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

A thesis presented in partial fulfilment of the requirements for the degree of  

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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*, *MESAPI1* 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 Southernns showed that *MEL* and *MESAPI1* 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 *API* is considered an indicator of floral determination, the expression of both *MEL* and *MESAPI1* 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 GA3 application caused upregulation of *MEL* but not *MESAPI1* 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. *MESAPI1* 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 *MESAPI1*. 
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Table of Contents

Abstract ii
Acknowledgements iii
Table of Contents iv
List of Figures v
List of Tables viii

Chapter 1. Introduction

1.1 Overview 1
1.2 Pathways to flowering 2
   1.2.1 Photoperiod and flowering 5
   1.2.2 Temperature and flowering 7
   1.2.3 Hormonal control of flowering 9
      1.2.3.1 Cytokinins and flowering 9
      1.2.3.2 Gibberellins and flowering 11
1.3 Floral and inflorescence meristem identity genes and flowering in herbaceous plants 14
   1.3.1 LEAFY and APETALA1 15
   1.3.2 TERMINAL FLOWER1 20
1.4 Expression patterns of LEAFY and APETALA1 in woody perennials 22
1.5 The interaction between flowering time and floral meristem identity genes 25
1.6 Herbaceous models in flowering 28
1.7 Flowering in Metrosideros and related species 32
1.8 Summary 34
1.9 Aims of the study 35

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

2.1 Introduction 37
2.2 Microscopy techniques for viewing meristems of M. excelsa 37
2.2.1 Plant material and study site 37
2.2.2 Techniques 38
2.2.3 Results 43
2.2.4 Discussion 47

2.3 Calendar of floral development in *Metrosideros excelsa* 49
2.3.1 Materials and methods 49
2.3.2. Results 49
2.3.4. Discussion 55

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

3.1 Introduction 58
3.2 Materials and Methods 59
3.2.1 Extraction and purification of DNA 59
3.2.2 Extraction of RNA 61
3.2.3 Polymerase chain reaction (PCR) 63
3.2.4 Reverse transcription-polymerase chain reaction (RT-PCR) 64
3.2.5 Isolation of the partial orthologue of *LEAFY (MEL)* from *M. excelsa* 64
3.2.6 Isolation of the partial orthologues of *APETALA1* from *M. excelsa (MESAP1)* and *M. collina* ‘Tahiti’ (*MTAPI1*) 65
3.2.7 Isolation of the partial orthologue of *TERMINAL FLOWER1* from *M. excelsa (METFL1)* and *M. collina* ‘Tahiti’ (*MTTFL1*) 65
3.2.8 Cloning of the gene fragments 66
3.2.9 DNA plasmid preparation 67
3.2.10 Sequencing 67
3.2.11 Southern Analysis 68

3.3 Results 70
3.3.1 Sequences of *MEL* 70
3.3.2 Alignment of the amino acid sequence of MEL with other LFY-like amino acid sequences 72
3.3.3 Sequences of MESAP1 and MTAP1 74
3.3.4 Alignment of the deduced amino acid sequences of MESAP1 and MTAP1 with other API-like amino acid sequences 76
3.3.5 Sequences of METFLI and MTTFLI 79
3.3.6 Alignment of the deduced amino acid sequences of METFLI and MTTFLI with other TFL1-like proteins 80
3.3.7 Southern analysis 82

3.4 Discussion 82

Chapter 4. Temporal expression patterns of floral and inflorescence meristem identity genes in M. excelsa 89

4.1 Introduction 89
4.2 Materials and Methods 90
  4.2.1 Sampling of buds and RNA extraction 90
  4.2.2 Northern analysis 91
    4.2.2.1 RNA electrophoresis and blotting 91
    4.2.2.2 Probe synthesis 92
    4.2.2.3 Hybridisation and detection of radioactive probe 92
  4.2.3 RT-PCR analysis 92
    4.2.3.1 Isolation and sequencing of a fragment of actin for loading control 92
    4.2.3.2 RT-PCR of MEL, MESAP1, METFLI and actin 93
    4.2.3.3 Polymerase chain reaction using RNA 93
  4.2.4 RT-PCR of vegetative tissue 94
  4.2.5 Expression Southerns for MEL, MESAP1 and METFLI 94
    4.2.6 Restriction enzyme analysis of MESAP1 94
4.3 Results

4.3.1 Northern analysis 95

4.3.2 RT-PCR analysis 95

4.3.2.1 Isolation and sequencing of a fragment of actin for loading control 95

4.3.2.2 Temporal expression patterns of MEL, MESAP1 and METFL1 97

4.3.2.3 PCR of RNA 99

4.3.3 MEL, MESAP1 and METFL1 expression in vegetative tissue 99

4.3.4 Expression Southerns 99

4.3.5. Restriction analysis of MESAP1 101

4.4 Discussion 101

Chapter 5. Spatial expression patterns of floral and inflorescence meristem identity genes in M. excelsa 112

5.1 Introduction 112

5.2 Materials and methods 113

5.2.1 Fixation of tissue 115

5.2.2 Dehydration 115

5.2.3 Clearing 115

5.2.4 Infiltration 115

5.2.5 Embedding and sectioning 116

5.2.6 Probe Synthesis 116

5.2.7 DNase treatment 117

5.2.8 Probe precipitation 117

5.2.9 Probe hydrolysis 117

5.2.10 Probe quantification 117

5.2.11 Prehybridisation treatments 118

5.2.12 Hybridisation and washes 118

5.2.13 Immunological detection 119
5.3 Results

5.3.1 The ideal fixative 120
5.3.2 Yield of probes 120
5.3.3 Spatial expression patterns of $MEL, MESAPI$ and $METFL1$ 121

5.4 Discussion

Chapter 6. Interaction of floral meristem identity genes and gibberellins 134
6.1 Introduction 134
6.2 Materials and methods 134
6.3 Results 136
6.4 Discussion 136

Chapter 7. Final discussion and conclusions 140

References 148

Appendices
Appendix I Recipes for common buffers and media 169
Appendix II Vector circle maps of pGEM$^R$-T and pGEM$^R$-T Easy vectors 170
Appendix III Preparation of competent cells 171
Appendix IV DNA plasmid preparations 172
Appendix V GAs in juvenile seedlings 173
Appendix VI Endogenous gibberellins in $M. excelsa$ in winter (June) and in summer (November) 174
Appendix VII Primer Regions 175
List of Figures

Figure 1.1 Pathways to flowering in *Arabidopsis thaliana*. 3

Figure 1.2 Genetic interactions between flowering time, meristem identity and floral organ identity genes; the *Arabidopsis* model. 31

Figure 2.1 Flow chart of tissue dehydration and wax infiltration for paraffin embedding of buds of *M. excelsa*. 41

Figure 2.2 Staining schedule with safranin and fast green. 42

Figure 2.3 Images of meristems of *M. excelsa* generated through different microscopy techniques. 45

Figure 2.4 Comparison of fixation protocol for softer tissues and that of *M. excelsa*. 46

Figure 2.5 Comparison of browning caused by different fixatives. 46

Figure 2.6 Development of floral buds of *M. excelsa* from March to August. 52

Figure 2.7 Development of floral buds of *M. excelsa* from August to October. 53

Figure 2.8 Development of vegetative buds of *M. excelsa* from June to September. 54

Figure 2.9 Photoperiod greater than 107.6 lux (hours) in Palmerston North, New Zealand. 54

Figure 3.1 Isolation of *MEL, MESAPI* and *METFLI* by RT-PCR. 71
Figure 3.2 Southern analysis on *M. excelsa* genomic DNA after restriction digestion with *BamH*I.

