceding section except that a volume of 3 µl of 1 µg 20 µl^{-1} RNA was used as the template for amplification. PCR products (12 µl) were run on a 2% agarose gel in the case of *MEL* and *METFL1* and 1.5% gel in the case of *MESAP1* along with 0.8 µl of 1 Kb plus DNA ladder^{TM} (Life Technologies) and visualised after EtBr staining.

4.2.4 RT-PCR of vegetative tissue

RNA extracted from very young vegetative buds that were collected on 14 October was used for RT-PCR for *MEL, MESAP1* and *METFL1* as described in Section 4.2.3.2. The final PCR products (12 µl) were run on 2% agarose gels along with 0.8 µl of 1 Kb plus DNA ladder^{TM} (Life Technologies) and visualised after EtBr staining.

4.2.5 Expression Southerns for *MEL, MESAP1* and *METFL1*

Southerns were carried out to see whether probes of *MEL, MESAP1* and *MTTFL1* prepared from cloned and sequenced fragments would hybridise to the bands seen after RT-PCR, and thereby confirm that they were amplified from mRNA of *MEL, MESAP1* and *METFL1*. Probe synthesis was carried out as described in Section 3.2.11. RT-PCR products (4 µl) of *MEL, MESAP1* and *METFL1* were prepared as described in Section 4.2.3.2 for the 12 sampling periods and from the vegetative tissues (Section 4.2.4) and were electrophoresed on 2% agarose gels at 100V for 1 h. The DNA was then blotted onto Hybond^{TM}-N+ nylon membranes as described in Section 3.2.11, but without the depurination step with HCl, as the DNA fragments were small enough to transfer without this. Hybridisation and detection of radioactive probes were conducted as described in Section 3.2.11, but membranes were exposed to the films for only 30 min to 1 h.

4.2.6 Restriction enzyme analysis of *MESAP1*

In order to confirm the identity of the PCR products after RT-PCR for *MESAP1*, a restriction analysis was carried out. A restriction map of *MESAP1* was formed using Webcutter 2.0 for identifying enzymes that cut the probe once. *BglII* was chosen as a suitable enzyme. RT-PCR was carried out for *MESAP1* as in Section 4.2.3.2. The resultant products were electrophoresed on a 1.5% agarose gel at 80 V for 1 h. The gel was

visualised after EtBr staining and the bright bands of the appropriate size were cut out into two equal lots in Eppendorf tubes. The DNA was purified out of the gel slices using Concert™ Rapid PCR purification system (Life Technologies). Fifty µl of the final products in each tube was reduced to 25 µl volumes after vacuum drying. The purified DNA (10 µl) was restriction digested with 2 µl of the enzyme *BglII* (Roche) and buffer in a final volume of 20 µl at 37°C for 4 h. An aliquot (10 µl) of the digest was electrophoresed on a 1.5% agarose gel along with undigested, purified DNA (5 µl) and 0.8 µl of Kb plus DNA ladder™.

4.3 Results

4.3.1 Northern analysis

Both the Northerns for *MEL* were unsuccessful. Bands did not develop on films even after exposures of up to 2 weeks and even under conditions of low stringency washes.

4.3.2 RT-PCR analysis

4.3.2.1 Isolation and sequencing of a fragment of actin for loading control

A single bright band of approximately 450 bp in size was visible in each lane (Figure 4.1) when aliquots of the RT-PCR products for actin were run on a gel and visualised after EtBr staining. A 423 bp fragment of the constitutively expressed gene, actin, was successfully isolated and sequenced. The nucleotide sequence is given below:

```
ACCACTGCTGAGCGGGAAATTGTCCGTGACATGAAGGAAAAGCTGGCATATGTTGCCCTTGACT
ATGAGCAAGAGCTGGAAACTGCTAAGAGCAGCTCGTCTGTTGAGAAAAACTATGAGCTGCCCG
ATGGACAAGTCATCACAATTGGGGCCGAGAGGTTCCGTTGTCCAGAAGTCCTCTTCCAGCCATC
ATTGATTGGAATGGAAGCTGCTGGAATTCATGATACCACCTACAACTCCATCATGAAGTGCGAT
GTGGATATCAGGAAGGATCTTTATGGCAATATTGTGCTTAGTGGTGGTTCCACTATGTTCCCTGG
TATTGCAGACAGGATGAGCAAGGAGATTACTGCTCTTGCTCCAAGCAGCATGAAGATTAAGGT
GGTGGCTCCTCCTGAGAGGAAGTACAGTGTTTGGATTGGG
```
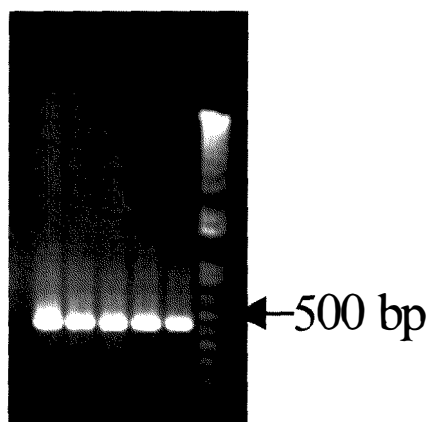
Figure 4.1. RT-PCR for isolation of actin fragment from *M. excelsa*

Amino acid sequence and homology: The deduced amino acid sequence of the isolated gene fragment and the four sequences with the highest homology identified by blast search are presented below.

```
M.excelsa actin (MeA):
        1 TTAEREIVRDMRKEKLAYVALDYEQELETAKSSSSVEKNYELPDGQVITIGAERFRCPEVL 180
Malva pusilla actin Accession No. AF112538. Identities = 140/141 (99%)
      204 TTAEREIVRDMRKEKLAYVALDYEQELETAKSSSSVEKNYELPDGQVITIGAERFRCPEVL 263
Helianthus annuus actin Accession No.AF282624. Identities = 140/141 (99%)
      204 TTAEREIVRDMRKEKLAYVALDYEQELETAKSSSSVEKNYELPDGQVITIGAERFRCPEVL 263
Gossypium hirsutum actin Accession No.D88414. Identities = 139/141 (98%)
       49 TTAEREIVRDMRKEKLAYVALDYEQELETAKSSSSVEKNYELPDGQVITIGAERFRCPEVL 108
Nicotiana tabacum actin Accession No.U60495. Identities = 137/141 (97%)
      184 TTAEREIVRDMREELAYVALDYEQELDTAKSSSSVEKNYELPDGQVITIGAERFRCPEVL 243


MeA     :    181 FQPSLIGMEAAGIHDTTYNSIMKCDVDIRKDLYGNIVLSGGSTMFPGIADRMSKEITALA 360
M.pusilla:264 FQPSLIGMEAAGIHETTYNSIMKCDVDIRHDLYGNIVLSGGSTMFPGIADRMSKEITALA 323
H.annuus:  264 FQPSLIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGSTMFPGIADRMSKEITALA323
G.hirsutum:109 FQPSFIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGSTMFPGIADRMSKEITALA 168
N.tabacum 244 FQPSMIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIVLSGGSTMFPGIADRMSKEITALA 303


MeA:       361 PSSMKIKVVAPPERKYSVWIG 423
M.pusilla:324 PSSMKIKVVAPPERKYSVWIG 344
H.annuus:  324 PSSMKIKVVAPPERKYSVWIG 344
G.hirsutum:169 PSSMKIKVVAPPERKYSVWIG 189
N.tabacum:304 PSSMKIKVVAPPERKYSVWIG 324
```

It was thus confirmed that the isolated gene fragment was that of actin from *M. excelsa* as it had a very high homology (>90%) at the amino acid level with actin isolated from other plant species.

4.3.2.2 Temporal expression patterns of *MEL*, *MESAP1* and *METFL1*

RT-PCR analysis (Figure 4.2) revealed that *MEL* was expressed as early as March at low levels and was upregulated during May to early June (during cymule primordia initiation) and again later during organogenesis. *MESAP1* showed a similar bimodal pattern except that its expression was markedly higher during organogenesis. *METFL1* appeared to be expressed at relatively lower levels than *MEL* or *MESAP1* as two rounds of PCR were needed to visualise its products. *METFL1* was expressed throughout the process of floral development studied from March to November. The levels of *METFL1* were slightly higher during the early stages (March-May) and towards the very late stages (30 September-November). The levels of expressed actin were uniform for the RT-PCR

A

12/3 9/4 7/5 4/6 20/6 4/8 18/8 1/9 16/9 30/9 14/10 12/11

MEL
188 bp

MESAP1
343 bp

Actin
423 bp

B

Vegetative 12/3 9/4 7/5 4/6 20/6 4/8 18/8 1/9 16/9 30/9 14/10 12/11
meristem
14/10

METFL1
175 bp
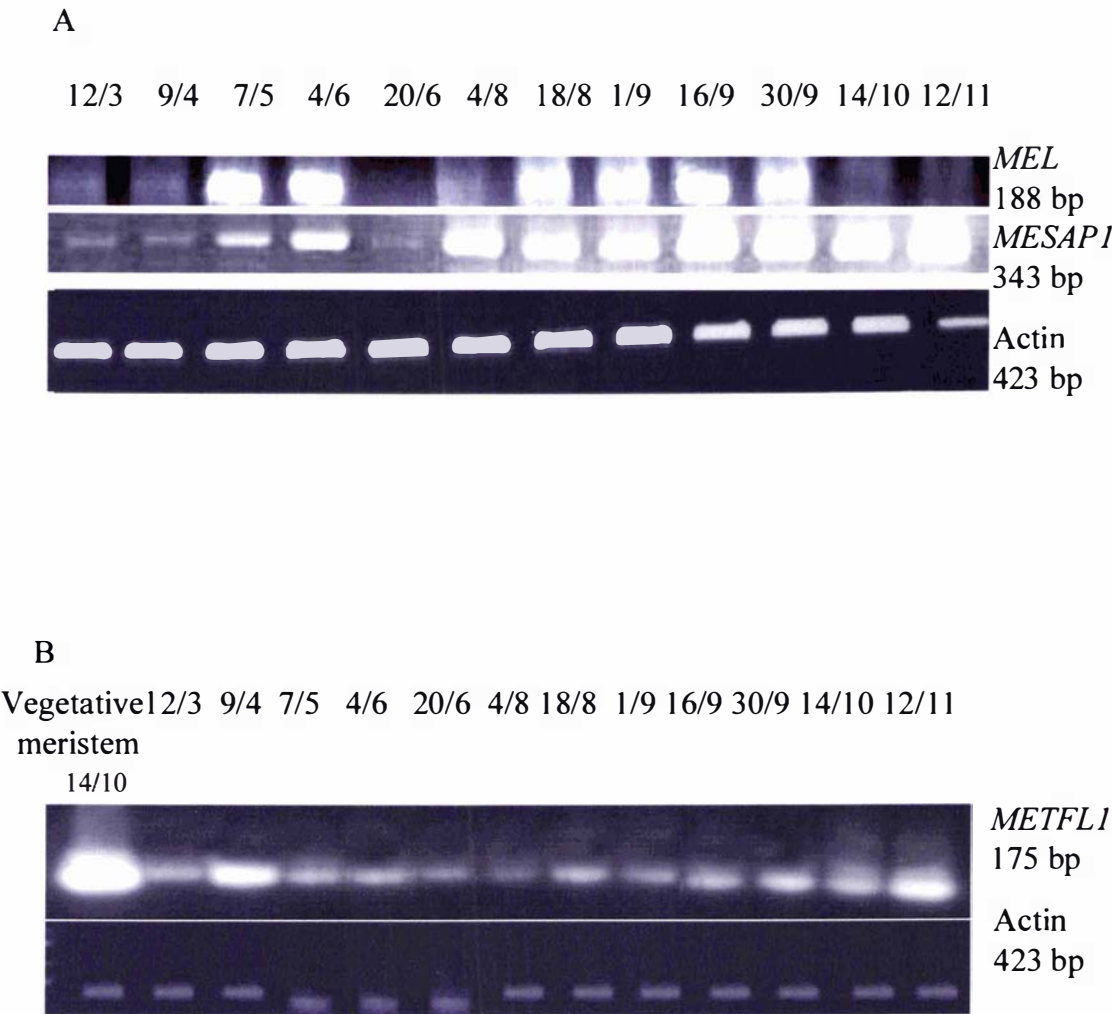
Actin
423 bp

Figure 4.2 Temporal expression patterns of floral and inflorescence meristem identity genes in *M. excelsa* buds from March to November. (A) RT-PCR products of *MEL* and *MESAP1* electrophoresed along with actin as loading control. (B) RT-PCR products of *METFL1* electrophoresed along with actin as loading control.

products of all the RNA samples indicating that same quantities of cDNA were used for RT-PCR for *MEL*, *MESAP1* and *METFL1* for all dates of sampling.

4.3.2.3 PCR of RNA

Bands did not form after PCR in any of the samples of RNA that were used as templates without reverse transcription. This indicated that *MEL*, *MESAP1* and *METFL1* were not amplified from any possible DNA contamination.

4.3.3 *MEL*, *MESAP1* and *METFL1* expression in vegetative tissue

A bright band of 175 bp in size was amplified from the RNA from young vegetative buds after RT-PCR in the case of *METFL1* (Figure 4.2 B). No amplification was seen for *MESAP1* from vegetative tissue (data not shown). For *MEL*, also, a band could not be clearly visualised on the gel just by EtBr staining after RT-PCR, but could be seen by expression Southern (see Section 4.3.4 below)

4.3.4 Expression Southerns

Bands developed on films prepared as described in Section 4.2.5 even with exposure for just 30 min. The *MEL* probe hybridised to bands of approximately 180 bp (Figure 4.3 A) and the *MESAP1* probe hybridised to bands of approximately 340 bp (Figure 4.3 B) seen in the RT-PCR analysis (section 4.3.2.2). *MTTFL1*, a heterologous probe from *M. collina* 'Tahiti' was used successfully for expression Southerns for detecting *METFL1*. The *MTTFL1* probe hybridised to bands of approximately 175 bp (Figure 4.3 C) amplified in the RT-PCR analysis. The identities of the bands visualised after RT-PCR and considered as *MEL*, *MESAP1* and *METFL1* were thus confirmed. No bands were formed for *MESAP1* from vegetative tissue. However, there was strong expression of *METFL1* in vegetative tissue. *MEL* also was expressed in vegetative tissue as the 188 bp band could be seen on the film after expression Southern, although it could not be visualised on a gel after RT-PCR.

Vegetative 12/3 9/4 7/5 4/6 20/6 4/8 18/8 1/9 16/9 30/9 14/10 12/11
meristem
14/10

A   188 bp

12/3 9/4 7/5 4/6 20/6 4/8 18/8 1/9 16/9 30/9 14/10 12/11

B   343 bp

Vegetative 12/3 9/4 7/5 4/6 20/6 4/8 18/8 1/9 16/9 30/9 14/10 12/11
meristem
14/10

C   175 bp
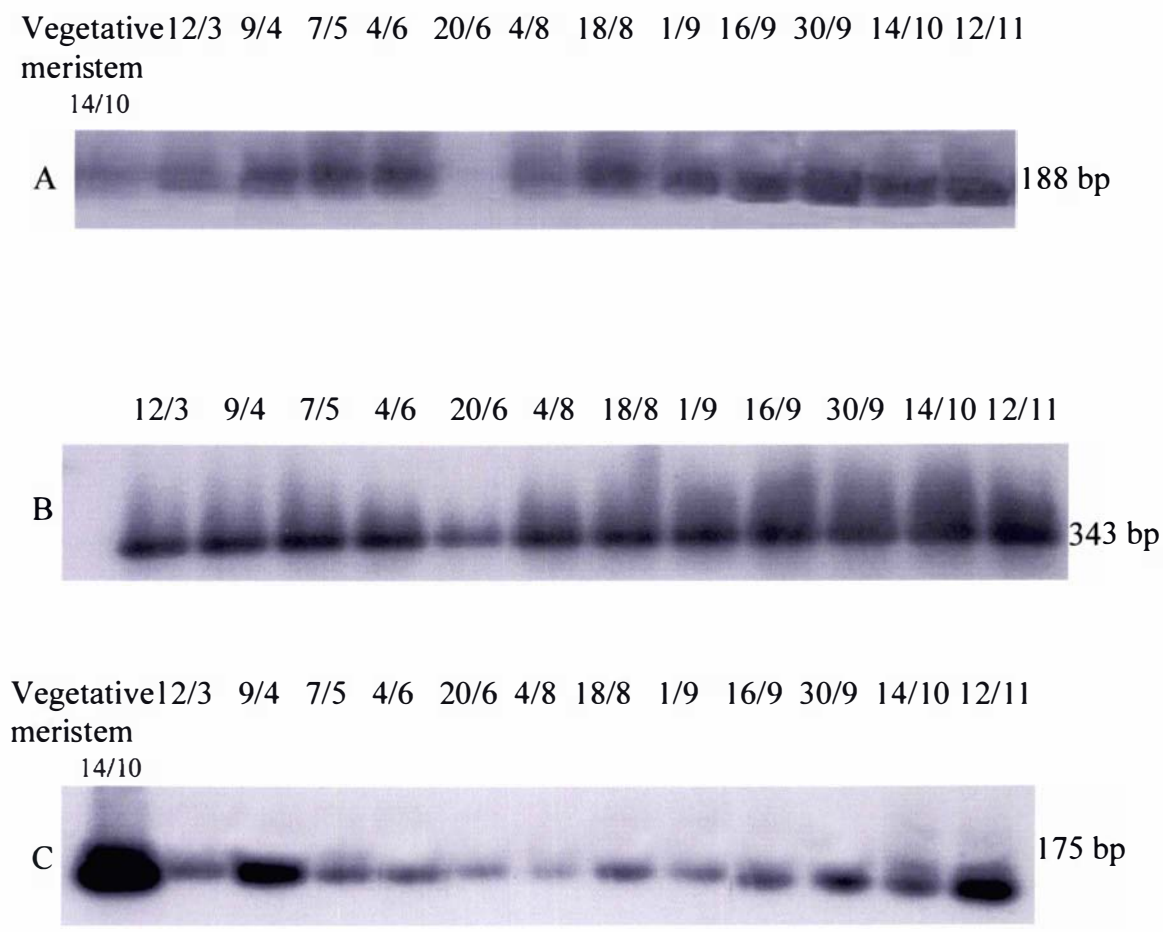
Figure 4.3 Expression Southerns of floral and inflorescence meristem identity genes in *M. excelsa* buds from March to November (A) *MEL* probe hybridised to the 188 bp fragments. (B) *MESAP1* probe hybridised to the 343 bp fragments. (C) *MTTFL1* probe hybridised to the 175 bp fragments.

