# A Molecular Analysis of Flowering in *Metrosideros*

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

Master of Science in Plant Biology

at Massey University, Palmerston North, New Zealand

Alexa Jury

2005

# Abstract

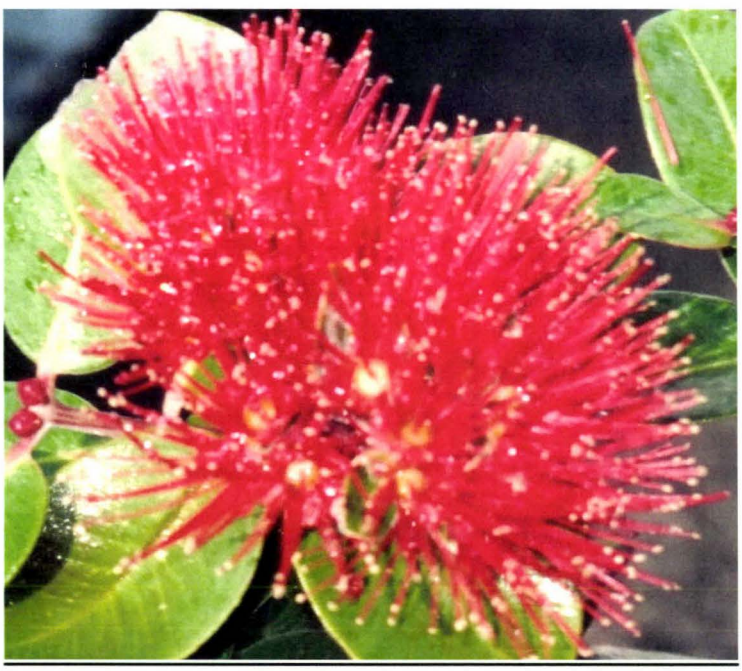
A calendar of floral and vegetative developmental events has been previously determined for the woody perennial *Metrosideros excelsa*. *M. excelsa* flowers just once a year in summer and bud development has been related to seasonality and gene expression. *M. collina* cv. Tahiti is closely related to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

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

# Acknowledgements

I would like to express my appreciation for the encouragement, support and patience of my supervisor, Professor Paula Jameson, for the duration of my Masters, and especially for speedy reading skills at the end. Many thanks to John Clemens for helping me out of the mess of tangled data, and also for rapid reading. Thanks to Ann and Cynthia for distracting me when I should have been writing, but also for their support.

I am very grateful to my friends, especially Sasha, Kitty, and Enid, my current lab (particularly Maria and Jules), and my family for their support, and for knowing when not to talk about this specific subject. They have also put up with my moaning and 'I hate my thesis' moments, of which there were many. I have particular gratitude for Liz who got excited for me near the end, which allowed me to concentrate and finish. Finally, special thanks to my fairies, spiders and plants.