Figure 4.1. RT-PCR for isolation of actin fragment from *M. excelsa*.

Figure 4.2 Temporal expression patterns of floral and inflorescence meristem identity genes in *M. excelsa* buds from March to November.

Figure 4.3 Expression Southern of floral and inflorescence meristem identity genes in *M. excelsa* buds from March to November.

Figure 4.4 Restriction map of *MESAPI* with sites of 6 bp cutters cutting once and *API*-specific primers.

Figure 4.5 Restriction analysis of *MESAPI*.

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

Figure 5.2 Spatial expression patterns of *MEL* during early stages of floral development.

Figure 5.3 Spatial expression pattern of *MEL* during cymule development in August.

Figure 5.4 Spatial expression pattern of *MEL* during later stages of floral development.

Figure 5.5 Spatial expression pattern of *MESAPI* during early stages of floral development.
Figure 5.6 Spatial expression pattern of MESAP1 during later stages of floral development. 127

Figure 5.7 Spatial expression pattern of METFL1. 128

Figure 6.1 Growth of juvenile M. excelsa plants as affected by growth regulators and GA3. 137

Figure 6.2 MEL expression in buds of juvenile M. excelsa plants as affected by growth regulators and GA. 138

Figure 7.1 Semi-quantitative estimate of MEL and MESAP1 expression. 143
List of Tables

Table 1.1 *Arabidopsis* and *Antirrhinum* genes controlling meristem identity. 22

Table 2.1 Composition of fixatives and stains 39

Table 2.2 Timing and progress of floral development in distal axillary buds of *M. excelsa*. 50
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)*, *APETALAL (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 al 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 API. 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 API 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 API expression is delayed under SD (120 hours after DEX treatment), suggesting that API expression requires other factors that are present in LD grown plants but accumulate slowly under SD. The fact that API 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 SOCI 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. fkm 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 GA₃ 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 *gal-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 *gal-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, *gal*, 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 *hyl*, 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 *AP1*. 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 (luel) and two late flowering mutants (fpal-3 and fpal-4). Mapping and complementation analyses showed that the late flowering mutants were allelic to fpal. 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 apetalal (apl) 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 *TFL1*, required to maintain inflorescence meristem identity (Ratcliffe et al., 1999). A considerable body of knowledge has accumulated on the roles of *LFY*, *API* 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
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 LpTFL1 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 \textit{LpTFL1} was a repressor of flowering and a controller of axillary meristem identity in ryegrass.

Ferrandiz et al. (2000) found that mutations in the \textit{FRUITFUL (FUL)} MADS-box gene, when combined with mutations in \textit{API} and \textit{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 \textit{TFL1} gene. Their results suggested that the \textit{FUL}, \textit{API} and \textit{CAL} genes acted redundantly to control inflorescence architecture by affecting the domains of \textit{LFY} and \textit{TFL1} expression as well as the relative levels of their activities.

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

Recent mutational studies and the genome project revealed that \textit{TFL1} belongs to a small gene family in \textit{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 \textit{LFY}, \textit{API} and \textit{TFL1} from studies on \textit{Arabidopsis} and \textit{Antirrhinum}. 
### Table 1.1. *Arabidopsis* and *Antirrhinum* genes controlling meristem identity

<table>
<thead>
<tr>
<th><em>Arabidopsis</em> genes</th>
<th><em>Antirrhinum</em> genes</th>
<th>Mutant phenotypes in meristem identity</th>
<th>Phenotypes of ectopic transgensics</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFY</td>
<td>FLO</td>
<td>Conversion of flower to shoot or shoot-like structures; floral reversion (lfyI+)</td>
<td>Conversion of lateral shoots to flower; terminal flower; early flowering</td>
</tr>
<tr>
<td>API</td>
<td>SQUA</td>
<td>Conversion of flower to shoot; branched flowers (api)</td>
<td>Conversion of lateral shoots to flower; terminal flower; early flowering</td>
</tr>
<tr>
<td>TFL1</td>
<td>CEN</td>
<td>Terminal flower; early flowering (tfll)</td>
<td>No flowering; continuous production of leafy shoots in place of flowers</td>
</tr>
</tbody>
</table>

### 1.4 Expression patterns of LEAFY and APETALAl 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/FLY 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-transcriptional 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/GI (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 fwallfy and fllfy 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 fcallfy 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 *API* 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, *API* 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).
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 \(-80^\circ C\) 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

<table>
<thead>
<tr>
<th>Fixative / Stain</th>
<th>Composition</th>
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<tbody>
<tr>
<td>FAA</td>
<td>70% ethyl alcohol---------90ml&lt;br&gt;glacial acetic acid----------5ml&lt;br&gt;37% formalin ---------5ml</td>
</tr>
<tr>
<td>FPA</td>
<td>70% ethyl alcohol---------90ml&lt;br&gt;propionic acid -----------5ml&lt;br&gt;37% formalin ----------5ml</td>
</tr>
<tr>
<td>Carnoy’s Fluid</td>
<td>100% ethyl alcohol--------60ml&lt;br&gt;glacial acetic acid------10ml&lt;br&gt;chloroform ----------30ml</td>
</tr>
<tr>
<td>Safranin</td>
<td>1% safranin in 70% ethyl alcohol</td>
</tr>
<tr>
<td>Picro- alcohol</td>
<td>0.5% picric acid in 95% ethyl alcohol</td>
</tr>
<tr>
<td>Fast green</td>
<td>clove oil -------15ml&lt;br&gt;methyl cellosolve-------15ml&lt;br&gt;95% ethyl alcohol------90ml&lt;br&gt;glacial acetic acid---------30ml&lt;br&gt;fast green -----------0.3 g</td>
</tr>
<tr>
<td>Clove oil/Absolute alcohol/</td>
<td></td>
</tr>
<tr>
<td>Histoclear</td>
<td>50:25:25</td>
</tr>
<tr>
<td>Histoclear/Absolute alcohol</td>
<td>50:50</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>1.5% in 96% alcohol</td>
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(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 fast green 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*