4.3.5. Restriction analysis of *MESAP1*

The restriction map of *MESAP1* is presented in Figure 4.4 along with the sites of *AP1*-specific primers. It was revealed that *BglII* had a restriction site at the 65 bp region on the cloned fragment isolated and sequenced as in Section 3.4.3.

When the *BglII* digest in Section 4.2.6 was visualised after electrophoresis on the gel (Figure 4.5), it was seen that the DNA was cut and formed a smaller (approximately 312 bp) band compared to the undigested DNA (343 bp).

4.4 Discussion

Molecular and genetic studies have shown that the mechanisms controlling flower development are largely conserved across distantly related angiosperm species (Ma, 1994). *LFY*, *AP1* and *TFL1*, or their equivalents, have been isolated and characterised in a number of annuals, while the former two have been isolated and studied in a limited number of woody perennials. The three genes are also reported to interact with each other in floral and inflorescence meristem specification. The partial equivalents of these genes were isolated from *M. excelsa* (Chapter 2) and their expressions were tracked from floral initiation to floral organ differentiation and development. This analysis thus has the advantage of correlating the expression of these genes with the actual developmental events taking place in the plant buds (as revealed through microscopy) and is the first such study on a woody perennial involving all three genes simultaneously.

Northern analysis did not yield successful results, possibly because *MEL* had very localised expression and whole *M. excelsa* buds were used for RNA extraction. This could have lowered the concentration of *MEL* in the total RNA as the buds were covered by at least 6-8 pairs of scales. Moreover, only 10 μg of RNA was loaded in the gels for each sample. RT-PCR was thus adopted as the preferred technique as it could help to detect even lowly expressed genes such as *TFL1* with very localised expression in the inflorescence meristem. However, the limitation of RT-PCR for accurate quantification of

AP1 specific Forward   *BssSI*

1 bp   ⟶   *BglII*

aacaagatcaataggcaagttacgt t c tcgaagc**ggagatgtgggctgctgaagaag**gcccac**gagatct**ccgtc
ttgttct agtt at ccgtt caatgcaagagctt c gcctctacacccgacgactt c t t ccgggtgctctagaggcag

                                                        BsiI

                  StyI   *BcgI*
             Bbv16II   *Ksp632I*
     DrdI   *BbsI EcoT14I*   *SapI*

ctctg*c*gacgcggaggtcgccctcatcgtctt c t ccaccaagggcaagctcttcgagtacgccaccgatgcttgc
gagacgctgcgcctccagcgggagtagcagaagaggtggttcccgtt c gagaagctcatgcggtggctacgaacg

                  BpiI   *BssTII*   *Eam1104I*
                  BpuAI Eco130I   *EarI*
                      ErhI

           HaeII
           Eco47III
           AfeI   *NdeI*

atggagaagatccttgagcgctatgaaagatattcatatgcagagagacagattctc acaaacaacgccgaaacc
tacctcttctaggaactcgcgatacttt c t ataagtatacgtctctctgtct aagagtgttt gttgcggcttt gg

           Aor51HI   *FauNDI*
           Bsp143II
           BstH2I

           NspI

aatggga actggactttggaacatgcaaaactcaaggccaggatggagattttgcagaaaaatcaaaagaatctg
ttacccttgacctgaaaccttgtacgttttgagttccggtcctacctctaaaacgtctttttagttttcttagac

          BfrI
          Vha464I   *Alw21I*
          MspCI   *ApoI*   *AspHI*

atgggagaagaacttgattccttaagcctcaaagagcttcagaatttggagcaccag   357 bp
taccctcttcttgaactaaggaattcggagtttctcgaa**gtcttaaacctcgtggtc**

         AflII   *AcsI*   *BsiHKAI*
         BspTI   *Bbv12I*
         Bst98I   ⟵

                     AP1 specific Reverse

Figure 4.4 Restriction map of *MESAP1* with sites of 6 bp cutters cutting once and *AP1*-specific primers

D = RT-PCR products purifed and digested with *BglII*

UD = RT-PCR products purified and undigested with enzymes
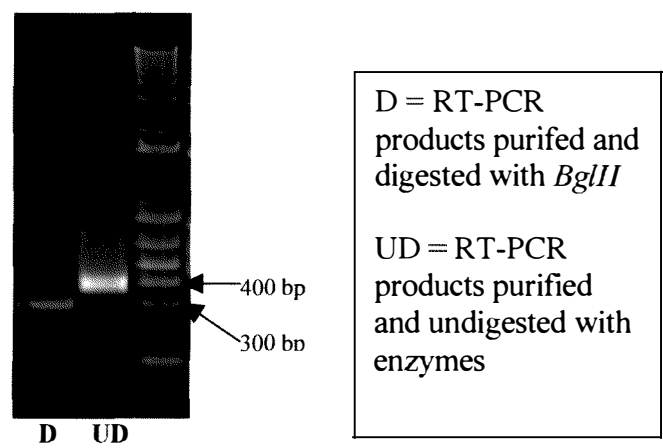
Figure 4.5 Restriction analysis of *MESAP1*

gene expression (Bustin, 2000) is acknowledged. It is conceded that even with a loading control, RT-PCR analysis should only be considered to be semi-quantitative.

The constitutively expressed gene, actin, has been used as a control in other studies (Shirley and Meagher, 1990; Cotton et al., 1990). Its promoter has also been used for creating expression vectors and transformations for constitutive expression (McElroy et al., 1991). In the present study, the 423 bp fragment of actin that was successfully isolated was used as an indicator of loading. In the RT-PCR analysis, the actin levels were similar in all the samples indicating the results were not caused by difference in cDNA quantities in the PCR.

It was also necessary to check whether the hot-borate method that was adopted for RNA extraction in the present study left DNA contaminants in the RNA samples and thereby provided templates for the amplification of the gene fragments. It was to address this issue that PCR was performed with the appropriate primers on RNA samples diluted to the extent they would be if reverse transcription had been carried out. The fact that no bands were formed from any of the samples confirmed that the bands observed in the RT-PCR analysis originated from mRNA.

Another potential problem with RT-PCR analysis is that the primers can amplify something other than the genes under consideration, especially when there is low concentration of their templates in the reaction. To overcome this concern, expression Southerns were conducted using probes with known sequences. The fact that the probes hybridised only to one band in each case, and that this band was of the correct size, confirmed the results from the RT-PCR.

The *MESAP1* probe included part of the MADS domain and it is possible that this probe could hybridise to other MADS box genes. The possible way to clarify this issue was restriction analysis of the RT-PCR products of *MESAP1* to confirm their identity. The choice of restriction enzyme was limited by the small size of the probe. It was also ideal to choose a 6 bp cutter that cut only once within the fragment. *BglII* was chosen as a suitable

enzyme for this purpose as it cut the probe only once and at a site that made it possible to visualise the larger fragment of the two pieces after digestion.

The fact that *BglII* cut the purified RT-PCR products of *MESAP1* (Figure 4.5) to form a resultant smaller fragment of 312 bp (the remaining 31 bp fragment could not be seen due to its small size), is explained by the fact that *AP1* specific primers (designed internally to the originally cloned and isolated fragment of *MESAP1*) were used for PCR in the RT-PCR analysis. These results confirmed that the bands seen on the gel in the RT-PCR analysis of *MESAP1* (Figure 4.2 A) were products amplified from the *AP1* equivalent in *M. excelsa* rather than from any other MADS box genes.

It was evident from the calendar of floral development formed for *M. excelsa* in Chapter 2, that the cymule primordia were visible as early as May. This indicated that floral commitment and evocation would have occurred in *M. excelsa* before that. In *A. thaliana*, the establishment of floral meristems is primarily regulated by *LFY* and *AP1* and their expression is observed prior to visible morphological changes (Yanofsky, 1995). Hempel et al. (1997) demonstrated the utility of *AP1* expression as an indicator of floral determination in *Arabidopsis* as its expression could be detected only in meristems after floral induction. *MEL* and *MESAP1* were detected in the RT-PCR analysis and the expression Southerns as early as March. Thus it became evident that floral determination in *M. excelsa* would have occurred by then.

MEL and *MESAP1* were upregulated during the period of cymule primordia initiation in early May to early June. Their expression was downregulated during late June (winter). Sections of floral buds during this period revealed undifferentiated groups of cells growing in the axils of scales, with bracteoles differentiating in some of them (Figure 2.6 D). These masses of cells could perhaps be the future pedicels of the cymules. In *lfy* and *apl* mutants, shoot-like structures are formed instead of flowers in *Arabidopsis* and *Antirrhinum* (Ma, 1998). It could be speculated that with downregulation of *MEL* and *MESAP1* during winter, a similar effect is produced to form shoot-like structures which become the pedicels in *M. excelsa.* It might further be speculated that this is how flower pedicels are formed

in plants generally.

The expression of both *MEL* and *MESAP1* expressions was upregulated again from August-September with the onset of longer days and warmer temperatures. *MESAP1* expression reached its peak during this period. It was only then that floral organs began to develop rapidly in *M. excelsa* and all the organs were fully differentiated by the end of September (Chapter 2).

The bimodal pattern of expression of *LFY* and *AP1* equivalents as reported here has also been observed in *A. deliciosa* by Walton et al. (2001). In the latter, the first period of expression was early in first-order bud development (late spring of the first growing season), when second-order meristems are initiated, and the second, approximately 10 months later, when those meristems differentiated flowers. This was the only other study where the expression of these genes has been tracked progressively from floral meristem formation to organ differentiation in a woody perennial, and the observations by Walton et al. (2001) support the results obtained in *M. excelsa.*

The timing of flower differentiation is also variable between species. Flower differentiation may occur during the first growing season [e.g. in apple, cherry, peach pear and plum (Tufts and Morrow, 1925)] but in others it is delayed until early in the second growing season [e.g. citrus (Lord and Eckard, 1985), grape (Pratt, 1971) and olive (Hartmann, 1951)]. It took only $1-1^{1}/_2$ months for all the floral organs to be differentiated in *M. excelsa.* If this had happened soon after the formation of the cymule primordia in the axils of the scales in early May, young flowers would have had to remain in a differentiated form in chilling temperatures. It could thus mean that the survival mechanism of the plant was programmed for certain other requirements (onset of longer days or chilling in winter) to be satisfied before the floral meristem identity genes could act on organ identity genes for organogenesis to progress.

It was evident that mere upregulation of *MEL* was alone insufficient for *MESAP1* to reach peak levels and for organogenesis to progress in *M. excelsa* prior to the cold period. It

may be that competence to respond to floral meristem identity genes is acquired gradually. From their experiments on transgenic plants in which *LFY* from *Arabidopsis* was constitutively expressed in *Arabidopsis* and aspen, Weigel and Nilsson (1995) reported *LFY* to be "a developmental switch sufficient for flower initiation in diverse plants". The authors reported from studies, where *35S::LFY* transgene was crossed into various mutant backgrounds, that combined activities of *LFY* and *AP1* were more effective in transforming shoot meristems into flower meristems than *LFY* activity alone. They also suggested that competence to respond to floral meristem identity genes was acquired gradually. In young meristems, competence appeared to be low, and both *LFY* and *AP1* were required to promote flower development over that of shoots. Competence increased later in the life cycle and *LFY* alone became sufficient to induce flower development. Their results also indicated that floral induction regulated an additional, parallel pathway that affected the competence of meristems to respond to the activity of floral meristem identity genes. Weigel and Nilsson (1995) suggested that a variety of possibilities for this mechanism could be envisaged, among them that the response of floral target genes was regulated and these genes underwent a change from being completely repressed to partly derepressed. They favoured this hypothesis because certain floral traits eventually developed even in *lfy/apl* double mutants. Perhaps chilling and/or onset of longer days were also needed for *MESAP1* to upregulate to peak levels.

The fact that *MEL* and *MESAP1* expressions followed a similar sequential pattern temporally is interesting. It has been suggested that the close timing of *LFY* and *AP1* activation in wild type *Arabidopsis* plants implied that regulation of *AP1* by *LFY* could be direct (Simon et al., 1996; Hempel et al., 1997). Indeed the *LFY* protein has been shown to bind to the *AP1* promoter (Parcy et al., 1998). Liljegren et al. (1999) found that in *Arabidopsis* the onset of *AP1* expression is delayed in *lfy* mutants, indicating that *LFY* is formally a positive regulator of *AP1*, which is consistent with molecular and genetic evidence provided by previous gain-of-function experiments.

In *Arabidopsis*, Bowman et al. (1993) observed that mutations in the *AP1* gene disturbed two phases of flower development, flower meristem specification and floral organ

specification and further showed that the two functions were separable. Results for *M. excelsa* show that despite a substantial temporal separation of these events, *MESAP1* is expressed both during meristem specification, and at a higher level, during floral organogenesis, thus supporting observations by Bowman et al. (1993).

Published work on expression patterns of the *TFL1* gene in perennials is as yet not available and its isolation from perennials has not been reported in the databases. RT-PCR analysis showed that *TFL1*-like genes were predominantly expressed in flowers in *Brassica* species (Mimida et al., 1999). In *Arabidopsis*, *TFL* mRNA was detected from the second or third day after induction, but expression was weak up to the point of commitment (day 7), after which it increased (Bradley et al., 1997). However, it has also been shown that *Arabidopsis tfl* mutants flower early, suggesting a role for *TFL* during vegetative development in influencing the timing of the transition to flowering.

The results of RT-PCR and expression Southerns for *METFL1* showed that in *M. excelsa*, *TFL1* is expressed throughout the process of floral development although its level may be slightly higher during the very early stages and towards the very late stages. It thus appeared that *METFL1* was upregulated during the early stages of inflorescence development and also when the inflorescence meristem resumed further vegetative development towards the end of flowering. The increased level of *METFL1* expression in both these stages implicates its importance in transition from vegetative to reproductive phase and from reproductive to vegetative phase. Studies on *Arabidopsis* mutants have shown that the *TFL1* gene product is involved in establishing the timing of phase transition during shoot development from the early inflorescence phase (characterised by nodes bearing coflorescences and leaves) and the late inflorescence phase (characterised by nodes bearing flowers) and thus it influences Controllers of Phase Switching (COPS) activity (Schultz and Haughn, 1993). Flowering time genes were considered likely to be involved in the increased expression of *TFL* that occurred at the time of commitment to flowering. The activation of *CO* led to increased expression of *TFL1* (Simon et al., 1996; Bradley et al. 1997). Results of studies by Ferrandiz et al. (2000) suggested that the *FUL, AP1* and *CAL* genes acted redundantly to control inflorescence architecture by affecting the

domains of *LFY* and *TFL1* expression as well as the relative levels of their activities in *Arabidopsis*.