*M. collina* cv. Tahiti

# Table of Contents


Abstract ii

Acknowledgements iii

Table of Contents iv

List of Figures ix

List of Tables xii

**Chapter One Introduction**

1.1 Overview 1

1.2 Pathways that regulate flowering 2

1.2.1 Regulation of flowering by photoperiod 5

1.2.2 Regulation of flowering by gibberellins 7

1.2.3 Regulation of flowering by temperature 12

1.2.4 Regulation of flowering by nutrients and sucrose 14

1.2.5 Autonomous floral development 18

1.3 Flowering time genes 20

1.3.1 *FLOWERING LOCUS C* 21

1.4 Inflorescence meristem identity genes 23

1.4.1 *TERMINAL FLOWER 1* 23

1.5 Floral meristem identity genes 25

1.5.1 *LEAFY* 25

1.5.2 *APETALA1* 29

1.6 The genetic interactions of floral genes in *Arabidopsis* 32

1.7 Flowering in *Metrosideros* 34

1.8 Rationale and aims of this research 36

1.8.1 Specific objectives 37

1.8.2 Presentation of results 38

## Chapter Two Materials and methods


2.1 Plant material and measurements of shoot growth 39

2.2 Plant growth regulator and plant hormone applications 39

2.3 Measurements of bud growth across the season 41

2.4 Methods for extracting genomic DNA 43

2.4.1 CTAB maxi preparation 45

2.4.2 Cesium chloride density centrifugation 46

2.4.3 Modified CTAB mini preparation 48

2.4.4 Quantification of DNA 49

2.5 RNA extraction methods 49

2.5.1 'Hot Borate' maxi preparation 49

2.5.2 Modified 'Hot Borate' mini preparation 51

2.5.3 Tri reagent mini preparation 52

2.5.4 RNA quantification 53

2.6 *DNase1* treatment of RNA 53

2.7 Reverse transcriptase polymerase chain reaction (RT-PCR) 54

2.7.1 RT-PCR of *M. excelsa* and *M. collina* equivalents of *LEAFY* 54

2.7.2 RT-PCR of *M. excelsa* and *M. collina* equivalents of *APETALA1* 55

2.7.3 RT PCR of *ACTIN* 55

2.8 Polymerase chain reaction (PCR) of FLOWERING LOCUS C (*FLC*) 56

2.9 Sequencing of isolated DNA and cDNA fragments 59

2.10 Restriction enzyme digests of genomic DNA from *Meterosideros* 59

2.11 Southern blotting 60

2.11.1 Probe synthesis 61

2.11.2 Probe labeling 63

2.11.3 Probe hybridisation and stringency washes 63

2.11.4 Detection of labelled probe 64

2.12 Histological techniques 65

2.12.1 Processing and embedding of *M. collina* samples 65

2.12.2 Schedule for staining slides 67

**Chapter Three Growth and development of *Metrosideros.***

3.1 Introduction 69

3.2 The effect of plant growth regulators and hormones on plant growth 70

3.3 Bud growth measurements of adult *M. collina* cv. Tahiti 75

3.4 Histological investigation of bud growth and development of *M. collina* cv. Tahiti 83

3.4.1 Vegetative bud development 83

3.4.2 Floral bud development 87

3.5 Discussion 90

3.5.1 Morphological and physiological changes resulted from applications of $GA_3$ and paclobutrazol 90

3.5.2 *M. collina* bud development may be linked to temperature 91

3.5.3 *M. collina* bud developmental sequence 93

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

4.1 Introduction and hypotheses 96

4.2 Development and optimisation of protocols 97

4.2.1 Comparison of DNA extraction method 97

4.2.2 Comparison of RNA extraction methods 98

4.3 Nucleotide sequences 100

4.3.1 *LFY*-like sequences 100

4.3.2 *AP1*-like sequences 103

4.4 Amino acid sequences 103
4.4.1 LFY-like sequences 103
4.4.2 AP1-like sequences 105
4.5 Phylogenetic relationships 107
4.6 Southern blotting of *M. excelsa* and *M. collina* genomic DNA 116
4.6.1 Detection of *LFY*-, *AP1*-, and *TFL-1*-like genes 116
4.7 Gene expression in response to growth regulator and hormone applications in *Metrosideros* 116
4.7.1 Expression of *MEL* and *MESAP1* in adult, seedling, and micropropagated *M. excelsa* 116
4.7.2 Expression of *MCL* and *MTAP1* genes in micropropagated and adult *M. collina* 120
4.7.3 Temporal expression of *MCL* and *MTAP1* in adult *M. collina* 120
4.8 Discussion 120
4.8.1 *MCL* and *MTAP1* were isolated from *M. collina* cv. Tahiti 120
4.8.2 *MEL* and *MCL* were downregulated by $GA_3$ application 124

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

5.1 Introduction and hypotheses 127
5.2 Initial isolation and analysis of *FLC*-like sequences 127
5.2.1 Nucleotide sequences 128
5.2.2 Amino acid sequences 133
5.3 Discussion 133

**Chapter Six Final discussion**

6.1 Final discussion 136
6.2 Future directions 138

**Bibliography** 139

## Appendices

Appendix I Recipes for common buffers and media 148

Appendix II List of abbreviations for Figure 4.5 150

Appendix III List of abbreviations for Figure 4.6 151

# List of Figures

## Chapter One


**Figure 1.1** The input pathways that regulate the transition from vegetative to reproductive phase in *Arabidopsis.* 4

**Figure 1.2** The interactions of proteins involved in regulating the photoperiodic pathway of flowering in *Arabidopsis.* 6