<table>
<thead>
<tr>
<th>Date of sampling</th>
<th>Number of buds examined</th>
<th>Number of buds in which floral development was evident</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 March '98</td>
<td>12</td>
<td>0</td>
<td>Bud scales were forming. No other activity could be observed</td>
</tr>
<tr>
<td>9 April '98</td>
<td>12</td>
<td>0</td>
<td>Bud scales were forming. No other activity could be observed</td>
</tr>
<tr>
<td>7 May '98</td>
<td>15</td>
<td>2</td>
<td>Small bulges in the axils of scales with actively dividing cells</td>
</tr>
<tr>
<td>4 June '98</td>
<td>12</td>
<td>5</td>
<td>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</td>
</tr>
<tr>
<td>20 June '98</td>
<td>15</td>
<td>9</td>
<td>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</td>
</tr>
<tr>
<td>4 August '98</td>
<td>15</td>
<td>10</td>
<td>The bracts subtending the three flowers could be seen to develop in some floral buds. Vegetative buds still not much elongated</td>
</tr>
<tr>
<td>18 August '98</td>
<td>15</td>
<td>9</td>
<td>&quot; &quot; &quot; &quot; &quot; &quot;</td>
</tr>
<tr>
<td>1 September '98</td>
<td>15</td>
<td>9</td>
<td>Cymules continued to develop in floral buds while vegetative buds started to elongate</td>
</tr>
<tr>
<td>16 September '98</td>
<td>5</td>
<td>5</td>
<td>Floral organs could be seen developing in the individual flowers. Stopped tracking development of vegetative buds</td>
</tr>
<tr>
<td>30 September '98</td>
<td>5</td>
<td>5</td>
<td>All floral organs were fully differentiated in individual flowers</td>
</tr>
<tr>
<td>14 October '98</td>
<td>5</td>
<td>5</td>
<td>Floral organs fully differentiated and growing</td>
</tr>
<tr>
<td>12 November '98</td>
<td>5</td>
<td>5</td>
<td>Organs fully differentiated and developing. Ovules and pollen visible.</td>
</tr>
</tbody>
</table>
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.
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.
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
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 *METFLI* and *MTTFLI*, 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.
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 $1 \frac{A_{260}}{A_{230}/A_{260}/A_{280}}$ 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 dithiotreitol (DTT)
- 1% Nonidet P-40
- 2% (w/v) polyvinylpyrrolidone

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' GCGAATTTCACIAAYCARGTITTYMGIYAYGC 3'

**LFY Right primer:** 5' CGGATCCGTGICKIARIYKIGTIGGIACRTA 3'

The composition of the PCR mix is given below.

- 10 x buffer : 5 µl
- MgCl₂ (25mM) : 5 µl
- dNTPs (2.5 mM) : 5 µl
- Platinum Taq DNA Polymerase (5 U/µl⁻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
- 72°C - 1 min
- 72°C - 3 min
- 4°C

29 cycles

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' GCGAATTCCAAGCCGAAGATGAGGCTAC 3'

LFY Specific Reverse Primer: 5' GCATTGAGATAGCGTCGATG 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* (MESAPI) and *M. collina* ‘Tahiti’ (MTAPI)

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' CAAGCTTGAAGAGTAGAG 3'

*AP1* Reverse Primer: 5' GCTTAAGAGCATATCAAGCTG 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). Multiple alignment of sequences for designing the primers and the primer regions are presented in Appendix VII. The primer sequences were:

\[
\begin{align*}
TFL1 \text{ outer forward primer: } &5'\ ARSAARSNNGTYWNYAATGGN CRTG 3' \\
TFL1 \text{ outer reverse primer: } &5'\ SMRTTRAAGWARACNGCNACNGG 3' \\
TFL1 \text{ nested forward primer: } &5'\ GGTTATGACAGACCCAGATGT 3' \\
TFL1 \text{ nested reverse primer: } &5'\ CGAACCTGTGGGATACCAATG 3'
\end{align*}
\]

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 \textit{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 \textit{M. excelsa} is a native species. The fragment from \textit{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).

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

\[\begin{align*}
2 \times \text{Rapid ligation buffer, T4 DNA ligase} & : 5 \mu l \\
pGEM-T or pGEM-T Easy vector (25 ng µl}^{-1}) & : 2 \mu l \\
\text{PCR product (20-50 ng µl}^{-1}) & : 1-2 \mu l \\
\text{T4 DNA Ligase (3 Weiss units µl}^{-1}) & : 1 \mu l \\
\text{Sterile milliQ water to a final volume of 10 µl} & \text{The reactions were mixed by pipetting and were then incubated overnight at 4°C.}
\end{align*}\]
**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 DH5α 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-β-D-thiogalactopyranoside (IPTG) and 15 μl of 50 mg ml⁻¹ 5-bromo-4-chloro-3-indoyl-β-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. \textit{TFLI} nested forward primer and \textit{TFLI} 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 \textit{M. excelsa} using radio-labeled probes prepared from the cloned fragments of \textit{MEL}, \textit{MESAP1} and \textit{MTTFLI} 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:** \textit{M. excelsa} genomic DNA (30 μg) was digested with the restriction enzyme \textit{BamHI} 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\textsuperscript{TM}-N+; positively charged nylon membrane from Amersham Life Science with 20 x SSC (3 M NaCl, 0.3 M Na\textsubscript{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 \textit{MEL}, \textit{MESAP1} and \textit{MTTFLI} in the pGEM-T vectors. \textit{MEL} and the primers used were designed based on the original \textit{M. excelsa LFY} and \textit{API} sequences. The sequences of the \textit{LFY-}specific primers are given in Section 3.2.5. The \textit{API} specific primer sequences are
given below.

**API** specific Forward Primer: 5' GCGAATTCCGAGATGTGGGCTGAAGAAG 3'

**API** specific Reverse Primer: 5' GCGGGATCCCAAGCTGGCTCCAAATTCTG 3'

**TFL1** nested primers as described in Section 3.2.7 were used to amplify **MTTFLI**.

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 T7QuickPrimekit 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 ~32P-(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 solution 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 cassettes 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
TGAGGCACTACGTCCACTGCTACGCGCTACGCCCTGCACTGCA
TGAGGCACTACGTCCACTGCTACGCCCTGCACTGCA
TGAGGCACTACGTCCACTGCTACGCCCTGCACTGCA
```

**Amino acid sequence:**

```
KKAGAS YINKPKMRHYVHCYALHCMDHASNALR
```

```
Figure 3.1 Isolation of *MEL, MESAPI* 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 *MESAPI*. (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:** CAAGCCGAAGATGAGGCACTACGCTCCACTGCTACGCCCT GCACCTGACATGGAGCAGACGCACGCCTCAAACGCCCTCCGCAAGAGCCTTCAAGGAGC GCAGGGAAACGTCGGGCGCTGGAGGCAAGGCTTAGCTACCACCCCTGTTACC ATCGCCGCGAGCCGCCTGGCTGGACATCGACGCTATCTCTCAATGC

**Amino acid sequence:** KPKMRHYVHCYALHCDEHASNLRKSFKERENVGAWRQACYHPLVTIAGRRAWDIDAI INF

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 amino acids 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 database 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. *Eucalyptus globulus* (ELF1) Accession No. 7227892 Identities = 79/80 (98%), Positives = 80/80 (99%)

![Comparison with Eucalyptus globulus](Image)

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

![Comparison with Arabidopsis thaliana](Image)

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

![Comparison with Nicotiana tabacum](Image)
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**: AACAAGATCAATAGGCAAGTTACGTTCGAGCCGAGATGTGGG
CTGCTGAAGAAGGCCACGAGATCTCCGTTCGACGAGCGAGGTGGCCCT
CATCGTCTTCACCAAGGGCTATCTGAGCTACGCCACCGATGTGGCAT
GGAGAAGACGCTTGGAGACTGACGACTTTGGAACATGAAAAACT
CAAGGCCAGGATGGAGATTGTCAGAAAAATCAGAGCTCTGATGGAGA
GAACTTGATTCTTTAAGCTCAAAAGAGCAGACTCAGAGGCACACAG