A point to be considered is whether *METFL1* expression was influenced by temperature and /or photoperiod changes. It has been observed by Shannon and Meeks-Wagner (1991) that the effects of *tfl1* mutations were weakened by environmental conditions such as SD that delayed the onset of flowering. Also, all *tfl1* mutants in the study by Schultz and Haughn (1993) were responsive to environmental conditions. Assuming that at least some of the *TFL1* alleles were null, or close to null alleles, this indicated that *TFL1* was not required for photoperiodic response of COPS. Perennial ryegrass requires a cold treatment of at least 12 weeks to induce flowering. During this period a decrease in *LpTFL1* message was detected in the ryegrass apex (Jensen et al., 2001). However, upon subsequent induction with elevated temperatures and long-day photoperiods, *LpTFL1* message levels increased and reached a maximum when the ryegrass apex had formed visible spikelets. In the present study, the levels of *METFL1* were lowest during the coldest and the shortest day length period indicating temperature and/or photoperiod could be influencing *METFL1* expression.

LFY has been found to be expressed throughout plant development in *Arabidopsis,* even during the early stages of vegetative development, but its expression increases sharply around the time that flowering occurs (Blasquez et al., 1997). Under SD conditions *LFY* is expressed at initially low levels and increases gradually during the long period of vegetative growth (Blasquez et al., 1997). Very small amounts of *MEL* were detected from very young vegetative tissue of *M. excelsa* through expression Southern. Southerton et al. (1998) had observed that *ELF*, the *Eucalyptus* orthologue of *LFY*, was expressed in young leaves and leaf primordia. A comparison between young vegetative shoots and floral buds also suggested that *ELF* expression was higher in floral tissue than in vegetative tissue (Southerton et al., 1998). These findings are similar to the results that have been obtained for *MEL*.

METFL1 was found to be strongly expressed in young vegetative buds of *M. excelsa*. The

expression detected in vegetative tissue is in agreement with the reports on *TFL1* equivalents from other herbaceous plants. The *Antirrhinum* gene *CEN* and the *Arabidopsis* gene *TFL1* were shown to be homologous, which suggested that a common mechanism influenced indeterminacy in these plants. The *Arabidopsis TFL1* is also expressed during the vegetative phase, where it delays the commitment to inflorescence development and thus affects the timing of the formation of the inflorescence meristem as well as its identity (Bradley et al., 1997). The tobacco *TFL1* homologue (*NCH*) was expressed at a low level throughout plant development (Kato et al., 1998). The *SELF-PRUNING* gene, which is the *TFL1* orthologue in tomato, is expressed in shoot apices and leaves from very early stages, and later in inflorescence and floral primordia as well (Pnueli et al., 1998).

The overall morphology of an *Arabidopsis* plant depends on the behaviour of its meristems. Meristems derived from the shoot apex can develop into either shoots or flowers. The distinction between these alternative fates requires separation between the function of floral meristem identity genes and the function of an antagonistic group of genes, which includes *TFL1*. Ratcliffe et al. (1999) showed that the activities of these genes are restricted to separate domains of the shoot apex by different mechanisms. Meristem identity genes, such as *LFY*, *AP1* and *CAL*, prevent *TFL1* transcription in floral meristems on the apex periphery. *TFL1*, in turn, can inhibit the activity of meristem identity genes at the centre of the shoot apex in two ways: firstly, by delaying their upregulation, and secondly, by preventing the meristem from responding to *LFY* or *AP1*. Ratcliffe et al. (1999) suggested that the wild type pattern of *TFL1* and floral meristem identity gene expression depended on the relative timing of their upregulation.

Quantitative regulation of flowering genes could also play a very important role for proper regulation of flowering time. Expression of *CO* and *LFY* was considered to be tightly regulated so that small changes in their activity affected flowering time or shoot morphology (Pineiro and Coupland, 1998). Quantitative regulation of *LFY* expression was also important for the proper regulation of flowering time and the node at which flowers are first formed. The changes in levels of *MEL*, *MESAP1* and *METFL1* could thus have significance in the flowering process in *M. excelsa*.

The results fom the temporal expression analysis of *MEL* and *MESAP1* showed that the expresssion of both *MEL* and *MESAP1* genes could be detected as early as March in *M. excelsa* indicating that floral determination would have occurred by then, thereby confirming the hypothesis. The hypothesis that the expression pattern of floral and inflorescence meristem identity genes in woody perennials could be influenced by temperature and photoperiod could also be accepted because of the correlation between the bimodal pattern of expression of *MEL* and *MESAP1* with these environmental factors. In the case of *METFL1*, its predicted expression throughout the period of floral development was confirmed. However, it was revealed that upregulation of *METFL1* also occurred when the the inflorescence meristem resumed vegetative growth after flowering in addition to the increased levels seen at the early stages of floral development.

Chapter 5. Spatial expression patterns of floral and inflorescence meristem identity genes in *M. excelsa*

5.1 Introduction

During the transition from vegetative to reproductive growth, the vegetative meristem, which gives rise to the leaves, is transformed into an inflorescence meristem, which gives rise to floral meristems. In many species, the inflorescence meristem itself is transitional and rapidly becomes a floral meristem. In *Arabidopsis*, the inflorescence meristem is indeterminate and initiates multiple floral meristems while retaining its identity throughout reproductive development. In *Arabidopsis*, the *TFL1* gene was found to be required for normal inflorescence meristem function, and *LFY* and *AP1* for normal floral meristem function. Shannon and Meeks-Wagner (1993) presented evidence that inflorescence meristem identity was promoted by *TFL1* and that floral meristem identity was promoted by parallel developmental pathways, one defined by *LFY* and the other defined by *AP1/AP2*.

LFY, *AP1* and *TFL1* have been found to have specific regions of expression in plants. *LFY* and *AP1* are expressed predominantly in floral meristems, while *TFL1* is expressed in the inflorescence meristems. Among woody perennials, both the *AP1* homologues in *Eucalyptus* were found to be predominantly expressed in flower buds through RNA blot analysis (Kyozuka et al., 1997). *NLY*, the orthologue of *LFY* in *P. radiata*, was found to be expressed both in reproductive and vegetative meristems (Mouradov et al., 1998). *ELF1*, the *LFY* homologue in *Eucalyptus*, was strongly expressed in the early floral primordium and then successively in the primordia of sepals, stamens and carpels (Southerton et al., 1998). *PTLF*, the *P. trichocarpa* homologue of *LFY* was found to be expressed most strongly in developing inflorescences (Rottman et al., 2000). *In situ* hybridisation studies on axillary meristems in kiwifruit showed *ALF* (*LFY* equivalent) expression throughout the developing first-order buds and *AAP1* (*AP1* equivalent) expression to be localised in developing second-order meristems (Walton et al., 2001).

There are no reports of studies conducted on the spatial expression of *TFL1* equivalents in woody perennials preceding the current study. However, in *Arabidopsis*, Bradley et al. (1997) and Liljegren et al. (1999) have shown *TFL1* expression to be localised in the inflorescence meristem and to be absent from floral meristems where *LFY* and *AP1* are expressed, indicating an antagonism between *TFL1* and *LFY* and *AP1* (Shannon and Meeks-Wagner, 1993).

If the antagonism between *TFL1*, and *LFY* and *AP1* occurred in *M. excelsa* one would expect *MEL* and *MEASP1*, being floral meristem identity genes, to be expressed in the cymule and floral primordia but not in the inflorescence apex where *METFL1*, as an inflorescence meristem identity gene, would be expressed. Likewise, *METFL1* was not expected to be expressed in the floral primordia, the region of expression of *MEL* and *MESAP1*. It was also hypothesised that the expression of *MESAP1*, if it is a Class A gene, would be restricted to the outer two whorls of the developing flowers because of possible antagonism with probable *AG* equivalents in *M.excelsa*.

The technique most commonly used by other research workers for locating tissues where these genes are expressed is *in situ* hybridisation. Hence, *in situ* hybridisation was chosen as the technique for locating gene expression in this study and the present chapter details how this was done and discusses the results obtained. *In situ* hybridisation techniques allow specific nucleic acid sequences to be detected in morphologically preserved chromosomes, cells or tissue sections. Using nucleic acid probes with a stable non-radioactive label removes all the problems associated with radioactive probes such as safety requirements and scatter inherent in radioactive decay, and hence, in the present study, digoxygenin (DIG) labeled probes were used for *in situ* hybridisation.

5.2 Materials and methods

The general procedure followed for *in situ* hybridisation was a modification of the procedures of Drews et al. (1991) and Jackson (1991) and is presented as a flow chart in Figure 5.1. Each step is described in detail below.

Fixation and embedding of tissue
⇩
Sectioning and affixing sections to slides
⇩
Prehybridisation treatments
⇩
Hybridisation ⇐ Probe synthesis
⇩
Low stringency washes
⇩
RNase treatment
⇩
Low stringency and high stringency washes
⇩
Blocking
⇩
BSA wash
⇩
Antibody reaction
⇩
Antibody washes
⇩
Colour development
⇩
Rinses
⇩
Dehydration
⇩
Slide mounting
⇩
Microscopy

Immunological detection

Figure 5.1 Flow chart of operations for *in situ* hybridisation

5.2.1 Fixation of tissue

Young flower buds were randomly harvested from the five trees used for the study (Section 2.2.1) and immediately immersed in liquid nitrogen. They were stored at -80°C until ready for fixation. Three fixatives were tested for *in situ* hybridisation. They were FAA, Carnoy's fluid (compositions given in Table 2.1) and 4% paraformaldehyde in 1 x phosphate buffered saline (PBS). The fixatives were chilled on ice and buds from -80°C were transferred into them. The buds were then vacuum-fixed for 2 h. After replacing with fresh ice-cold fixatives, the buds were fixed for a further period of 7 h at 4°C.

5.2.2 Dehydration

Following fixation, the buds were dehydrated through an ethanol series (15, 30, 50,70, 85, 95 and 100% ethanol) with at least 1.5-2 h in each solution. The buds were then left in 100% ethanol at 4°C overnight. The next morning, buds were dehydrated in renewed 100% ethanol for 2 h at room temperature.

5.2.3 Clearing

Dehydrated tissue was subsequently permeated with Histoclear. The 100% ethanol was replaced sequentially with 75% ethanol: 25% Histoclear, 50% ethanol: 50% Histoclear, 25% ethanol: 75% Histoclear and 100% Histoclear with tissues being left in each solution for 1.5-2 h. Histoclear (100%) treatment was repeated 2 times further.

5.2.4 Infiltration

Molten paraplast was prepared by placing paraplast chips in a 60°C oven overnight. Paraplast chips were added to the universal bottles with the buds in Histoclear, until the volume was increased by 25% and the bottles were incubated in a water bath at 42°C for 30 min. This process was repeated three times with more paraplast until the volume was doubled. The samples were then put in a 60°C oven to melt all the paraplast. The paraplast: Histoclear solution was then poured off and replaced with fresh, molten paraplast. This solution was changed after 3-4 h and the samples were incubated at 60°C overnight in molten paraplast. The tissue was infiltrated with paraplast at 60°C. The

paraplast was changed six times with at least 4 h in each solution.

5.2.5 Embedding and sectioning

RNase free baked metal blocks and embedding plastic blocks were used. Fresh, molten paraplast was poured into the metal blocks, buds were oriented in the paraplast and the embedding plastic blocks were put on top and pressed down. More molten paraplast was poured on top and the embedded tissue was allowed to harden on the cold plate on the embedding machine (Leica EG1160). The embedded buds were stored at 4°C and subsequently sectioned to a thickness of 8-10 µM. The procedure followed was as described in Procedure 5 of Section 2.2.2.

5.2.6 Probe Synthesis

Probes were labeled with digoxygenin-UTP and *in vitro* transcribed from cloned inserts of *MEL*, *MESAP1* and *MTTFL1* in pGEM-T vectors. The plasmids (1 µg) with the inserts were linearised using *Sph1* or *Sal1* restriction enzymes (Roche). The templates were purified using the Concert^{TM} Rapid PCR purification system (Life Technologies) and sense (- strand) and anti sense (+ strand) probes were synthesised using a DIG RNA labeling kit (Roche). The transcription reaction mix comprised the following components:

Water	12 µl
10x transcription buffer	2 µl
RNase inhibitor (20 U µl^{-1})	1 µl
NTP labeling mix	2 µl
[10x (10mM ATP, CTP and GTP	
and 6.5 mM UTP, 3.5mMDIG-11-UTP)]	
Linearised and purified template DNA(1µg µl^{-1})	1 µl
T7 or SP6 RNA polymerase (20 U µl^{-1})	2 µl
	20 µl

The reaction mix was incubated at 37°C for 4-6 h.

5.2.7 DNase treatment

RNase free DNase 1 (2 µl of 10 U µl^{-1}) was added to the 20 µl reaction mix to remove DNA from the final products and the mix was incubated at 37°C for 30 min. Following this, the reaction was stopped by adding 2 µl of 200 mM EDTA.

5.2.8 Probe precipitation

The transcribed and DIG labeled RNA probes were precipitated by adding 2.5 µl of 4 M LiCl and 75 µl of ethanol. The mix was incubated overnight at 20°C and the probes pelleted by centrifugation. The pellets were washed twice in 70 % ethanol, vacuum dried and resuspended in 50 µl of RNase free water for 2 h on ice. Aliquots (4 µl) were electrophoresed on RNase free agarose (1%) gels to check recovery. The probes were stored frozen at -80°C until ready for hydrolysis or hybridisation.

5.2.9 Probe hydrolysis

Small probes (50-100 nt) are believed to give higher signals (Drews et al., 1991) than larger probes (>150 nt). Hence half of the probes of *MEL* and *MESAP1* were hydrolised in carbonate buffer (pH 10.2) to reduce their size. To 25 µl of the RNA probes, 10 µl of 200 mM NaHCO$_3$ was added first, followed by 15 µl of 200 mM Na$_2$CO$_3$. The mixture was left at 60°C for 10 min and the reaction was then stopped by adding 5 µl 1 M NaOAc, pH 4.7. Probes were then precipitated by adding 5µl of 4 M LiCl and 150 µl of ethanol and incubated over night at -20°C. The next morning they were spun down and washed twice in 70% ethanol, vacuum dried and re-suspended in 25 µl of RNase-free water.

5.2.10 Probe quantification

Each of serial dilutions of the synthesised probes (1 µl) and dilutions of DIG labeled control RNA (1 µg 10 µl^{-1}) from the RNA labeling kit were spotted on and UV cross-linked to a Boehringer Mannheim nylon membrane. Probes were then quantified by colorimetric detection as described in the non-radioactive *in situ* hybridisation application manual (Boehringer Mannheim, 1996).

5.2.11 Prehybridisation treatments

Removal of paraplast and hydration: This was a necessary step to make tissues amenable for proteinase K digestion. Slides of tissue sections prepared as in Section 5.2.5 were taken through the following solutions for removal of the paraplast and for hydration. Histoclear→Histoclear→100% ethanol→100% ethanol→ 95% ethanol→85% ethanol→70% ethanol→60% ethanol→30% ethanol→15% ethanol →water→water. Slides were immersed in Histoclear for 10 min each time and in the ethanol series and water for 1 min each.

Proteinase K digestion: This step was vital for making mRNAs in tissues accessible to the probes. After hydration the slides were incubated for 35 min at 37°C in 1 μg ml^{-1} proteinase K in pre-warmed proteinase K solution. Subsequently, the slides were rinsed for 2 min in 0.2% glycine in 1 x PBS and washed twice with 1 x PBS followed by a rinse in RNase-free water.

Acetylation reaction: This reaction was used to acetylate any remaining positive charges in the tissue or on the slides (Jackson, 1991) and to reduce background. Slides were incubated for 5 min in freshly prepared acetic anhydride solution.

Washes and dehydration: The slides were then washed in 2 x SSC and dehydrated once again through an ethanol series to 100% ethanol using the same ethanol solutions as used earlier except for the 100% ethanol. Subsequently, the slides were vacuum-dried for at least 1 h in a dessicator.

5.2.12 Hybridisation and washes

Probes were diluted to 200 ng ml^{-1} per kb of probe complexity in hybridisation solution. The solution was warmed to 42°C in a water bath for easy application and 150-200 μl was applied per slide to wet all tissue. The slides were placed elevated in a preheated humid plastic box and incubated overnight at 42°C.