**Figure 1.3** The interactions of proteins involved in regulating the gibberellin pathway of flowering in *Arabidopsis.* 11

**Figure 1.4** The interactions of proteins involved in regulating the vernalisation pathway of flowering in *Arabidopsis.* 15

**Figure 1.5** The interactions of proteins involved in regulating the sucrose pathway of flowering in *Arabidopsis.* 17

**Figure 1.6** The interactions of proteins involved in regulating the autonomous pathway of flowering in *Arabidopsis.* 19

**Figure 1.7** The interactions between flowering time gene products, inflorescence meristem identity genes, and floral meristem identity genes in *Arabidopsis* to promote and repress flowering. 33


## Chapter Two


**Figure 2.1** Measurement of shoot growth. 40

**Figure 2.2** *M. collina* floral and vegetative bud measurements. 44

**Figure 2.3** Flow chart for tissue dehydration and infiltration for paraffin embedding of *M. collina* buds. 66

**Figure 2.4** Schedule for staining *M. collina* bud sections. 68


## Chapter Three


**Figure 3.1** The inhibitory effect of paclobutrazol on plant growth. 72

**Figure 3.2** Observable physiological response of micropropagated *M. excelsa* plants to paclobutrazol, other plant growth regulators and plant hormone one month after the last application. 73

**Figure 3.3** Proportions of *M. collina* bud type development and dates of bud development. 77

**Figure 3.4** Percentage of adult *M. collina* vegetative bud break in each of the three sample sets in relation to bud diameter. 80

**Figure 3.5** Percentage of adult *M. collina* Set 1 vegetative bud break and the percentage of Set 2 floral bud break in relation to bud diameter. 82

**Figure 3.6** Histological analysis of vegetative bud development of M. *collina*. 86

**Figure 3.7** Histological analysis of floral bud development of *M. collina*. 90

## Chapter Four

**Figure 4.1** Isolation of a partial *LFY* equivalent from *M. collina*. 101

**Figure 4.2** Isolation of a partial *AP1* equivalent from *M. collina*. 102

**Figure 4.3** Comparison of deduced amino acid sequence of the putative LFY-like sequence from *M. collina* and LFY sequences from other plant species. 104

**Figure 4.4** Comparison of deduced amino acid sequence of the putative AP1-like sequence from *M. collina* and AP1 sequences from other plant species. 110

**Figure 4.5** The phylogenetic relationship of the putative LFY-like sequence from *M. collina* and LFY sequences from other plant species. 113

**Figure 4.6** Phylogenetic relationship of the putative AP1-like sequence from *M. collina* and AP1 sequences from other plant species. 115

**Figure 4.7** Southern blots of *M. excelsa* and *M. collina LFY-*, *AP1-*, and *TFL1*-like genes. 117

**Figure 4.8** Expression of *MEL* and *MESAP1* in response to the exogenous applications of growth regulators and plant hormones to adult, seedling, and micropropagated *M. excelsa*. 118

**Figure 4.9** Expression of *MCL* and *MTAP1* genes in response to the exogenous application of $GA_3$ to micropropagated and adult *M. collina*. 121

**Figure 4.10** Temporal expression of *MCL* and *MTAP1* in adult *M. collina* in response to the exogenous application of water. 122

**Figure 4.11** Temporal expression of *MCL* and *MTAP1* in adult *M. collina* response to the exogenous application of $GA_3$. 123

## Chapter Five

**Figure 5.1** Isolation of *FLC* from *Arabidopsis* using FLC-1 primers. 129

**Figure 5.2** Isolation of *FLC* from *Arabidopsis* using FLC-3 primers. 132

# List of Tables

## Chapter Two

**Table 2.1** Details of the first foliar applications of the growth regulators and hormones applied to *Metrosideros* plants. 42