**MTAP1**: AACAAGATCAATAGGCAAGTTACGTTCGAGCCGAGATGTGGG
CTGCTGAAGAAGGCCACGAGATCTCCGTTCGACGAGCGAGGTGGCCCT
CATCGTCTTCACCAAGGGCTATCTGAGCTACGCCACCGATGTGGCAT
GGAGAAGACGCTTGGAGACTGACGACTTTGGAACATGAAAAACT
CAAGGCCAGGATGGAGATTGTCAGAAAAATCAGAGCTCTGATGGAGA
GAACTTGATTCTTTAAGCTCAAAAGAGCAGACTCAGAGGCACACAG

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

MESAP1: NKINROYTFSKRRCGLLKKAHEISVLCDAEVALIVFSTKGKLFEY
ADACMEKILERYERYSAERQLTNNAETNGNWTELHAKLKRMEILQKNQK
NLMGEELDSLSLKELQNLHQ

MTAP1: NKINRxVTFSKRCGRKLKKAHEISVLCDAEVALIVFSTKGKLFEYAT
DGCMKELERYERYSAERQLTNNAETNGNWTELHAKLKRMEILQKNQKNLM
GEELDSLSLKELQNLHQ

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 residues 1-48 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.
There was one amino acid difference in between MESAPI and MTAPI caused by the C-G difference at position 146 of the nucleotide sequences. Thus the GCT sequence (position 145-147) in MESAPI that codified for Alanine was substituted by GGT (position 145-147) in MTAPI that codified for Glycine. This change fell immediately outside the MADS box region.

3.3.4 Alignment of the deduced amino acid sequences of MESAPI and MTAPI with other AP1-like amino acid sequences

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

<table>
<thead>
<tr>
<th>MESAPI</th>
<th>NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTAPI</td>
<td>Identities = 118/119 (99%)</td>
</tr>
<tr>
<td></td>
<td>NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
<tr>
<td>Eucalyptus globulus EAP2L Accession No.1120557 Identities = 114/119 (95%), Positives = 117/119 (97%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
<tr>
<td>Eucalyptus globulus EAP2S Accession No.11037010 Identities = 114/119 (95%), Positives = 117/119 (97%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
<tr>
<td>Betula pendula MADS Accession No.1483232 Identities = 100/119 (84%), Positives = 113/119 (94%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
<tr>
<td>Eucalyptus globulus EAPI Accession No.10946429 Identities = 93/119 (78%), Positives = 108/119 (90%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
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<tr>
<td>Malus domestica Accession No.3947985 Identities = 95/119 (79%), Positives = 110/119 (91%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
<tr>
<td>Nicotiana tabacum NAP1-L Accession No.4102111 Identities = 95/119 (79%), Positives = 107/119 (89%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
<tr>
<td>Populus tremuloides female Apetalal Accession No.6465897 Identities = 87/119 (73%), Positives = 106/119 (88%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
<tr>
<td>Populus tremuloides male Apetalal Accession No.6465895 Identities = 85/119 (71%), Positives = 107/119 (89%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
<tr>
<td>Brassica oleracea BOAPI Accession No.887392.Identities = 84/119 (70%), Positives = 105/119 (87%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
<tr>
<td>Antirrhinum majus SQUAMOSA Accession No.82313 Identities = 87/119 (73%), Positives = 110/119 (92%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 NKINRQVTFSKRRGGLKKAAHEISVLCDAEVALIVFSTKGKLFYEYATDGMCEKILERYER</td>
</tr>
</tbody>
</table>
Arabidopsis lyrata Apetalal Accession No. 6707086 Identities = 85/119 (71%), Positives = 108/119 (90%)

13 NKINRQVTFSRRAGLKKAHEISVLCDAEVALVFSHKKLFYSTDSCMEKILERYER 72

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

13 NKINRQVTFSRRAGLKKAHEISVLCDAEVALVFSHKKLFYSTDSCMEKILERYER 72

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

13 NKINRQVTFSRRAGLKKAHEISVLCDAEVALVFSHKKLFYSTDSCMEKILERYER 72

MESAPI
61 YSYAERQILTNNAEAGNWTLEHKRAKEMELQKNQKNGEELDLSKELQNEHQ 119

NTAPI
61 YSYAERQILTNNAEAGNWTLEHKRAKEMELQKNQKNGEELDLSKELQNEHQ 119

Eucalyptus globulus EAP2L
73 YSYAEOQYLTNNAEAGNWTLEHKRAKEMELQKNQKNGEELDLSKELQNEHQ 131

Eucalyptus globulus EAP2S
73 YSYAEOQYLTNNAEAGNWTLEHKRAKEMELQKNQKNGEELDLSKELQNEHQ 131

Betula pendula MAD2S
73 YSYAERQILANDLEQNSWTLHEAKLARLVEQROQNHHLYGEELDLSKELQNEHQ 131

Eucalyptus globulus EAP1
73 YSYAERQILASETESIGSWTLHEAKLARLVEQROQNHHLYGEELDLSKELQNEHQ 131

Malus domestica
73 YSYAERQILANDNESTQSWTLHEAKLARLVEQROQNHHLYGEELDLSKELQNEHQ 131

Nicotiana tabacum NAPI-1
73 YSYAERQILATDHETPSWTLHEAKLGRLEVQROQNHHLYGEELDLSKELQNEHQ 131

Populus tremuloides female Apetalal
62 YPYAERQLVATDSQGMWLYKRLLQNHHRHLYGEELDLSKELQNEHQ 120

Populus tremuloides male Apetalal
62 YPYAERQLVATDSQGMWLYKRLLQNHHRHLYGEELDLSKELQNEHQ 120

Brassica oleracea BOAPI
73 YSYAERQILAPESDVNVNSYMEYRLKAKELRNRHLYGEELDLSKELQNEHQ 131

Antirrhinum majus SQUAMOSA
73 YSFAERQLVESFQPSWNTLEYSKLKARLQNHHRHLYGEELDLSKELQNEHQ 131

Arabidopsis lyrata Apetalal
73 YSYAERQILAPESDVNVNSYMEYRLKAKELRNRHLYGEELDLSKELQNEHQ 131

Sinapis alba Saapl
73 YSYAERQILAPESDVNVNSYMEYRLKAKELRNRHLYGEELDLSKELQNEHQ 131

Arabidopsis thaliana API
73 YSYAERQILAPESDVNVNSYMEYRLKAKELRNRHLYGEELDLSKELQNEHQ 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

| -------- | NKinRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| API SRF Arabidopsis Accession No | 543813 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| AGL2 Accession No | 113512 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| AGL1 Accession No | 113511 |
| 16 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| FB8 Floral binding protein Petunia hybrida Accession No. | 729446 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| AGL2 Accession No. | 113512 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| AGL1 Accession No. | 113511 |
| 16 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| Floral homeotic protein DEFICIENS Antirrhinum majus Accession No. | 118426 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| Consensus | 1 |
| AGL1 Accession No. | 113511 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| AGL2 Accession No. | 113512 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| AGL1 Accession No. | 113511 |
| 16 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| Floral homeotic protein AGL15 Accession No. | 3831486 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| Cucumis sativus Agamous like putative transcription factor CAGL Accession No. | 4103342 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| Consensus | 1 |
| AGL1 Accession No. | 113511 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| AGL2 Accession No. | 113512 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| AGL1 Accession No. | 113511 |
| 16 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| Floral homeotic protein DEFICIENS Antirrhinum majus Accession No. | 118426 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |
| Consensus | 1 |
| AGL1 Accession No. | 113511 |
| 1 | MGRGKVQLKRIENKINRQVTFSKRRCGLLKKKAHEISVLCDAEVALIVFSTKGLYEYAT 47 |