Low stringency washes and RNase treatment: After hybridisation, slides were washed three times in 2 x SSPE for 10 min each time. Following this, they were incubated for 30 min at 37°C in 5 μg ml^{-1} RNaseA in RNase buffer.

Post-RNase low and high stringency washes: After RNase treatment, the slides were rinsed twice in 2 x SSPE and then washed for a further period of 30 min with 2 x SSPE. This was followed by two high stringency washes in 0.2 x SSPE at 57°C for 45 min each and two washes in 0.2 x SSPE at 37°C for 5 min each. A final rinse was given with 1x PBS. Slides were stored over-night at 4°C in 1 x PBS or were used for immunological detection straight away.

5.2.13 Immunological detection

Blocking and washing: Slides were incubated in 1 x blocking reagent (Roche) in maleic acid buffer for 45 min. Following this, they were washed for 45 min in Bovine Serum Albumin (BSA) wash solution.

Antibody reaction: Anti- DIG- AP, Fab fragments from Roche (150 U 200 μl^{-1}) was diluted in BSA wash solution (1:600 to 1:3000 dilution). The BSA wash solution from the previous step was replaced with this antibody solution and the slides were incubated in it for 2 h with gentle agitation.

Post-antibody BSA washes: The slides were removed from antibody solution and put in BSA wash solution and incubated for 30 min with gentle agitation. Thereafter the solution was replaced with fresh BSA wash solution and this was repeated for a total of 3 BSA washes of 30 min each.

Colour development: The slides were washed twice for 5 min each in TNM-50 buffer. A colour solution was prepared containing 10 ml of TNM-50 buffer, 45 μl of nitroblue tetrazolium salt (NBT) solution and 35 μl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution. The colour subtrate was applied on top of the sections on the slides and the slides were incubated at room temperature in the dark for > 36 h.

Slide mounting: When the colour reaction was completed, slides were washed 3 times for 5 min each in T_{10} E_1 Buffer and then dehydrated through an ethanol series (water→70% ethanol→95% ethanol→100% ethanol→ 100% ethanol). They were then immersed in Histoclear or xylene briefly, DPX mountant was applied on top and coverslips put on. Slides were then dried in a fume hood for at least 2 h. Slides were viewed under a light microscope and images recorded using a digital camera.

Acetic anhydride solution : 1.25 ml 500 ml^{-1} of 100 mM triethanolamine (pH 8)

BCIP solution : 50 mg BCIP ml^{-1} in 100% dimethylformamide

10 x Blocking reagent : 10% blocking reagent in maleic acid buffer

BSA wash solution : 1% BSA, 0.3% Triton-x-100, 100 mM Tris-HCl (pH 7.5), 150 mM NaCl

Hybridisation solution : 50% formamide, 300 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 5% dextran sulfate, 1% blocking reagent, 150 µg ml^{-1} tRNA

Maleic acid buffer : 100 mM Maleic acid (pH 7.5), 150 mM NaCl

NBT solution : 75 mg NBT ml^{-1} in 70% dimethylformamide

10 x PBS : 1.3 M NaCl, 30 mM NaH$_2$PO$_4$, 70 mM Na$_2$HPO$_4$

Proteinase K solution : 100 mM Tris (pH 7.5), 50 mM EDTA

RNase buffer : 10 mM Tris (pH 7.5), 1 mM EDTA, 500mM NaCl

20 x SSPE : 0.2 M NaH$_2$PO$_4$, 20 mM EDTA, 3 M NaCl

TNM-50 buffer : 100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM MgCl$_2$

T$_{10}$ E$_1$ buffer: 10 mM Tris (pH 8.0), 1 mM EDTA

5.3 Results

5.3.1 The ideal fixative

Among the three fixatives tried in the initial experiments, it was found that paraformaldehyde gave the best results with the strongest signals. FAA was found to cause relatively more browning of tissues, and signals were also weak. Although sections with the best morphological preservation could be obtained with Carnoy's fluid, the signal output from *in situ* hybridisation was nil. Consequently, paraformaldehyde was chosen as the ideal fixative for all further experiments.

It was also revealed that *in situ* hybridisation could be successfully achieved using plant tissue frozen at -80°C and fixed subsequently, as good signals were obtained from *M. excelsa* buds with this procedure.

5.3.2 Yield of probes

The 20 µl labeling reactions yielded 5-10 µg of DIG-labeled RNA probes as estimated semi-quantitatively through the procedure outlined in Section 5.2.10. Hydrolysis of the

probes seemed to reduce the final yield of probes, and signals obtained also weakened in the case of *MEL* and *METFL1*. Hence these probes were not hydrolised for subsequent hybridisations.

5.3.3 Spatial expression patterns of *MEL*, *MESAP1* and *METFL1*

Expression of *MEL* was detected as a dark purple-blue colour on the sides of the apex of the inflorescence in the very early stages of inflorescence development in May (Figure 5.2 A). Expression was seen on the cymule primordia as they were formed in May-June (Figure 5.2 B) and it was not seen on the inflorescence apex at this time. From late June to August, little expression of *MEL* could be detected. The slight expression found at this stage was in the in the sub-apical region of the cymule primordia as a small area where bracteoles were differentiating (Figure 5.2 C). As the cymules developed (August), expression of *MEL* disappeared from the inflorescence apex and was localised on the three floral primordia emerging within each cymule (Figure 5.4 A) and on the subtending bracteoles where it was mainly seen in the axils (Figures 5.3 A and B). Expression was detected in the inner walls of the cup-like structures formed by the developing floral primordia (Figures 5.3 A and B). No expression was detected on the stalk-like structures supporting the developing floral primordia. As the floral primordia developed, *MEL* expression was seen in the sepals and petals from where it moved into the stamens and the inner regions of the gynoecium. By the end of September, strong expression of *MEL* was detected in the anthers and the ovules inside the ovary (Figures 5.4 B and C). No expression was detected in sections probed with the sense probe (Figure 5.4 D).

Unlike *MEL*, *MESAP1* expression was not detected in cells of the inflorescence apex at any time (Figure 5.5 A). However, its expression was detected on the cymule primordia as they formed (May-June) although the signal was weaker than that of *MEL* at this stage (Figure 5.5 B and C). In early August the expression of *MESAP1* was confined to the differentiating bracteoles in the cymule primordia (Figure 5.5 D). Its expression was similar to that of *MEL* and as strong in the floral primordia that formed from the cymule primordia in August (Figure 5.5 E) and its expression was absent from the inflorescence apex at this phase as well.

CP = Cymule
     primordia
IA = Inflo-
     rescence apex
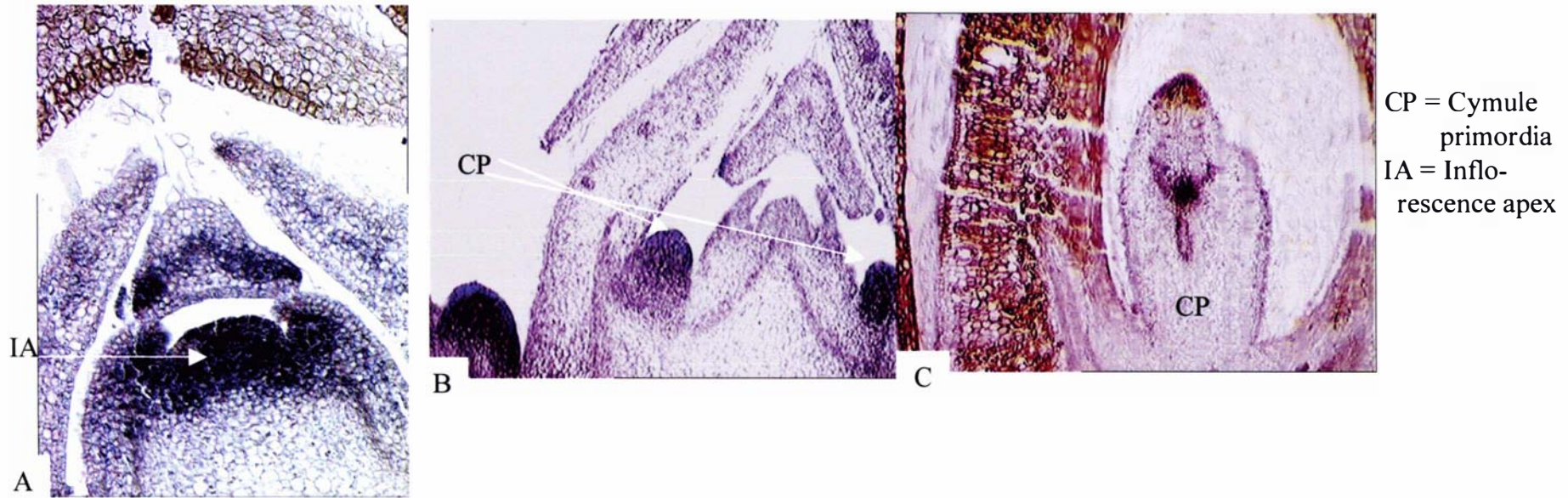
Figure 5.2 Spatial expression patterns of *MEL* during early stages of floral development. (A) Expression of *MEL* in the apex of the inflorescence in May at the time of floral initiation. (B) Expression of *MEL* in cymule primordia during early June. (C) Expression of *MEL* confined to a small area in the sub-apical region of the cymule primordia in late June.
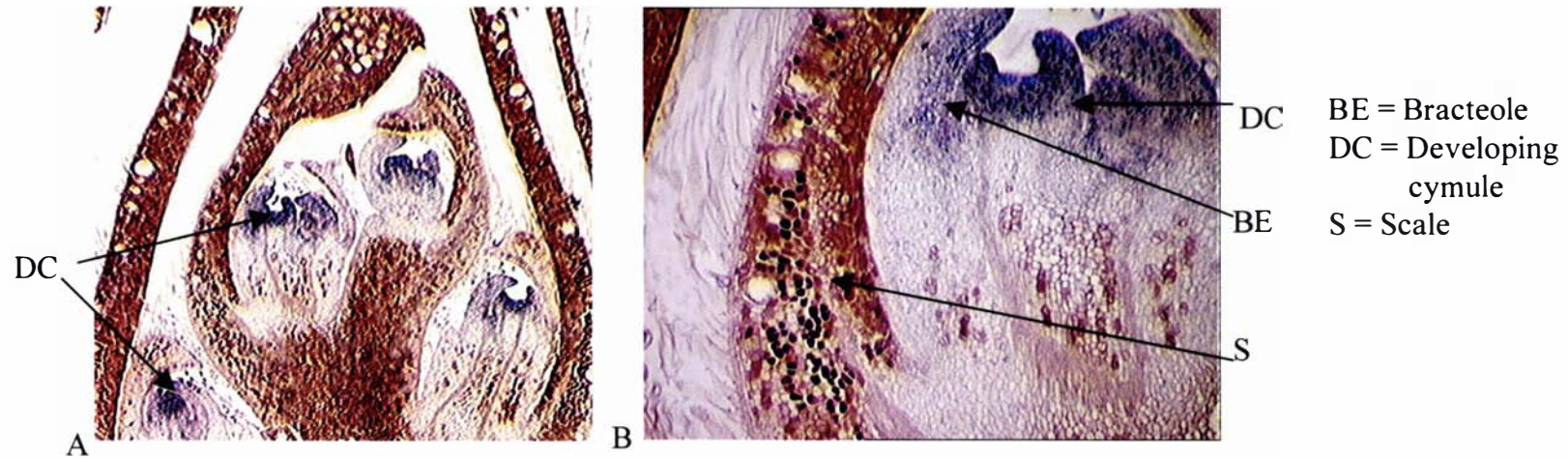
Figure 5.3 Spatial expression pattern of *MEL* during cymule development in August.
(A) *MEL* expression in the floral primordia forming in each cymule and in the bracteoles but not in the cymule stalks. (B) *MEL* expression also seen near the axils of scales subtending the developing cymule and in the bracteoles and floral primordia.

AN = Anthers
DC = Developing
      cymule
IA = Inflorescence
      apex
P = Petals
SE = Sepals
O = Ovule
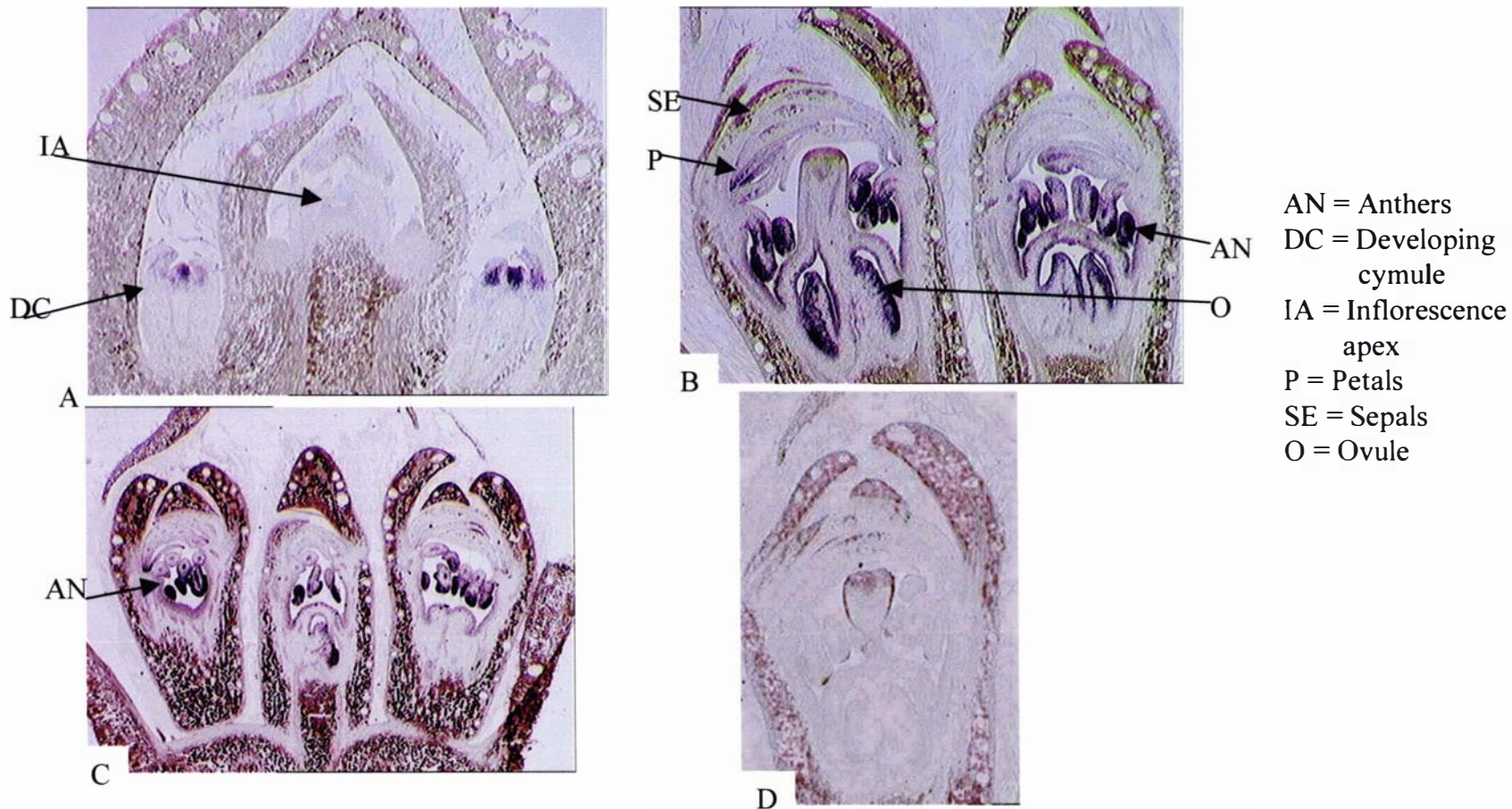
Figure 5.4 Spatial expression pattern of *MEL* during later stages of floral development. (A) *MEL* expression in the three flowers forming in each cymule in August and no expression in the vegetative inflorescence apex. (B) and (C) Strong *MEL* expression in the anthers and the gynoecium and ovules and slight expression in the sepals and petals in late September. (D) Flower probed with a sense probe of *MEL* (control).
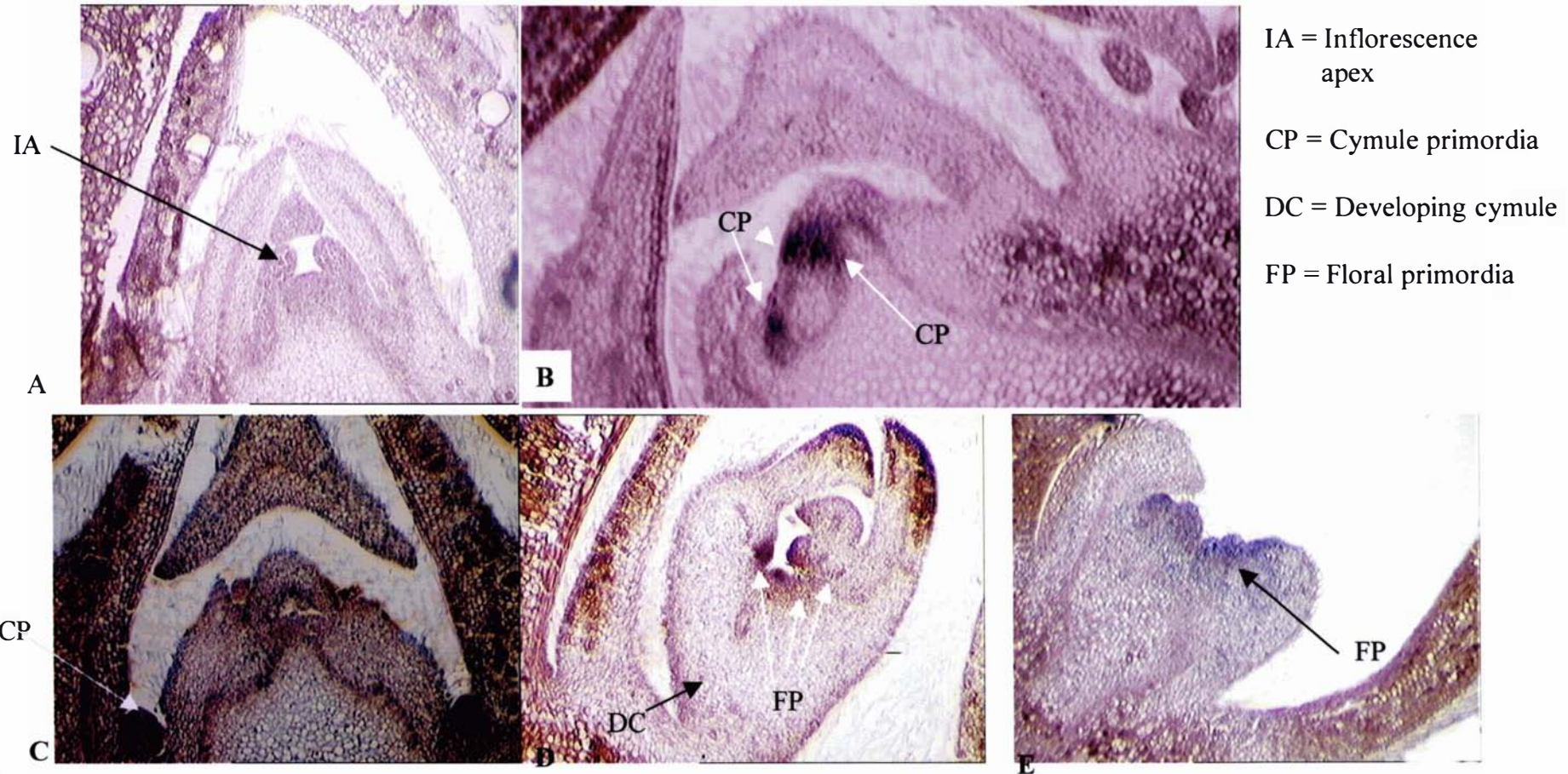
Figure 5.5 Spatial expression pattern of *MESAP1* during early stages of floral development. (A) No expression of *MESAP1* in apex in May. (B) Cross section showing *MESAP1* expression in May localised on periphery of apex in points where cymule primordia are initiated. (C) *MESAP1* expression in cymule primordia in early June. (D) *MESAP1* expression in early August when bracteooles and flowers start differentiating in the cymule primordia. (E) *MESAP1* expression confined to floral primordia in the cymule in late August