## Chapter Three

**Table 3.1** Percentage of vegetative and floral buds of the *M. collina* buds collected for histological studies. 84

## Chapter Four

**Table 4.1** A comparison of DNA yield and quality extracted using the methods described in Chapter Two. 99

**Table 4.2** A comparison of RNA yield and quality extracted using the methods described in Chapter Two. 99

**Table 4.3** The percent amino acid identity and similarity of the MCL sequence with other *LFY*-like sequences. 106

**Table 4.4** The percent amino acid identity and similarity of the MTAP1 sequence with other AP1-like sequences. 108

## Chapter Five

**Table 5.1** The homology of both the large and small DNA fragments isolated using the FLC-1 primers. 131

**Table 5.2** The homology of both the medium and small DNA fragments isolated using the FLC-3 primers. 134

# CHAPTER ONE

# Introduction

## 1.1 Overview

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

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

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

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

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

## 1.2 Pathways that regulate flowering

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

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

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

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

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

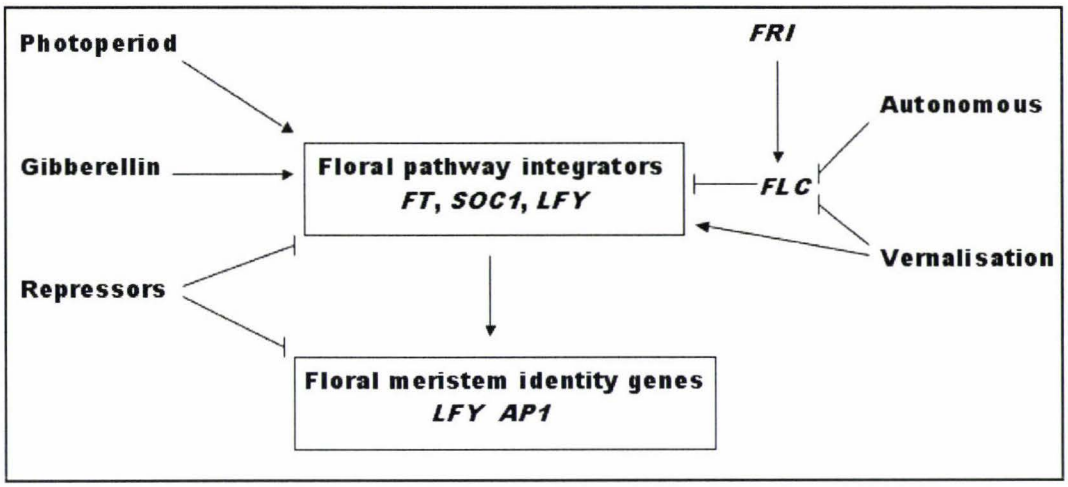



**Figure 1.1** The input pathways that regulate the transition to flowering in Arabidopsis. Modified from simpson and Dean, (2003).

### 1.2.1 Regulation of flowering by photoperiod

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

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

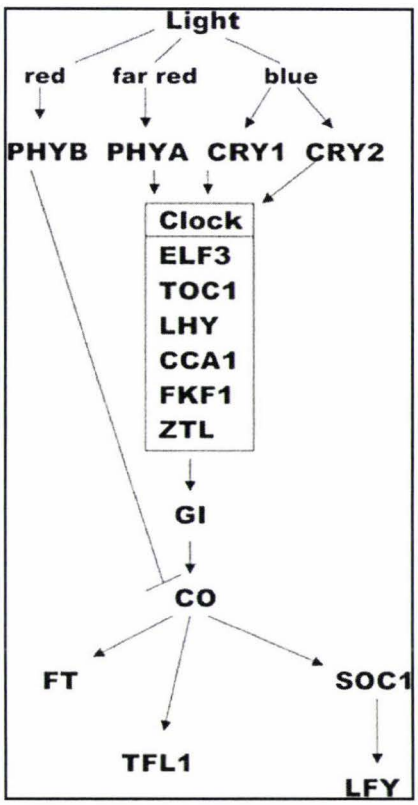



**Figure 1.2** The interactions of proteins involved in regulating the photoperiodic pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