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MESAP1</td>
<td>NKINRQVTFKRCGLKKAAHEISVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>Eucalyptus EAP2</td>
<td>NKINRQVTFKRCGLKKAAHEISVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>EAP1</td>
<td>NKINRQVTFKRCGLKKAAHEISVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>SQUAMOSA</td>
<td>NKINRQVTFKRCGLKKAAHEISVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>AP3</td>
<td>NTTNRQVTFCKRNRGLKKAHEVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>AGL1</td>
<td>NTTNRQVTFCKRNRGLKKAHEVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>AGL2</td>
<td>NTTNRQVTFCKRNRGLKKAHEVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>AGL3</td>
<td>NTTNRQVTFCKRNRGLKKAHEVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>AGL4</td>
<td>NTTNRQVTFCKRNRGLKKAHEVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>AGL5</td>
<td>NTTNRQVTFCKRNRGLKKAHEVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>AGL6</td>
<td>NTTNRQVTFCKRNRGLKKAHEVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>AP3</td>
<td>NTTNRQVTFCKRNRGLKKAHEVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
<tr>
<td>PI</td>
<td>NTTNRQVTFCKRNRGLKKAHEVLCDAEVALIVFSTSGKLFYATD</td>
</tr>
</tbody>
</table>

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**: ACTAGTGATTGGTTATGACAGACACCAGATGTGCTGGTCTAGTGA
TCCATATCTAAAGGAGACACTTGCACTGGATGTCGACGGACATCACCAGGCAAAA
CAGATGCACATGGGAAAGGAGGTGTTGAGTCGAGATGCGAGGCGAGAC
CATTGGTATCCACAGGGTCGAATC

**MTTFL1**: ACTAGTGATTGGTTATGACAGACACCAGATGTGCTGGTCTAGTGA
TCCATATCTAAAGGAGACACTTGCACTGGATGTCGACGGACATCACCAGGCAAAA
CAGATGCACATGGGAAAGGAGGTGTTGAGTCGAGATGCGAGGCGAGAC
CATTGGTATCCACAGGGTCGAATC

The alignment of the nucleotide sequences of METFL1 and MTTFL1 is presented below.
There was 99% homology between the nucleotides of the two fragments (Identities = 174/175).

Amino acid sequences

The deduced amino acid sequences of METFLI and MTTFLI are presented below.

**METFLI**: LVMTDPDVPGPSDPYLREHLHWMVTDIPTDDATFGKEVVEYEMPRIPNGIHREFE

**MTTFLI**: LVMTDPDVPGPSDPYLREHLHWMVTDIPTDDATFGKVEVVEYEMPRIPNGIHREFE

### 3.3.6 Alignment of the deduced amino acid sequences of METFLI and MTTFLI with other TFLI-like proteins

The alignment of the deduced amino acid sequences of METFLI and MTTFLI with other selected TFLI-like amino acid sequences in the GenBank data base of NCBI is given below.

**METFLI**

<table>
<thead>
<tr>
<th>METFLI</th>
<th>LVMTPDVPGPSDPYLREHLHWMVTDIPTDDATFGKEVVEYEMPRIPNGIHREFE</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTTFLI</td>
<td>LVMTPDVPGPSDPYLREHLHWMVTDIPTDDATFGKVEVVEYEMPRIPNGIHREFE</td>
<td>55</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>72</th>
<th>LVMTPDVPGPSDPYLREHLHWMVTDIPTDDATFGKVEVVEYEMPRIPNGIHREFE</th>
</tr>
</thead>
</table>

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

<table>
<thead>
<tr>
<th>68</th>
<th>LVMTPDVPGPSDPYLREHLHWMVTDIPTDDATFGKVEVVEYEMPRIPNGIHREFE</th>
</tr>
</thead>
</table>

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

<table>
<thead>
<tr>
<th>67</th>
<th>LVMTPDVPGPSDPYLREHLHWMVTDIPTDDATFGKVEVVEYEMPRIPNGIHREFE</th>
</tr>
</thead>
</table>
Arabidopsis thaliana Terminal flowerl Accession No. 15237535 Identities = 46/54 (85%),
Positives = 52/54 (96%)
70  LVM1DPDVPGSPSDFPLKEHLHIVTNIPGTTDATFGKEVSYELPRPSIGIHRF 123
Lolium perenne terminal flower 1-like protein Accession No. 11139708 Identities = 45/54
(83%), Positives = 51/54 (94%)
67  LVM1DPDVPGSPSDFPLREHLHIVSNIPGTTDATFGKEVMSYPEFKPGIHRF 120
Nicotiana tabacum CEN-like protein 1 Accession No. 17367234 Identities = 42/54 (77%),
Positives = 51/54 (93%)
68  L1MDPDVPGSPSDFPLREHLHIVTDIPGSTDSSFGREIVSYEPPSPVEIHR 121
Brassica napus BNTFLl-1 Accession No. 3650419 Identities = 43/54 (79%), Positives = 50/54
(91%)
70  LVMTDPDVPSPDFPLKEHLHIVSNIPGTTDATFGKEVSYEPLPKPGIHRF 123
Brassica rapa BRTFLl-1 Accession No 3650425 Identities = 43/54 (79%), Positives = 50/54
(91%)
70  LVMTDPDVPSPDFPLREHLHIVSNIPGTTDATFGKEVSYEPLPKPGIHRF 123
Brassica oleracea BOTFL1-1 Accession No. 3650429 Identities = 40/53 (75%), Positives =
47/53 (88%)
70  LVMTDPDVPSPDFPLREHLHIVSNIPGTTDATLGEVSYEPLPKPGIHRF 122
Citrus unshiu Flowering Time gene (CiFT). Accession No. 4903139 Identities = 39/54 (72%),
Positives = 45/54 (83%)
67  LVMVDPAPSPSDFLREHLHIVTDIPATTGAFGQQEIVYRSPFTP1MGHIHR 120
Oryza sativa FT-like protein Accession No. 17221650 Identities = 39/53 (73%), Positives =
45/53 (84%)
68  LVMTDPDVPSPSNPLREYELHIVTDIPGTTGATFGQEVNCYEPRFPS1MGHIHR 120
Arabidopsis thaliana flowering locus T (FT) Accession No. 15218709 Identities = 37/53
(69%), Positives = 42/53 (78%)
67  LVMTDPDVPSPSNPLREYELHIVTDIPATTGTFGQEVNCYEPRFPS1MGHIHR 119
Arabidopsis thaliana TWIN SISTER OF FT (TSF) TFLl like protein Accession No. 15237061
Identities = 37/53 (69%), Positives = 41/53 (76%)
67  LVMTDPDVPSPSNPQREYELHIVTDIPATTGNAGFQEVNCYEPRFPS1MGHIHR 119

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

METFLl and MTTFLl 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. LVMTDPDVPGSYPVLREHLMWMVTDPGTDDKFGKVEYEE--MPRPNIGHRF 54
PEBP:   65 LVMTDPDSPSNKREWLHVVNPGTDDISKGNTEYEGPPKNTGLHRY 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 *MESAPI* fragment (Figure 3.2 B). A single band could also be seen when hybridised with the *M1TFL1* fragment (Figure 3.2 C).