No expression of *MESAP1* was detected on the stalk-like structures supporting the developing floral primordia. Expression could be detected on the subtending bracteoles as well and was mainly concentrated in their axils. As the floral primordia developed further, *MESAP1* expression was seen in the developing sepals, petals and the perianth (September) to which regions it was restricted (Figures 5.6 A, B and C). Little or no *MESAP1* expression was detected in the anthers, the gynoecium or the inner wall of operculum. No expression was detected in sections probed with the sense probe (Figure 5.6 D).

The expression of *METFL1* was detected in a small group of cells in the inflorescence meristem. This group of cells was found to be located in the sub-apical region on the inflorescence meristem (Figures 5.7 A, C and D). No expression of *METFL1* could be detected anywhere on the floral meristems. No expression was detected in sections probed with the sense probe (Figure 5.7 B).

5.4 Discussion

A successful protocol for *in situ* hybridisation was developed for recalcitrant species and in tissues with high levels of phenolics. Southerton et al. (1998) had reported from *in situ* hybridisation studies on *Eucalyptus* tissues that these tended to stain light brown during fixation, noticeably in oil glands, epidermal cells and stigmatic cells of more mature flowers. They had also reported that fully developed floral buds, that were quiescent over winter and flowered in the following spring, were extremely difficult to section and contained high levels of phenolics and oils that interfered with proper *in situ* development. However, these authors had used FAA as the fixative. From the current study, it has become clear that paraformaldehyde was a superior fixative, causing minimal browning of tissues and at the same time enabling strong signals.

In situ hybridisation was also successfully carried out on *M. excelsa* buds that had been stored at -80°C. Bearing in mind that soft tissues such as maize meristems also sectioned well after fixation and embedding from storage at -80°C (Section 2.2.3), it can be
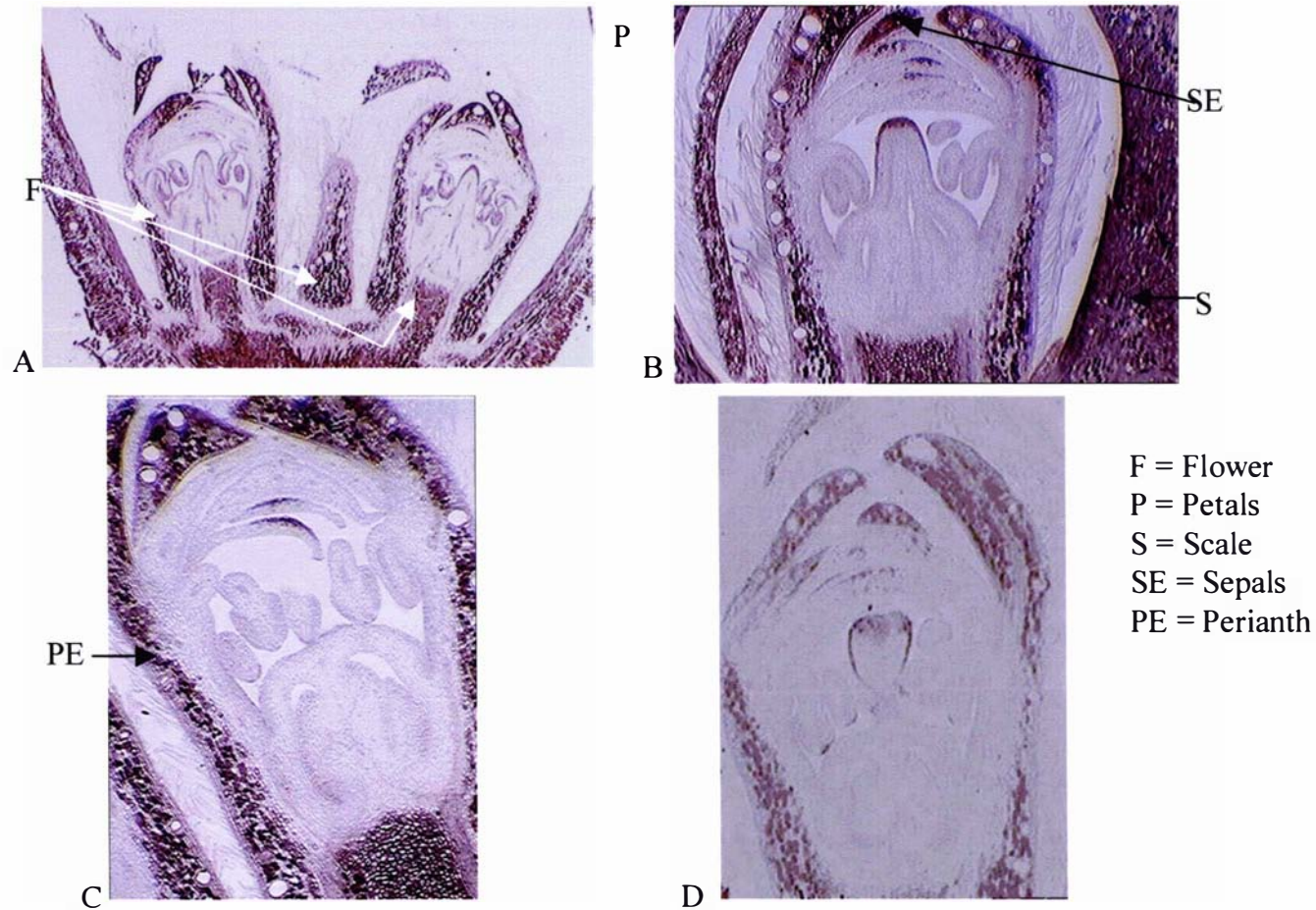
Figure 5.6 Spatial expression pattern of *MESAP1* during later stages of floral development. (A) A cymule showing *MESAP1* expression in the perianth, sepals and petals of the three flowers in late September. (B) A flower with *MESAP1* expression in the perianth, sepals, petals and axil of scale. (C) A flower with *MESAP1* expression confined to the perianth, sepals and petals. No expression of *MESAP1* in the anthers or gynoecium. (D) Flower probed with sense probe of *MESAP1* (control).

DC = Developing cymules

IA = Inflorescence apex

DS = Differentiating scale
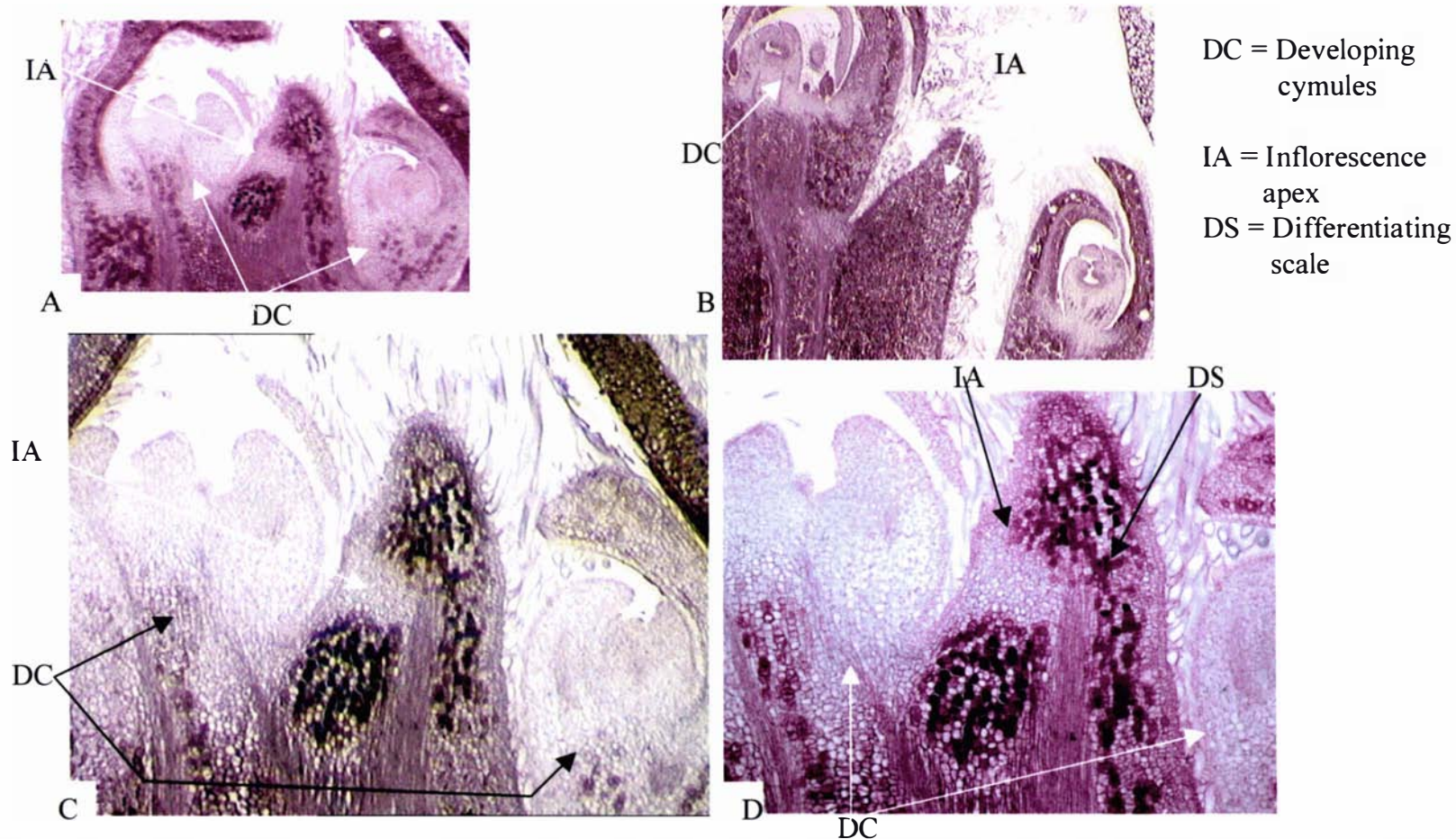
Figure 5.7 Spatial expression pattern of *METFL1*. (A), (C) and (D). Expression of *METFL1* detected with anti-sense probe of *MTTFL1* in the sub-apical region of the inflorescence meristem and the differentiating scale in mid August. No expression of *METFL1* was detected on the cymules or floral primordia. (B) Tissue probed with sense probe of *MTTFL1* (control).

concluded that this procedure could be universal in application. This means that tissues could safely be stored without mRNA degradation and loss of signal, and *in situ* hybridisations could be carried out on them at one's convenience, when the protocols had been refined and optimised to suit that tissue. This is especially useful when studying aspects such as flowering in perennials when it might be necessary to wait for a year at least to obtain samples across a full season. The clarity achieved with *in situ* hybridisation enabled a detailed study of the spatial expression patterns of *MEL*, *MESAP1* and *METFL1* and a comparison made with their counterparts in other species.

MEL expression was also detected in the bracts and the bracteoles apart from floral primordia in buds of *M. excelsa* (Figure 5.3 B). In this respect also, *MEL* was similar to *FLO* as *FLO* expression has also been detected in bracts and early floral meristems (Ma, 1998).

Expression of *MEL* in the buds was in a pattern very similar to *ELF* expression in two *Eucalyptus* species as observed by Southerton et al. (1998). In *E. macrandra*, *ELF* was first detected at multiple foci corresponding to the developing floral primordia before they had enlarged into distinct floral buds that burst through the covering bracts. Expression was observed briefly in sepal primordia and then in petal primordia. In *E. globulus*, strong expression was observed in sepal primordia that arose on the sides of the floral meristem and *ELF* expression declined in the sepals as they enlarged. *ELF* expression shifted to the petal primordia at that stage and when petal primordia enlarged, expression appeared in the centre of the floral meristem, where the carpels were formed, and in the stamen primordia. After this time *ELF* expression declined in the petals and was not observed again in the operculum tissues. Expression was, however, maintained during stamen development and in the region of the developing gynoecium, particularly in the developing ovules. *ELF* expression was not detected in fully developed floral buds that lay quiescent over winter.

The fact that *MEL* was expressed sequentially from the floral meristems to the outer whorls of the flowers (Figures 5.2, 5.3 and 5.4 A), and finally in the inner whorls such as androecium and gynoecium (Figures 5.4 B and C) indicated a role for this gene in the

development of all the floral organs and perhaps for the normal function of all the other organ identity genes as well. Proper development of petals and stamens in *Arabidopsis* flowers required the activities of *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), whose transcripts could be detected in the petal and stamen primordia (Medard and Yanofsky, 2001). Localised expression of *AP3* and *PI* required the activities of at least three genes: *APETALA1* (*AP1*), *LEAFY* (*LFY*), and *UNUSUAL FLORAL ORGANS* (*UFO*). It has been proposed that that *LFY* specified competence and *UFO* provided spatial cues for *AP3* and *PI* expression in the developing flower (Medard and Yanofsky, 2001).