### 3.4 Discussion

Sequencing and homology searches revealed that *MEL* was a partial orthologue of *LFY*. Similarly, *MESAPI* and *MTAPI* were confirmed to be partial equivalents of *API* and *METFL1* and *M1TFL1*, 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 oleracea* 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 *MESAPI* and *MTAPI* 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 *API* 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 *API*. 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 Å 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, MESAPI* 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 *METF1*, 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 Southern 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™-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⁻¹ethidium 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’ GCGAATTCTTCTACCACYACHGCYGCARCG 3’
Actin reverse primer: 5’ GCGGATCCCRATCCARACACTGTAYTTC 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™ Rapid Gel Extraction System (Life Technologies) and quantified by using High DNA Mass™ Ladder (Life Technologies). Sequencing was carried out at the Massey University Sequencing 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, MESAPl, METFL1 and actin: RT-PCR was performed on RNA prepared from the twelve sampling periods (Section 4.2.1) for MEL, MESAPl 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 MESAPl. The LFY-specific primers were used for MEL (Section 3.2.5). In the case of MESAPl, 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' GCGGGATCCCAAGCTGGTGCTCCA AATTCTG 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, MESAPl, 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 MESAPl and actin, along with 0.8 µl of 1 Kb plus DNA ladder™ (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™ (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™ (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 *METFL1* 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™-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:

```
ACCACTGCTGAGCGGGAAATTTGTCGGTGACATGAAGGAAAGCTGGGATATGTTGCCCTTGACT
ATGAGCAAGAGCTGGAAGACTGCTAAGAGCAGCTGGTCGTTGAGAGAATATGAGCTGCCCG
ATGGACAAGTCATCAACTATGGGGGAGGAGTGGTCCGGTGTTCCGAGAAGTCTCTTCAGCCATC
ATTGTGGGAATGGAAGCTGGTTGCTAATTCTGATACCATCAACTTACACCCATTATCGAGATTCGAT
GTGGATATCAAGGGATTCTTTTATGGCAATATTGGCTTTAGTGGTGTCCACTATGTTGCCCTGG
TATTGCGAGACAGGATGAGCAAGGATTACTGCTTTGGCTTTCCAGACGAGATTAAGGT
GGTGCGCTCTCCTGAGGAGGTACAGTGGTTGGATTTGGG
```
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 TTAEREIVRDMKEKLYALVADYEQELETKSSSVEKKNYELPDQVITIAGERFRCPEVL 180
```

Malva pusilla actin Accession No. AF12538. Identities = 140/141 (99%)

```
204 TTAEREIVRDMKEKLYALVADYEQELETKSSSVEKKNYELPDQVITIAGERFRCPEVL 263
```

Helianthus annuus actin Accession No. AF283624. Identities = 140/141 (99%)

```
204 TTAEREIVRDMKEKLYALVADYEQELETKSSSVEKKNYELPDQVITIAGERFRCPEVL 263
```

Gossypium hirsutum actin Accession No. D88414. Identities = 139/141 (98%)

```
49 TTAEREIVRDMKEKLYALVADYEQELETKSSSVEKKNYELPDQVITIAGERFRCPEVL 108
```

Nicotiana tabacum actin Accession No. U60495. Identities = 137/141 (97%)

```
184 TTAEREIVRDMKEKLYALVADYEQELETKSSSVEKKNYELPDQVITIAGERFRCPEVL 243
```

MeA: 181 FQPSLIGMEAAGIHDTYNSIMKCVDRLKLYGNI1VLSGGSTMFGIDRMSKEITALA 360

M. pusilla: 264 FQPSLIGMEAAGIHETYNSIMKCVDRLKLYGNI1VLSGGSTMFGIDRMSKEITALA 323

H. annuus: 264 FQPSLIGMEAAGIHETYNSIMKCVDRLKLYGNI1VLSGGSTMFGIDRMSKEITALA 323

G. hirsutum: 109 FQPSLIGMEAAGIHETYNSIMKCVDRLKLYGNI1VLSGGSTMFGIDRMSKEITALA 168

N. tabacum: 244 FQPSLIGMEAAGIHETYNSIMKCVDRLKLYGNI1VLSGGSTMFGIDRMSKEITALA 303

MeA: 361 PSSMKIKVAPPKESVWIG 423

M. pusilla: 324 PSSMKIKVAPPKESVWIG 344

H. annuus: 324 PSSMKIKVAPPKESVWIG 344

G. hirsutum: 169 PSSMKIKVAPPKESVWIG 189

N. tabacum: 304 PSSMKIKVAPPKESVWIG 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, MESAPI* and *METFLI*

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. *MESAPI* showed a similar bimodal pattern except that its expression was markedly higher during organogenesis. *METFLI* appeared to be expressed at relatively lower levels than *MEL* or *MESAPI* as two rounds of PCR were needed to visualise its products. *METFLI* was expressed throughout the process of floral development studied from March to November. The levels of *METFLI* 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
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, MESAPI 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, MESAPI and METFL1 were not amplified from any possible DNA contamination.

4.3.3 MEL, MESAPI 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 MESAPI 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 Southern

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 MESAPI probe hybridised to bands of approximately 340 bp (Figure 4.3 B) seen in the RT-PCR analysis (section 4.3.2.2). MTTFLL, a heterologous probe from M. collina ‘Tahiti’ was used successfully for expression Southern for detecting METFL1. The MTTFLL 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, MESAPI and METFL1 were thus confirmed. No bands were formed for MESAPI 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.
Figure 4.3 Expression Southern 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) *MESAPI* probe hybridised to the 343 bp fragments. (C) *MTTF1I* probe hybridised to the 175 bp fragments.
4.3.5. Restriction analysis of MESAPI

The restriction map of MESAPI 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
Figure 4.4 Restriction map of MESAPI with sites of 6 bp cutters cutting once and AP1-specific primers
Figure 4.5 Restriction analysis of *MESAPI*

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

UD = RT-PCR products purified and undigested with enzymes
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 Southernss 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 MESAPI 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 MESAPI 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 \emph{BglII} cut the purified RT-PCR products of \emph{MESAPI} (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 \emph{API} specific primers (designed internally to the originally cloned and isolated fragment of \emph{MESAPI}) 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 \emph{MESAPI} (Figure 4.2 A) were products amplified from the \emph{API} equivalent in \emph{M. excelsa} rather than from any other MADS box genes.

It was evident from the calendar of floral development formed for \emph{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 \emph{M. excelsa} before that. In \emph{A. thaliana}, the establishment of floral meristems is primarily regulated by \emph{LFY} and \emph{API} and their expression is observed prior to visible morphological changes (Yanofsky, 1995). Hempel et al. (1997) demonstrated the utility of \emph{API} expression as an indicator of floral determination in \emph{Arabidopsis} as its expression could be detected only in meristems after floral induction. \emph{MEL} and \emph{MESAPI} were detected in the RT-PCR analysis and the expression Southern's as early as March. Thus it became evident that floral determination in \emph{M. excelsa} would have occurred by then.

\emph{MEL} and \emph{MESAPI} 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 \emph{lfy} and \emph{ap1} mutants, shoot-like structures are formed instead of flowers in \emph{Arabidopsis} and \emph{Antirrhinum} (Ma, 1998). It could be speculated that with downregulation of \emph{MEL} and \emph{MESAPI} during winter, a similar effect is produced to form shoot-like structures which become the pedicels in \emph{M. excelsa}. It might further be speculated that this is how flower pedicels are formed.
in plants generally.

The expression of both MEL and MESAPI expressions was upregulated again from August-September with the onset of longer days and warmer temperatures. MESAPI 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 1-1½ 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 MESAPI 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, MESAPI 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 Southern 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, API* 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 tfll mutations were weakened by environmental conditions such as SD that delayed the onset of flowering. Also, all tfll 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 \textit{TFL1} equivalents from other herbaceous plants. The \textit{Antirrhinum} gene \textit{CEN} and the \textit{Arabidopsis} gene \textit{TFL1} were shown to be homologous, which suggested that a common mechanism influenced indeterminacy in these plants. The \textit{Arabidopsis} \textit{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 \textit{TFL1} homologue (\textit{NCH}) was expressed at a low level throughout plant development (Kato et al., 1998). The \textit{SELF-PRUNING} gene, which is the \textit{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 \textit{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 \textit{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 \textit{LFY}, \textit{AP1} and \textit{CAL}, prevent \textit{TFL1} transcription in floral meristems on the apex periphery. \textit{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 \textit{LFY} or \textit{AP1}. Ratcliffe et al. (1999) suggested that the wild type pattern of \textit{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 \textit{CO} and \textit{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 \textit{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 \textit{MEL, MESAP1} and \textit{METFL1} could thus have significance in the flowering process in \textit{M. excelsa}. 


The results from the temporal expression analysis of *MEL* and *MESAPI* showed that the expression of both *MEL* and *MESAPI* 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 *MESAPI* 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 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 MESAPI. It was also hypothesised that the expression of MESAPI, 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.
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, MESAPI and MTTFL1 in pGEM-T vectors. The plasmids (1 μg) with the inserts were linearised using Sphl or SalI restriction enzymes (Roche). The templates were purified using the Concert™ 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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>12 μl</td>
</tr>
<tr>
<td>10x transcription buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>RNase inhibitor (20 U μl⁻¹)</td>
<td>1 μl</td>
</tr>
<tr>
<td>NTP labeling mix</td>
<td>2 μl</td>
</tr>
<tr>
<td>[10x (10mM ATP, CTP and GTP and 6.5 mM UTP, 3.5mMDIG-11-UTP)]</td>
<td></td>
</tr>
<tr>
<td>Linearised and purified template DNA(1μg μl⁻¹)</td>
<td>1 μl</td>
</tr>
<tr>
<td>T7 or SP6 RNA polymerase (20 U μl⁻¹)</td>
<td>2 μl</td>
</tr>
<tr>
<td></td>
<td>20 μl</td>
</tr>
</tbody>
</table>

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 MESAPI 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 1 x 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) 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 substrate 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 T1 E1 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
10x 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
10x PBS: 1.3 M NaCl, 30 mM NaH₂PO₄, 70 mM Na₂HPO₄
Proteinase K solution: 100 mM Tris (pH 7.5), 50 mM EDTA
RNase buffer: 10 mM Tris (pH 7.5), 1 mM EDTA, 500 mM NaCl
20x SSPE: 0.2 M NaH₂PO₄, 20 mM EDTA, 3 M NaCl
TNM-50 buffer: 100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM MgCl₂
t₀E₁ 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, MESAPI 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, MESAPI 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 MESAPI 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.
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.
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 bracteoles and flowers start differentiating in the cymule primordia. (E) *MESAP1* expression confined to floral primordia in the cymule in late August.
No expression of *MESAPI* 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, *MESAPI* 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 *MESAPI* 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).
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: APETALAI (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 MESAPI 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 MESAPI was confined to the outer whorls, it implied that MESAPI would be acting in a similar way to API 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 MESAPI l 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). MESAPI l expression was more similar to that of AP1 than SQUA as it was not detected in the carpels. The fact that MESAPI l 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 MESAPI l showed that it was a Class A gene. The fact that MESAPI l 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, MESAPI* 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 *API1/ AP2* function. As a consequence, *TFL1* was considered not to influence floral meristem development, while *LFY, API* 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 *API1/ 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 *API* and *LFY* expression from the inflorescence meristem. This negative regulation could be mutual because *TFL1* expression was downregulated in plants constitutively expressing *API*. 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 *API*, together with negative interactions of these two genes with *TFL1*.