The shifts in expression also probably indicates that *MEL* has to be present in each specific region to activate the next set of genes for the particular development of those organs. Sector analysis to study (Sessions et al., 2000) whether *LFY* could participate in cell-to-cell signaling between and within different layers of the floral meristem showed that *LFY* signaled equally well from all layers and had substantial long-range action within layers. Non-autonomous action of *LFY* was accompanied by movement of the protein to adjacent cells, where it directly activated homeotic target genes. This was similar but not identical to *FLO* which was found to be non-autonomous between layers but exhibited some cell autonomy within cell layers (Carpenter and Coen, 1995). It could be that the cell-to-cell signaling capacity of *MEL* was not as efficient as *LFY* or *FLO*. It could also be that the much larger floral structures in *M. excelsa* compared to *Arabidopsis* or *Antirrhinum* necessitated *MEL* expression to shift from organ to organ as signals could not be transmitted over that long distances.

It appeared that *MESAP1* expression was restricted to the developing cymule primordia, the subtending bracteoles and the perianth region comprising of the sepals and petals. Even though the probe used included part of the MADS domain, it did not hybridise in tissues in the inner regions of the flower buds (such as anthers and ovary), where other MADS box genes are expressed, was further confirmation of its fidelity, which had already been established by Southerns. As the expression of *MESAP1* was confined to the outer whorls, it implied that *MESAP1* would be acting in a similar way to *AP1* in which the transcriptional activation of downstream genes for specifying the inner whorls was by

regulating the spatial domains of *AP3* and *PI* expression through *UFO* (Medard and Yanofsky, 2001). *AP1* had only limited non-autonomous effects, apparently mediated by downstream genes because activation of early target genes by *AP1* was cell-autonomous (Sessions et al., 2000).

The spatial expression pattern of *MESAP1* was very similar to the findings from other *in situ* studies on *AP1* and its equivalents in other plants. *AP1* and *SQUA* are initially expressed in the floral meristems and expression could not be seen in developing stamens even as soon as they were microscopically visible (Mandel et al., 1992). While *AP1* was not found to be expressed in carpels, the *SQUA* transcript was found to be present in the basal part of the carpel which gives rise to the ovary wall but not the style and the stigma. *AP1* and *SQUA* were expressed in the pedicel and *SQUA* in the receptacle (Huijser et al., 1992; Mandel et al., 1992). *MESAP1* expression was more similar to that of *AP1* than *SQUA* as it was not detected in the carpels. The fact that *MESAP1* expression was not detected on the inflorescence apex indicated its antagonism with *METFL1* similar to the antagonism between *AP1* and *TFL1*.

Among woody perennials, RNA blot analysis to study the expression patterns of the *AP1* equivalents in *Eucalyptus* showed that both *EAP1* and *EAP2* transcripts occurred in the operculum, which in *E. globulus* is formed from the outer two whorls of the floral organs Kyozuka et al. (1997). A relatively low level of *EAP1*, but not *EAP2* expression was detected in mRNA from the anthers. Both the genes were expressed in the floral receptacle which is prominent in the *Eucalyptus* flower. In *A. deliciosa*, Walton et al. (2001) found that *AAP1* expression was localised in meristems that could ultimately differentiate flowers. In sections where sepals had differentiated in a developing flower bud, transcript accumulation was concentrated in the region where petals were about to be initiated.

The expression pattern of *MESAP1* showed that it was a Class A gene. The fact that *MESAP1* was expressed only in the outer two whorls of the flowers indicated that as a Class A organ identity gene, it could be involved in the formation of sepals and petals, restricting Class C gene expression to the inner whorls. The model for control of floral

organ identity (Bowman et al., 1991; Coen and Meyerowitz, 1991) states that the expression of Class A and Class C genes are mutually exclusive, such that each gene class restricts the expression of the other.

METFL1 expression in *M. excelsa* could be detected even with the use of a heterologous probe (*MTTFL1*) which was the partial *TFL1* orthologue isolated from *M. collina* 'Tahiti'. This was possible because of the high homology between *METFL1* and *MTTFL1*. Southern hybridisation with *MTTFL1* on *M. excelsa* DNA had shown that the probe hybridised only to one gene, indicating that it could safely be used as a probe for *in situ* hybridisation.

METFL1 was found to be expressed in a group of a few cells located not at the tip of the inflorescence meristem, but in a sub-apical region in the inflorescence meristem. Bradley et al. (1997) and Liljegren et al. (1999) had also found that *TFL1* had a similar spatial expression pattern in *Arabidopsis*. As has been found in the case with *TFL1* in *Arabidopsis*, *METFL1* expression was also totally absent from all floral primordia. The localisation of expression of *METFL1* showed that it could be an inflorescence meristem identity gene but not a floral meristem identity gene.

Having observed where *MEL*, *MESAP1* and *METFL1* are expressed, it is worth speculating on the interactions between these genes. Shannon and Meeks-Wagner (1993) proposed that the *TFL1* function was mutually antagonistic with both *LFY* function and *AP1/AP2* function. As a consequence, *TFL1* was considered not to influence floral meristem development, while *LFY*, *AP1* and *AP2* were considered not to influence inflorescence meristem development. They also proposed that the interaction between *TFL1* and *LFY* occurred independently of the interaction between *TFL1* and *AP1/AP2*.

Liljegren et al. (1999) showed that vegetative shoot meristems maintained an identity distinct from that of floral meristems, in part through the action of genes such as *TFL1*, which barred *AP1* and *LFY* expression from the inflorescence meristem. This negative regulation could be mutual because *TFL1* expression was downregulated in plants constitutively expressing *AP1*. Liljegren et al. (1999) proposed that the normally sharp

phase transition between the production of leaves with associated shoots, and the formation of flowers, which occurs upon floral induction, is promoted by positive feedback interactions between *LFY* and *AP1*, together with negative interactions of these two genes with *TFL1*.

The spatial expression patterns of *MEL*, *MESAP1* and *METFL1* also confirmed that the fragments of these genes cloned from *M. excelsa* were the partial orthologues of *LFY*, *AP1* and *TFL1* as the expression patterns showed that these genes could also be interacting in a similar manner in *M. excelsa*. *METFL1* expression was localised exclusively in the inflorescence meristem, and *MEL* and *MESAP1* expression was detected in the floral meristems. It may be presumed that similar antagonistic reactions could be restricting their regions of expression. However, it was interesting to see that in the early stages of floral development in May, *MEL* was expressed at the inflorescence apex where it was not expected to be seen if it was strictly antagonistic to *METFL1*. In this respect, the interaction was very similar to that seen between *FLO* and *CEN* in *Antirrhinum* rather than the interaction between *LFY* and *TFL1* in *Arabidopsis*. Although the *Antirrhinum CEN* gene is the homologue of *TFL1* (Bradley et al., 1996; 1997) its interaction with *FLO* (the *LFY* homologue) is not the same as that between *TFL1* and *LFY*. Bradley et al. (1996) observed that *CEN* was dependent on *FLO* for its initial expression and subsequently *CEN* represses *FLO* expression at the centre of the inflorescence meristem, similar to the *TFL1* function.

The hypothesis that *MEL* and *MESAP1* were floral meristem identity genes was supported as they were expressed in floral primordia and floral tissues. The hypothesis that *MESAP1*, as a Class A gene, would be expressed only in the outer two whorls was also confirmed. The characteristic expression pattern of *METFL1* in the sub-apical region of the inflorescence meristem also confirmed it to be involved with inflorescence meristem specification. Its absence from floral primordia confirmed the hypothesis that it would not be expressed in those regions with high expression levels of *MEL* and *MESAP1* due to mutual antagonism.

Chapter 6. Interaction of floral meristem identity genes and gibberellins

6.1 Introduction

Genes involved in gibberellin (GA) biosynthesis and GA signal transduction have been suggested to form a distinct promotion pathway in floral transition (Koornneef et al., 1998; Simpson et al., 1999). In short day (SD) conditions, the *ga1-3* (*gibberellin requiring 1-3*) mutation, which has severely reduced endogenous GA levels, abolished the upregulation of *LFY* and prevented floral transition in *Arabidopsis*. The exogenous application of GA restored both (Wilson et al., 1992; Blasquez et al., 1998). *LFY* expression in the vegetative phase is believed to be regulated by both photoperiod and GA.

GAs vary in their florigenic activity, although for woody angiosperms including *M. collina*, applied GAs, especially GA_3 and GA_7, are generally inhibitory to flowering (Looney et al., 1985; Looney and Pharis, 1986; Oliveira and Browning, 1993; Clemens et al., 1995). Further, Moncur et al. (1994) and Moncur and Hasan (1994) have observed that *Eucalyptus nitens* treated with paclobutrazol, had a reduced concentration of endogenous GAs and enhanced umbel production and reproductive activity. The response to GAs in woody species is obviously in contrast to the response of herbaceous plants. The response of *MEL* to application of GA_3 was therefore of interest. Consequently, juvenile *M. excelsa* plants were grown in a glasshouse and treated with growth retardants with or without GA_3. As *MEL* was found to be expressed in young vegetative tissue, although at a low level (Figure 4.3 A), plants were treated with growth retardants to reduce endogenous GA levels and /or applied with GA_3 to enhance GA levels. As expression of *AP1* is regarded as indicative of floral commitment, and upregulation of *LFY* has been shown to cause upregulation of *AP1* (Ma, 1998), the expression of *MESAP1* was also monitored.

6.2 Materials and methods

Micropropagated plants of *M. excelsa* 'Lighthouse' supplied in 70 mm liner pots were

transplanted into 1.5 L containers containing peat/pumice mix amended with fertilisers, and grown in a temperature controlled glasshouse from August to December 1998. Plants were grown with four main branches arising from the two uppermost nodes. Apices were pinched to promote growth of distal lateral buds. The plants were assigned randomly to five blocks. Three growth regulator treatments (prohexadione, paclobutrazol and water control) in factorial combination with two GA (GA$_3$ application and water control) treatments were assigned randomly to the population of plants within each block. There were four replicates (plants) of each treatment within each block.

Shoot growth of the plants was measured as the length of the two distal lateral buds from the base (leaf axil) to the tip of the leaves emerging from them. Growth regulator treatments were then applied. Prohexadione and paclobutrazol were initially applied as foliar sprays at 500 mg a.i. l^{-1}. The growth of buds was monitored for two weeks but in this time period growth was not retarded. Hence, a second round of growth regulator applications was given. This time 1000 mg l^{-1} of prohexadione was sprayed to foliage and paclobutrazol (10 mg a.i. per 100 ml of water) was applied as a soil drench. Monitoring of bud growth was continued and when it was evident that growth had slowed, two foliar sprays of GA$_3$ (5 mg a.i. plant^{-1}) were applied seven days apart. Bud growth was monitored afterwards and all the buds from the plants were harvested into liquid nitrogen three days after the final spray of GA$_3$. The buds were transported to the laboratory where they were stored at -80°C until RNA extraction. The quantity of buds that could be obtained was small, and hence bud samples were pooled within treatments to give samples for water control, prohexadione, paclobutrazol, water + GA$_3$, prohexodione + GA$_3$ and paclobutrazol + GA$_3$.

RNA was extracted from these bud samples, using the hot-borate method as described in Section 3.2.1.2. and quantified using a spectrophotometer. RT-PCR was carried out as described earlier in Section 3.2.4 from 1 µg of RNA with the *LFY* specific primers (Section 3.2.5) for *MEL* and *AP1* specific primers (Section 4.2.3.2) for *MESAP1*, but at annealing temperatures of 50°C. PCR was also carried out for actin at the same time with actin primers (Section 4.2.3.1) from the same cDNAs and used as the loading control. Aliquots

(12 µl) of the RT-PCR products were electrophoresed on a 2% agarose gel and visualised after EtBr staining. The RT-PCR products (4 µl) of *MEL* were also electrophoresed on a 2% gel and expression Southern analysis was carried out as described in Section 4.2.5.

6.3 Results

Growth of shoots over the study period is presented in Figure 6.1. It was observed that the growth of plants sprayed with prohexadione and paclobutrazol was slower than the control plants (water). Plants treated with growth retardants gave lower values for bud growth with or without GA_3 application compared to the control plants (water). At the time of the final measurements, the lowest growth was recorded by plants treated with prohexadione, followed by those treated with paclobutrazol. Plants treated with GA_3 recorded higher growth than those without GA_3 sprays. However, it was also observed that plants treated with growth retardants had resumed growth again after the initial growth retardation, even without the application of GA_3.

RT-PCR and expression Southern showed that *MEL* was upregulated compared to the water control by both the growth retardants as well as by GA_3 (Figure 6.2). *MESAP1* expression was not detected in any of the treatments (data not shown).