The spatial expression patterns of *MEL, MESAPI1* and *METFL1* also confirmed that the fragments of these genes cloned from *M. excelsa* were the partial orthologues of *LFY, API* 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 *MESAPI1* 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 *MESAPI1* were floral meristem identity genes was supported as they were expressed in floral primordia and floral tissues. The hypothesis that *MESAPI1*, 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 *MESAPI1* 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 \textit{gal-3 (gibberellin requiring 1-3)} mutation, which has severely reduced endogenous GA levels, abolished the upregulation of \textit{LFY} and prevented floral transition in \textit{Arabidopsis}. The exogenous application of GA restored both (Wilson et al., 1992; Blasquez et al., 1998). \textit{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 \textit{M. collina}, applied GAs, especially GA3 and GA7, 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 \textit{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 \textit{MEL} to application of GA3 was therefore of interest. Consequently, juvenile \textit{M. excelsa} plants were grown in a glasshouse and treated with growth retardants with or without GA3. As \textit{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 GA3 to enhance GA levels. As expression of \textit{API} is regarded as indicative of floral commitment, and upregulation of \textit{LFY} has been shown to cause upregulation of \textit{API} (Ma, 1998), the expression of MESAPI was also monitored.

6.2 Materials and methods

Micropropagated plants of \textit{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 (GA₃ 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 GA₃ (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 GA₃. 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 + GA₃, prohexadione + GA₃ and paclobutrazol + GA₃.

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 MESAPI, 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 GA3 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 GA3 recorded higher growth than those without GA3 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 GA3.

RT-PCR and expression Southern showed that MEL was upregulated compared to the water control by both the growth retardants as well as by GA3 (Figure 6.2). MESAPI 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 GA3. The upregulation of MEL with GA3 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 GA19 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 GA$_3$
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 GA₃ 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 (Henriot 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*, *API* 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 *API* (*MESAPI* and *MTTAPI*) 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 *API*, *TFL1* and *LFY*. Putative conserved domains were detected for *MESAPI*, *MTTAPI*, *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 cymeule 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 from the temporal expression analysis of MEL and MESAP1 (Figures 4.2 A, 4.3 A and 4.3 B) showed that the expression 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 MESAPI expression
excelsa trees in June at the time when the level of MEL expression was low (Figure 7.1). GA1 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 Southern 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 GA3 (plus appropriate controls). The buds when analysed for MEL and MESAP1 expression revealed that MEL expression was upregulated with GA3 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 meristerns are formed, and FLO is found to be expressed in the bracts and early floral meristems (Ma, 1998). 

In situ hybridisation for MESAPI 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). MESAPI 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 Southern, 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 *TFL1* 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 *API* (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 *MESAPI* 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 *MESAPI*. *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 $MESAPI$ 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 $M. \text{excelsa}$ fit a broader herbaceous model encompassing $\text{Antirrhinum}$ as well as $\text{Arabidopsis}$.

For future work, it would be interesting to clone full-length genes of $MEL$, $MESAPI$ and $METFL1$ and conduct complementation studies in $\text{Arabidopsis}$ 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 $M. \text{excelsa}$ and see how they differed in their expression patterns from the long day annual $\text{Arabidopsis}$. Another area of future research is to conduct similar studies on $M. \text{collina}$ ‘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.
References


Rouse, D. T., Sheldon, C. C., Bagnall, D. J., Peacock, W. J. and Dennis, E. S. (2002) FLC,
a repressor of flowering, is regulated by genes in different inductive pathways. 


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$^{-1}$

<table>
<thead>
<tr>
<th>LB (Luria Broth):</th>
<th>Tryptone</th>
<th>10.0 g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td></td>
<td>MilliQ to</td>
<td>1.0 L</td>
</tr>
<tr>
<td>Sterilise by autoclaving</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>TAE buffer (10 x):</th>
<th>Tris</th>
<th>48.4 g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDTA</td>
<td>7.4 g</td>
</tr>
<tr>
<td></td>
<td>Glacial acetic acid</td>
<td>11.4 ml</td>
</tr>
<tr>
<td></td>
<td>MilliQ to</td>
<td>1.0 L</td>
</tr>
<tr>
<td>pH to 8.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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<sup>R</sup>-T and pGEM<sup>R</sup>-T

Easy vectors

![Diagram of vector circle maps](image-url)
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 CaCl₂ and resuspend cells.
Add additional 10 ml 60 mM CaCl₂. 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 CaCl₂, 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 -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

<table>
<thead>
<tr>
<th>GA (ng.g FW⁻¹)</th>
<th>29 + 8</th>
<th>1</th>
<th>20</th>
<th>29</th>
<th>53</th>
<th>8</th>
<th>19</th>
<th>44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.25</td>
<td>1.14</td>
<td>2.65</td>
<td>0.48</td>
<td>NF</td>
<td>2.77</td>
<td>1.62</td>
<td>NF</td>
</tr>
<tr>
<td>Prohexadione</td>
<td>3.86</td>
<td>1.40</td>
<td>2.60</td>
<td>0.50</td>
<td>NF</td>
<td>3.36</td>
<td>3.60</td>
<td>NF</td>
</tr>
<tr>
<td>Paclobutrazol</td>
<td>0.59</td>
<td>0.14</td>
<td>0.14</td>
<td>0.04</td>
<td>NF</td>
<td>0.55</td>
<td>3.14</td>
<td>NF</td>
</tr>
</tbody>
</table>

Clemens (1999). Unpublished data
Appendix VI

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

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Juvenile or mature</th>
<th>Part sampled</th>
<th>GA concentration (ng.g FW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GA₃₃</td>
</tr>
<tr>
<td>Winter (June)</td>
<td>juvenile</td>
<td>leaves</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>mature</td>
<td>leaves</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>mature</td>
<td>overwintering buds</td>
<td>1.08</td>
</tr>
<tr>
<td>Summer (November)</td>
<td>mature</td>
<td>leaves</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>mature</td>
<td>single flower buds</td>
<td>&gt;15</td>
</tr>
</tbody>
</table>

Clemens (1999). Unpublished data
Appendix VII
Primer regions

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

*KKAGASYINKPKDHRHYVCYALHCMDHASNLRKSFKGERGENVGAWRQACYHPLVTAGRARGVWDIDATFNAMHRPLCIW*

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

*MGGRVQLKRIENRQYFSTKRHLGFKKAHEISLVDCAEVALVVFHSGKFLFSETDFCPMEKILERERETRASYAERQLIAPSDVNTNWSMEYNNLRKAKIELRNQRYHLGEDLQAMSPKELNLEQLOLTALKHRSHGRKQLMYEINTLQRKKEKAIQNEQMSLKSQIKERENVLRAAQEQWDEQNEQHGNHNPFPFPQHHQIQHPYMLSHQPSFLMNGLYQEDQAMRRNGLDSLEPVYNCNLGSF AA*

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

### **Lolium perenne**

---

**QGGDLRSLFTLVMTDFVPGSDPYLELHHLWTVSNIPGTDPDDGFGEVMSYEPKSNIG**

**56**

### **Oryza sativa**

---

**QGGDMRSLFTLVMTDFVPGSDPYLELHVLTVTDIPGTDSDFGEVMSYEPKSNIG**

**56**

### **Nicotiana**

---

**HGGDDLRSFVTL LyftDFPVDGSPDPYLREHHLWTVTDIPGTDCSFGEVMSYEPKSNIG**

**117**

### **Lycopersicon esculentum**

---

**HGGDLRSLFTLVMTDFVPGSDPYLELHHLWTVTDIPGTDCSFGEVMSYEPKSNIG**

**119**

### **Arabidopsis thaliana**

---

**IHRFIFVLFKQKKRRQTVS-VPSSFRHDNTQFQAVNDLGFLVAAVYYFCRRTARAA--**

**173**

### **Citrus unshiu**

---

**IHRFVFVLFKQKKRRQAVV-VPSSFRHDNTQFQAEENELGLVAAVYYFCRRTARAA--**

**173**

## **Metasideros**

### **Lolium**

---

**QGGDLRSLFTLVMTDFVPGSDPYLELHVLWTVSNIPGTDPDDGFGEVMSYEPKSNIG**

**116**

### **Oryza**

---

**QGGDMRSLFTLVMTDFVPGSDPYLELHVLWTVTDIPGTDSDFGEVMSYEPKSNIG**

**117**

### **Nicotiana**

---

**HGGDDLRSFVTL LyftDFPVDGSPDPYLREHHLWTVTDIPGTDCSFGEVMSYEPKSNIG**

**119**

### **Lycopersicon**

---

**IHRFIFVLFKQKKRRQTVS-VPSSFRHDNTQFQAVNDLGFLVAAVYYFCRRTARAA--**

**173**

### **Arabidopsis**

---

**IHRFVFVLFKQKKRRQAVV-VPSSFRHDNTQFQAEENELGLVAAVYYFCRRTARAA--**

**173**

### **Citrus**

---

**IHRFVFVLFKQKKRRQTVS-VPSSFRHDNTQFQAVNDLGFLVAAVYYFCRRTARAA--**

**177**

---

**TFL1 outer forward primer** **TFL1 nested forward primer**

**TFL1 outer reverse primer**  **TFL1 nested reverse primer**