6.4 Discussion

It was surprising to see *MEL* upregulation with the application of growth retardants as well as with GA_3. The upregulation of *MEL* with GA_3 application is supported by work reported by Blasquez et al. (1998). Concurrent with the molecular analysis, Clemens (1999) measured endogenous GAs (unpublished data shown in Appendix V) in buds harvested from the treated plants. Plants treated with prohexadione showed unexpectedly high levels of endogenous GAs which were sometimes even higher than the water control. Paclobutrazol-treated plants, however, recorded lower values for endogenous GAs except for GA_{19} which was more than double the amount in the water control. Thus, although the upregulation of *MEL* in plants treated with growth retardants could be explained by the
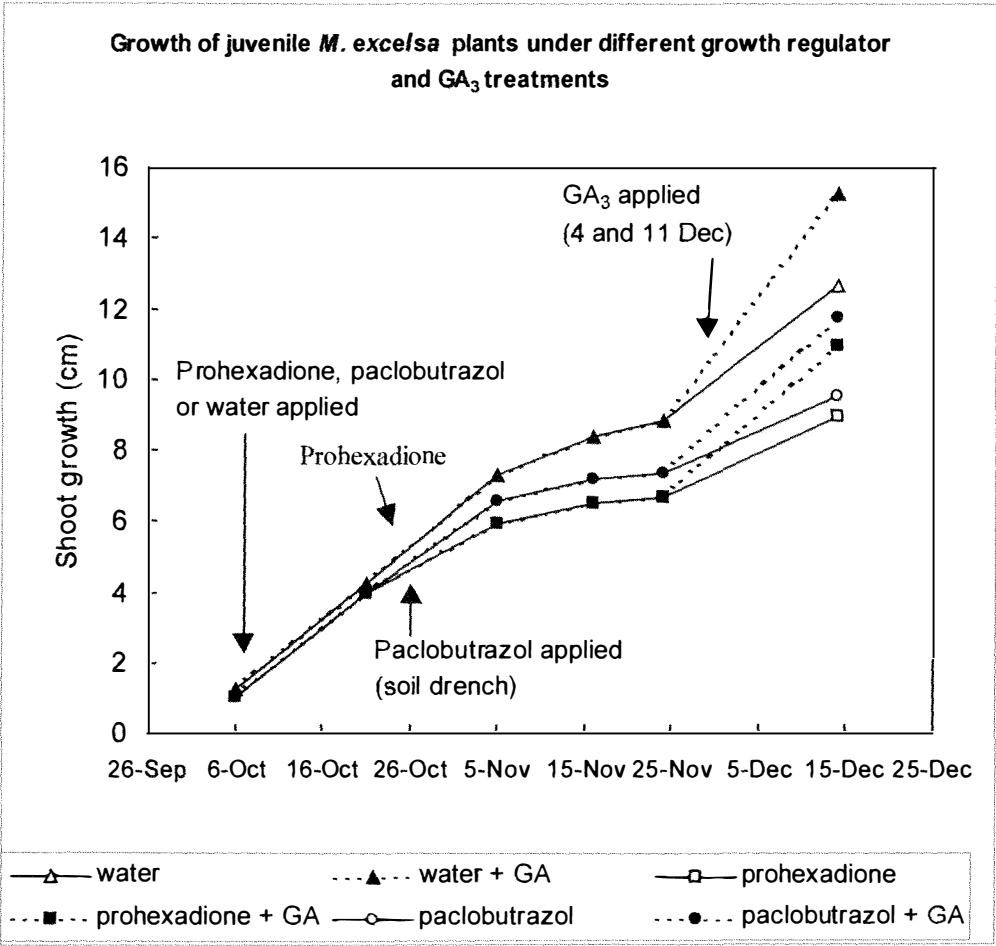
Figure 6.1 Growth of juvenile *M. excelsa* plants as affected by growth regulators and GA₃
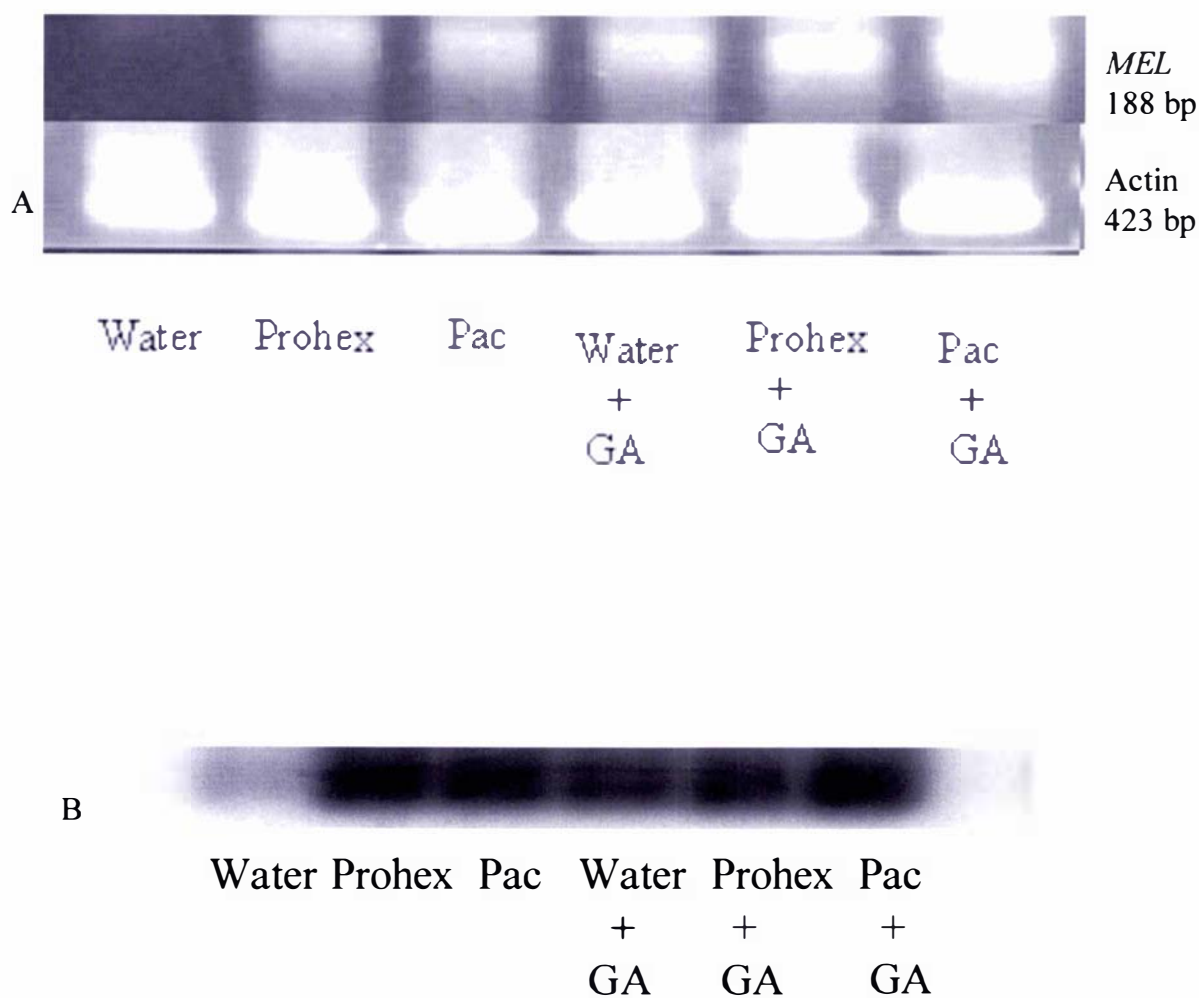
Figure 6.2 *MEL* expression in buds of juvenile *M. excelsa* plants as affected by growth regulators and GA. (A) RT-PCR products electrophoresed with actin as loading control. (B) *MEL* probe hybridised to the 188 bp fragments of the RT-PCR products in the expression Southern.

resumption of growth by these plants, (which could possibly occur by endogenous supply of GAs from other sources) it could not be linked to endogenous GA levels as results for these were inconsistent. This experiment is currently being repeated by another student.

There was no expression of *MESAP1* probably because the plants were juvenile. Blasquez et al. (1997) showed that the time to flowering is critically affected by levels of *LFY* expression in its normal pattern, as determined with plants that carry one, two, three, or four copies of wild type *LFY*. Increasing copy numbers of wild type *LFY* hastened flowering. However, in *M. excelsa*, mere upregulation of the *LFY* equivalent alone was not sufficient to trigger upregulated expression of the *AP1* orthologue and to promote flowering. The discussion on the competence of meristems to respond to increased levels of *LFY* (Section 4.4) is pertinent here as well. Weigel and Nilsson (1995) had demonstrated that flower initiation is promoted by a combination of *LFY* expression and competence to respond to *LFY* activity. In *Populus*, Rottman et al. (2000) had also observed that three year old transgenics with the *LFY* equivalent constitutively expressed showed little difference when compared to controls and concluded that *LFY* activity was dependent upon tree maturation. Presumably, juvenile *M. excelsa* plants are also not competent to respond to increased *LFY* activity.

It is concluded from this study that even though GA_3 does not promote flowering in several species of Myrtaceae, *MEL* is found to be responsive to GA. However, even with *MEL* upregulation, flowering could not be achieved in juvenile plants.

Chapter 7. Final discussion and conclusions

Genetic studies in model species such as *Arabidopsis* have led to the identification of a hierarchy of genes involved in the transition to flowering, in the establishment of floral and inflorescence meristem identity, and of floral organ identity. These studies have led to models that integrate environmental and genetic factors. Such a model is shown in Figure 1.2 which in this thesis has been referred to as the *Arabidopsis* or herbaceous model. Although information is accumulating on the genetic interactions and floral development in herbaceous annuals, such information is scanty for woody perennials. The genes chosen for the present study of the woody perennial *M. excelsa* were the equivalents of the floral meristem identity genes, *LFY* and *AP1* and the inflorescence meristem identity gene, *TFL1*. These genes were chosen as they occupy a central position in the hierarchy of genes involved from transition to meristem specification and organ identity.

Arabidopsis is a LD plant and flowering is seen to be promoted by GAs (Blasquez et al., 1997). *M. excelsa*, on the other hand, is a facultative SD plant (Henriod et al., 2000) and a close relative of *M. collina* in which flowering was found to be inhibited by exogenous GAs (Clemens et al., 1995). Hence, the behaviour of *M. excelsa* in terms of environmental response, response to gibberellins and its perenniality, provided a challenging test of the general applicability of the *Arabidopsis* model.

The programme of study required knowledge of events from floral initiation to floral organ development. Therefore microscopy techniques had to be optimised in order to study buds of *M. excelsa*. These were particularly difficult specimens as they were very waxy and pubescent and covered by at least six to eight pairs of scales. These techniques also formed the groundwork for the *in situ* localisation studies that were carried out subsequently. After developing suitable microscopy techniques, a calendar of floral development was formed from March to December, by studying distal axillary buds harvested during that period. Earlier studies on *M. excelsa* had not tracked the events from floral initiation to floral organogenesis. The present study has successfully filled this gap. It was revealed that the switch to flowering had occurred by May during the period of declining daylength or

earlier and also before the coldest days in June-July. It was known that the distal axillary buds on developing vegetative shoots developed into the inflorescences or the vegetative shoots of the next year (Dawson, 1968). From the present study it was revealed that floral and vegetative meristems developed in distinctly different patterns. While the apical meristem produced leaves in an opposite decussate manner in the case of vegetative buds, cymules comprising three flowers developed in the axils of the scales covering the apical meristem in the case of floral buds. Floral initiation was completed before late June as the percentage of floral buds, relative to the total buds examined, did not increase beyond that period. Development of the cymule primordia was seen to take place from August onwards, and by the end of September, all the floral organs were seen to be fully differentiated. Thus the present study revealed that, unlike *Eucalyptus* flowers that are fully differentiated before winter and remain quiescent in that differentiated condition until the onset of spring, the major floral organ differentiation in *M. excelsa* occurs only after the coldest period.

It was hypothesised that if there existed equivalents of the important floral meristem identity genes, *LFY*, *AP1* and the inflorescence meristem identity gene *TFL1*, in *M. excelsa* they would be expressed in the distal axillary buds from which inflorescences developed. *MEL*, a partial orthologue of *LFY* had been isolated earlier by PCR from *M. excelsa* genomic DNA (McKenzie et al., 1997). In the present study, partial orthologues of *AP1* (*MESAP1* and *MTTAP1*) and *TFL1* (*METFL1* and *MTTFL1*) were isolated from *M. excelsa* and *M. collina* 'Tahiti' through RT-PCR. *MEL* was also cloned from RT-PCR products. Their homology analysis confirmed that they were the fragments of the equivalents of *AP1*, *TFL1* and *LFY*. Putative conserved domains were detected for *MESAP1*, *MTTAP1*, *METFL1* and *MTTFL1*. The former two revealed a MADS box region with a SRF-type transcription factor and a K box region. A PEBP domain was detected in *METFL1* and *MTTFL1*. It is worth mentioning the high homology of *METFL1* and *MTTFL1* with *CiFT*, a flowering time gene from *Citrus*. What role *TFL* has in flowering as a repressor, other than specifying inflorescence meristem, is to be further probed. It could be speculated that it could act as a competitor to *FT*, the flowering time gene, and thus play a major role in timing of flowering.

Southern analysis revealed that there could be one homologue of *LFY* and *TFL1* in *M. excelsa* DNA, and one highly homologous sequence and a sequence similar to a lesser degree in the case of *AP1* (Figure 3.2). In *Eucalyptus*, which is a close relative of *Metrosideros*, two functional equivalents of *AP1* (*EAP1* and *EAP2*) have been detected (Kyozuka et al., 1997). *MESAP1* and *MTTAP1* had a very high homology to *EAP2* and a lesser homology to *EAP1*. Hence, it could be surmised that the second weaker band was the *EAP1* equivalent in *M. excelsa*. A restriction enzyme analysis (Figure 4.5) conducted on RT-PCR products of *MESAP1* (as the *MESAP1* fragment covered part of the MADS domain and the Southern hybridisation had revealed two bands) confirmed that the amplified bands were from *MESAP1* rather than any other gene. The isolated fragments of *MEL*, *MESAP1* and *MTTFL1* were considered suitable for use as probes for studying expression patterns of *LFY*, *AP1* and *TFL1* equivalents in *M. excelsa*.

To provide an integrated picture Figure 7.1 provides a schematic representation of the evidence obtained from both the temporal (RT-PCR) analysis and spatial (*in situ* hybridisation) analysis of *MEL* and *MESAP1*. A bi-modal pattern of expression of the *LFY*- and *AP1*- equivalents has also been reported from *A. deliciosa* (Walton et al., 2001) which supports the results obtained for *M. excelsa*.

Hempel et al. (1997) demonstrated the utility of *AP1* expression as an indicator of floral determination in *Arabidopsis* as its expression could be detected only in meristems after floral induction. As it was seen from the anatomical studies that cymule primordia initiation in *M. excelsa* began in May (Figure 2.6 B), it was hypothesised that *MEL* and *MESAP1* expression would be detected in distal axillary buds prior to May. The results fom the temporal expression analysis of *MEL* and *MESAP1* (Figures 4.2 A, 4.3 A and 4.3 B) showed that the expresssion of both these genes could be detected as early as March in *M. excelsa* indicating that floral commitment would have occurred by then. After downregulation in late June (winter), expression of both *MEL* and *MESAP1* was upregulated from August-September with the onset of longer days and warmer temperatures (Figure 7.1). In parallel research, Clemens (1999) showed that endogenous GA levels (unpublished data shown in Appendix VI) were lower in leaves of adult *M.*
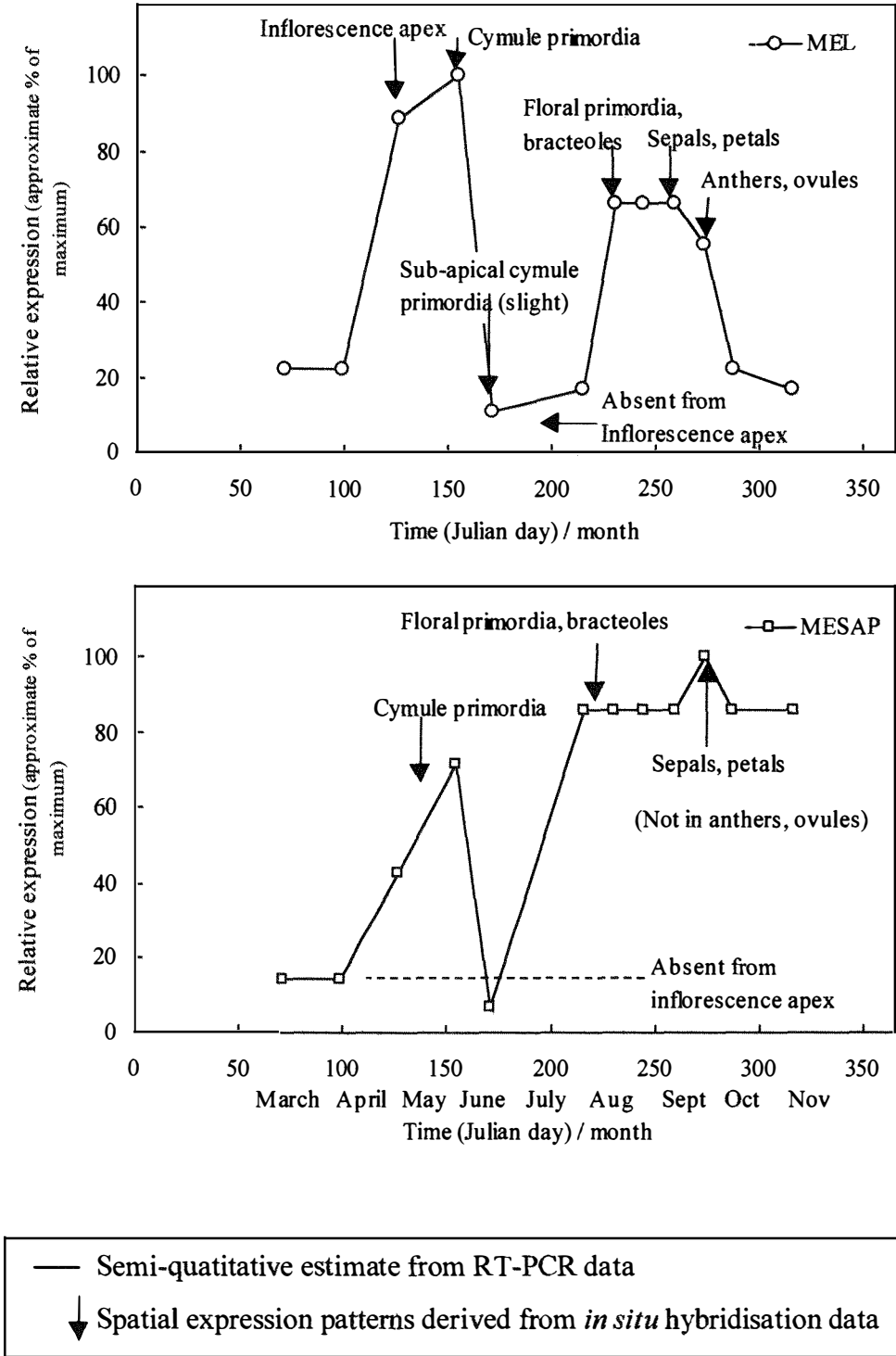
Figure 7.1 Semi-quantitative estimate of *MEL* and *MESAP1* expression

excelsa trees in June at the time when the level of *MEL* expression was low (Figure 7.1). GA_1 itself was not detected in floral buds in June which may be significant considering the interaction between gibberellins and *LFY* in *Arabidopsis*. *MESAP1* expression reached peak levels after August and it was interesting to note that the anatomical studies revealed that only then did floral organs begin to develop rapidly in *M. excelsa*, with all the organs being fully differentiated by the end of September.

Neither *MEL* nor *MESAP1* expression was detected in young vegetative tissue by RT-PCR although small amounts of *MEL* expression were detected by the expression Southerns of the RT-PCR products. *Arabidopsis LFY* has been found to be expressed throughout plant development and even during early stages of vegetative development, but its expression has been found to increase sharply around the time of flowering (Blasquez et al., 1997). Quantitative regulation of flowering genes could play a very important role in flowering and *LFY* seemed to be tightly regulated as small changes in its activity affected flowering time or shoot morphology (Pineiro and Coupland, 1998). In *M. excelsa* also, quantitative regulation of *MEL* seems important considering the low levels of expression in vegetative tissue and the higher levels seen during floral development, especially during floral primordia development and organogenesis.

The role of growth retardants and GA on expression of *MEL* and *MESAP1* in juvenile *M. excelsa* plants was also studied. Glasshouse grown juvenile plants were pre-treated with paclobutrazol or prohexadione and later treated with GA_3 (plus appropriate controls). The buds when analysed for *MEL* and *MESAP1* expression revealed that *MEL* expression was upregulated with GA_3 application (Figure 6.2). However, surprisingly, *MEL* expression was also upregulated by the growth retardant treatments. No *MESAP1* expression could be detected in any of the treatments. This again showed that upregulation of *MEL* alone was not sufficient to promote flowering. Probably the juvenile meristem was not competent to respond to the increased *MEL* activity as has been suggested for *Arabidopsis* (Weigel and Nilsson, 1995) and for *Populus* (Rottmann et al, 2000).

Analysis of the spatial expression patterns of *MEL* through *in situ* hybridisation revealed

that *MEL* expression shifted from the apex of the distal axillary bud in May to cymule primordia and the sepals, petals and to the anthers and the gynoecium and ovules in the later stages of floral development. Sessions et al. (2000) reported that *LFY* activity could move to adjacent cells and act non-autonomously between cell layers and over considerable distance within cell layers. In the case of *MEL*, the biological significance of the shift in its expression through different floral structures could be that it is necessary to be present in those specific regions to activate the next set of genes in the developmental events. This implies that *MEL* may not be able to do cell-to-cell signaling over large distances. Moreover, the floral structures in plants such as *Arabidopsis* are much smaller than those in plants such as *M. excelsa*. Hence, in *Arabidopsis*, *LFY* can be considerably non-autonomous and its activity could easily move to the different floral structures. On the other hand, .*MEL* would have to signal over a larger number of cells to activate other genes in the developmental process in the different floral structures if it did not shift its area of expression. Once again the importance of testing herbaceous models on larger woody perennials is highlighted.

MEL expression could also be detected on the bracts and bracteoles of the developing cymules (Figure 5.3 B). In this respect, *MEL* was similar to *FLO* the *LFY* equivalent in *Antirrhinum*. *LFY* expression is found to start before floral meristems are formed, and *FLO* is found to be expressed in the bracts and early floral meristems (Ma, 1998).

In situ hybridisation for *MESAP1* detected its expression in young floral meristems but, during the later stages of floral development, it was confined to the sepals, petals and the perianth region. Thus it was confirmed that it was a Class A gene and was restricted to the outer whorls. *AP1* is suggested to be restricted to the outer whorls because of antagonistic reaction with *AG*, which is expressed in the inner two whorls (Mandel et al., 1992). *MESAP1* was more similar to *AP1* than its *Antirrhinum* equivalent, *SQUA*. Unlike *SQUA*, it was not expressed in the carpels.

The temporal expression pattern of *METFL1*, studied through RT-PCR analysis and expression Southerns, showed that it was expressed throughout the process of floral

development in *M. excelsa*. However, *METFL1* levels were higher during the very early stages and towards the very late stages of floral development. Bradley et al. (1997) had suggested that *TFL* could have a role during vegetative development of *Arabidopsis* in influencing the timing of the transition to flowering. From the present study, it has been revealed that *METFL1* was upregulated during the transition from the vegetative phase to the reproductive phase, and also when the inflorescence meristem resumed further vegetative development towards the end of flowering. Such a role in transition, when the inflorescence meristem resumes further vegetative growth, cannot be studied in annual plants such as *Arabidopsis*. The high levels of expression of *METFL1* in young vegetative tissues also pointed to a major role for this gene in vegetative development in *M. excelsa*.

In situ hybridisation studies revealed that *METFL1* was expressed in the inflorescence meristem and not in the floral meristems. This is very much in agreement with the model in *Arabidopsis* where *TFL1* is restricted to the inflorescence meristem probably by the antagonistic reactions with *LFY* and *AP1* (Shannon and Meeks-Wagner, 1993). However, the interaction between *MEL* and *METFL1* seemed to be more similar to that between their *Antirrhinum* equivalents, *FLO* and *CEN*, rather than that between *LFY* and *TFL1*, as *MEL* expression was detected in the inflorescence apex (Figure 5.2 A) in the early stages of floral development (May). At that time period, like *CEN*, *METFL1* may also need its *LFY* equivalent to help its initial expression (Bradley et al., 1996).

Thus in this thesis a calendar of floral development for *M. excelsa* is presented. Evidence is provided on how similar or different the expression characteristics of the floral and inflorescence meristem identity genes are in this woody perennial in comparison to annual herbaceous plants such as *Arabidopsis* and *Antirrhinum*. The temporal expression of *MEL* and *MESAP1* showed a bimodal pattern that has not been seen in herbaceous plants but has been observed in another woody perennial, *Actinidia deliciosa*. As in the herbaceous models, meristem competence was necessary for *MEL* to upregulate *MESAP1*. *METFL1* was expressed throughout the process of floral development as seen in herbaceous plants and its expression was higher during the early stages of floral development which is similar to the *Arabidopsis* model. Spatial expression patterns of *MEL* showed that it had

greater similarity to *FLO* than *LFY* while *MESAP1* was more similar to *AP1* than *SQUA*. The interaction between *MEL* and *METFL1* was more similar to the interaction between *FLO* and *CEN* rather than *LFY* and *TFL1*. Consequently, the three genes from *M. excelsa* fit a broader herbaceous model encompassing *Antirrhinum* as well as *Arabidopsis*.

For future work, it would be interesting to clone full-length genes of *MEL*, *MESAP1* and *METFL1* and conduct complementation studies in *Arabidopsis* mutants to validate the functions of these genes, although this might be difficult under the current regulatory regime. It would also be interesting to isolate the genes involved in floral transition from the short day perennial *M. excelsa* and see how they differed in their expression patterns from the long day annual *Arabidopsis*. Another area of future research is to conduct similar studies on *M. collina* 'Tahiti' which flowers irrespective of daylength and temperature.

To be or not to be a pohutukawa flower is thus a question to be pondered further.

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Appendix I

Recipes for common buffers and media

Ethidium bromide stain: Make stock solution of 0.5 mg ml^{-1} in water. For use in staining solutions use at a final concentration of 0.5 µg ml^{-1}

LB (Luria Broth):   Tryptone   10.0 g
                    NaCl   5.0 g
                    Yeast extract   5.0 g
                    MilliQ to   1.0 L
                    Sterilise by autoclaving

LB Agar: As for Luria broth, but also add 15 g Bacteriological agar per litre.

Sterilise by autoclaving.

SDS loading dye: 20% Sucrose   4.0 g
                    5 mM EDTA   0.2 ml of 0.5M
                    1% SDS   2 ml of 10%
                    0.2% bromophenol blue   40 mg
                    MilliQ to total volume of 20 ml

TAE buffer (10 x): Tris   48.4 g
                    EDTA   7.4 g
                    Glacial acetic acid   11.4 ml
                    MilliQ to   1.0 L
                    pH to 8.5

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

Appendix II. Vector circle maps of pGEM[R]-T and pGEM[R]-T Easy vectors

Xmn1 1994

Sca1 1875

Nae1 2695

fl ori

Amp[r]

Lac Z

pGEM[R]-T vector
(3003 bp)

T

ori

T7 ↓		1 start
Apa1	14	
Aat11	20	
Sph1	26	
BstZ1	31	
Nco1	37	
Sac11	46	

Spe1	55	
Not1	62	
BstZ1	62	
Pst1	73	
Sal1	75	
Nde1	82	
Sac1	94	
BstX1	103	
Nsi1	112	
		126
↑ SP6		

Xmn1 2009

Sca1 1890

Nae1 2710

fl ori

Amp[r]

Lac Z

pGEM[R]-T Easy

vector (3018 bp)

T

ori

T7 ↓		1 start
Apa1	14	
Aat11	20	
Sph1	26	
Nco1	37	
BstZ1	43	
Not1	43	
Sac11	49	
EcoR1	52	

Spe1	64	
EcoR1	70	
Not1	77	
BstZ1	77	
Pst1	88	
Sal1	90	
Nde1	97	
Sac1	109	
BstX1	118	
Nsi1	127	
		141
↑ SP6		

Appendix III

Preparation of competent cells

Inoculate 10 ml LB medium (see Appendix I) with *E. coli* strain to be made competent. Incubate overnight at 37°C with shaking (225 rpm).

Take 400 µl overnight culture and inoculate into 40 ml LB medium. Incubate at 37°C for 2 h 20 min with shaking (260 rpm). Check growth and pour sample into labeled, sterile centrifuge tube (pre-chilled) and leave on ice to cool.

Centrifuge for 5 min at 4°C (3000 rpm) to pellet cells.

Remove supernatant.

Add 10 ml ice cold 60 mM $CaCl_2$ and resuspend cells.

Add additional 10 ml 60 mM $CaCl_2$. Cool on ice for 30 min.

Centrifuge for 5 min at 4°C (3000 rpm) and remove supernatant.

Resuspend pelleted cells in 4 ml 60 mM $CaCl_2$, 15% glycerol.

Aliquot at 300 µl into cryo-tubes and store at -80°C.

Appendix IV

DNA plasmid preparations

Inoculate 10 ml LB medium with bacterial culture and grow overnight at 37°C with shaking (225 rpm).

Next morning centrifuge bacterial culture at 5000 rpm for 15 minutes and remove supernatant.

Resuspend pellet in 200 μl of TEG buffer. Add 400 μl of freshly prepared NaOH/SDS solution and mix by slow inversion. Keep on ice for 5 min.

Add 300 μl of ice cold potassium acetate solution and shake vigorously. Keep on ice for 5 min.

Centrifuge for 5 min at 12000 rpm. Carefully, avoiding particulate matter, transfer supernatant (750 μl) to sterile Eppendorf tubes already with aliquots (750 μl) of isopropanol. Keep at at -20°C for 15 min.

Centrifuge for 10 min at 12000 rpm. Remove isopropanol.
Wash pellet with ice cold 70% ethanol.
Dry in speed vac for 2-3 min and dissolve in 50 μl of sterile milliQ water.

TEG buffer: 50 mM glucose
            10 mM EDTA
            25 mM Tris (pH 8.0)

NaOH/SDS solution: 0.2 N NaOH
                   1% SDS

Potassium acetate solution (pH 4.8)
To 60 ml of 5 M potassium acetate, add 11.5 ml of glacial acetic acid and 28.5 ml sterile water.

Appendix V

GAs in juvenile seedlings

GA (ng.g FW^{-1})	29 + 8	1	20	29	53	8	19	44
Control	3.25	1.14	2.65	0.48	NF	2.77	1.62	NF
Prohexadione	3.86	1.40	2.60	0.50	NF	3.36	3.60	NF
Paclobutrazol	0.59	0.14	0.14	0.04	NF	0.55	3.14	NF

Clemens (1999).Unpublished data

Appendix VI

Endogenous gibberellins in *M. excelsa* in winter (June) and in summer (November)

Sampling time	Juvenile or mature	Part sampled	GA concentration (ng.g FW^{-1})						
			GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_1	GA_8	GA_{29}
Winter	juvenile	leaves	0.13	1.73	3.93	0.35	0.14	3.11	0.73
(June)	mature	leaves	0.28	3.06	7.02	0.86	0.19	1.68	4.46
	mature	overwintering buds	1.08	0	6.27	0.14	0	0.06	0
Summer (November)	mature	leaves	0.15	3.21	3.76	4.00	2.56	NF	1.26
	mature	single flower buds	>15	>20	0.06	>5	1.53	2.82	1.13

Clemens (1999). Unpublished data

Appendix VII
Primer regions

(A) *LEAFY* **specific primer regions** underlined in the original sequence by McKenzie et al.(1997)

```
KKAGASYINKPKMRHYVHCYALHCMDEHASNALRKSFKERGENVGAWRQACYHPLVTIAGRRAGWDIDAIFNAHPR
LCIW
```

(B) The primer regions used for isolating *MESAP1* underlined in the amino acid sequence of *Brassica oleraceae AP1* gene.

```
MGRGRVQLKRIENKINRQVTFSKRRAGLFKKAHEISVLCDAEVALVVFSHKGKLFEYSTDPCMEKILERYERYSYA
ERQLIAPESDVNTNWSMEYNRLKAKIELLERNQRHYLGEDLQAMSPKELQNLEQQLDTALKHIRSRKNQLMYESIN
ELQRKEKAIQEQNSMLSKQIKERENVLRAQQEQWDEQNHGHNMPPPPPPQQHQIQHPYMLSHQPSPFLNMGGLYQE
EDQMAMRRNDLDLSLEPVYNCNLGSFAA
```

(C) Multiple sequence alignment for designing *TFL1* **primers for** *Metrosideros*

```
Lolium perenne          ----MSRSVEPLIVGRVIGEVLDPFNPCVKMVATYNSNKLVFNGHELYPSAVVSKPRVEV 56
Oryza sativa            ----MSRSVEPLVVGRVIGEVIDSFNPCTKMIVTYNSNKLVFNGHEFYPSAVVSKPRVEV 56
Nicotiana tabacum       ---MASRVVEPLVVARVIGEVVDSFNPSVKLNVIYNGSKQVFNGHELMPAVIAAKPRVEI 57
Lycopersicon esculentum MASKMCEPLVIGRVIGEVVDYFCPSVKMSVVYNNNKHVYNGHEFFPSSVTSKPRVEV 57
Arabidopsis thaliana    MENMGTRVIEPLIMGRVVGDVLDFFTPTTKMNVSYN-KKQVSNGHELFPSSVSSKPRVEI 59
Citrus unshiu           ---MSSRERDPLIVGRVVGDVLDNFTRTIPMRITYS-NKDVNNGRELKPSEVLNQPRAEI 56
                          ::   :**:.**:*:*:* *      :   *...* * **:*: *: :  :**.*:

Lolium       QGGDLRSLFTLVMTDPDVPGPSDPYLREHLHWIVSNIPGTTDASFGGEVMSYESPKPNIG 116
Oryza        QGGDMRSFFTLVMTDPDVPGPSDPYLREHLHWIVTDIPGTTDASFGREIISYESPKPSIG 116
Nicotiana    GGEDMRSAYTLIMTDPDVPGPSDPYLREHLHWIVTDIPGSTDSSFGREIVSYESPKPVIG 117
Lycopersicon HGGDLRSFFTLIMIDPDVPGPSDPYLREHLHWIVTDIPGTTDCSFGREVVGYEMPRPNIG 117
Arabidopsis  HGGDLRSFFTLVMIDPDVPGPSDPFLKEHLHWIVTNIPGTTDATFGKEVVSYELPRPSIG 119
Citrus       GGDDLRTFYTLVMVDPDAPSPSDPSLREYLHWLVTDIPATTGASFGQEIVNYESPRPTMG 116
              * *:*: :**:* ***.*.**** *:*:***:*::**.:*...:** *::.** *:* :*

Lolium       IHRFIFVLFKQKRRQTVS-VPSFRDHFNTRQFAVDNDLGLPVAAVYFNCQRETAARRR-- 173
Oryza        IHRFVFVLFKQKRRQAVV-VPSSRDHFNTRQFAEENELGLPVAAVYFNAQRETAARRR-- 173
Nicotiana    IHRYVLLLYKQSGRQTVK-PAATRDHFNTRRYTAENGLGSPVAAVYFNAQRETAARRR-- 174
Lycopersicon IHRFVFLLFKQKKRQTISSAPVSRDQFSSRKFSEENELGSPVAAVFFNCQRETAARRR-- 175
Arabidopsis  IHRFVFVLFRQKQRRVIFPNIPSRDHFNTRKFAVEYDLGLPVAAVFFNAQRETAARKR-- 177
Citrus       IHRFVFVLFRQLGRQTVY-APGWRQNFSTRDFAELYNLGPPVAAVYFNCQRESGSGGRPV 175
              ***::::*::*  *:.:  .    *::*.:* ::    ** *****:**.***:.: *

Lolium       --
Oryza        --
Nicotiana    --
Lycopersicon --
Arabidopsis  --
Citrus       RR 177
```

→	*TFL1* outer forward primer		⋯▸	*TFL1* nested forward primer
←	*TFL1* outer reverse primer		◂⋯	*TFL1* nested reverse primer