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

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

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

### 1.2.2 Regulation of flowering by gibberellins

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

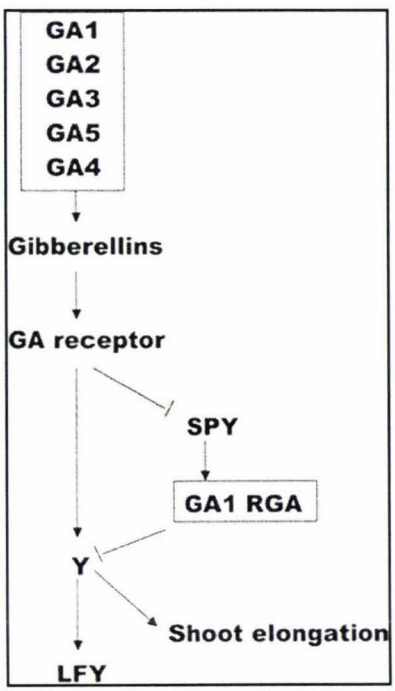



**Figure 1.3** The interactions of proteins involved in regulating the gibberellin pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

### 1.2.3 Regulation of flowering by temperature

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

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

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

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

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

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

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

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

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

### 1.2.4 Regulation of flowering by sucrose

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

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

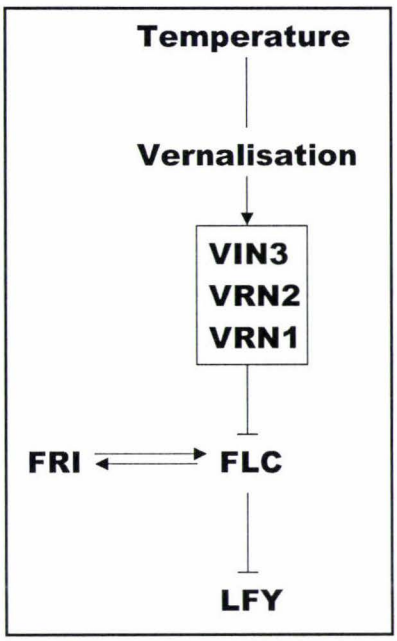



**Figure 1.4** The interactions of proteins involved in regulating the vernalisation pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

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

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

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

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

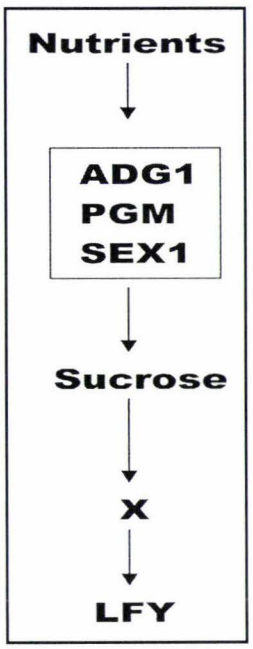



**Figure 1.5** The interactions of proteins involved in regulating the nutrients and sucrose pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

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

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

### 1.2.5 Autonomous floral development

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

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

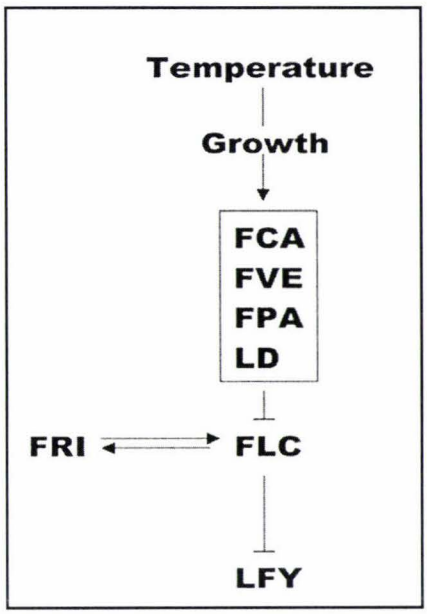



**Figure 1.6** The interactions of proteins involved in regulating the autonomous pathway of flowering in *Arabidopsis*. Diagram modified from Blazquez (2000).

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

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

## 1.3 Flowering time genes

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

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

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

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

### 1.3.1 *FLOWERING LOCUS C*

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

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

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

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

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

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

## 1.4 Inflorescence meristem identity genes

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

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

### 1.4.1 *TERMINAL FLOWER 1*

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

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

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

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

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

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

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

## 1.5 Floral meristem identity genes

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

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

### 1.5.1 *LEAFY*

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

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

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

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

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

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

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

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

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

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

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

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

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

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

### 1.5.2 *APETALA1*

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## 1.6 The genetic interactions of floral genes in *Arabidopsis*

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

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

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

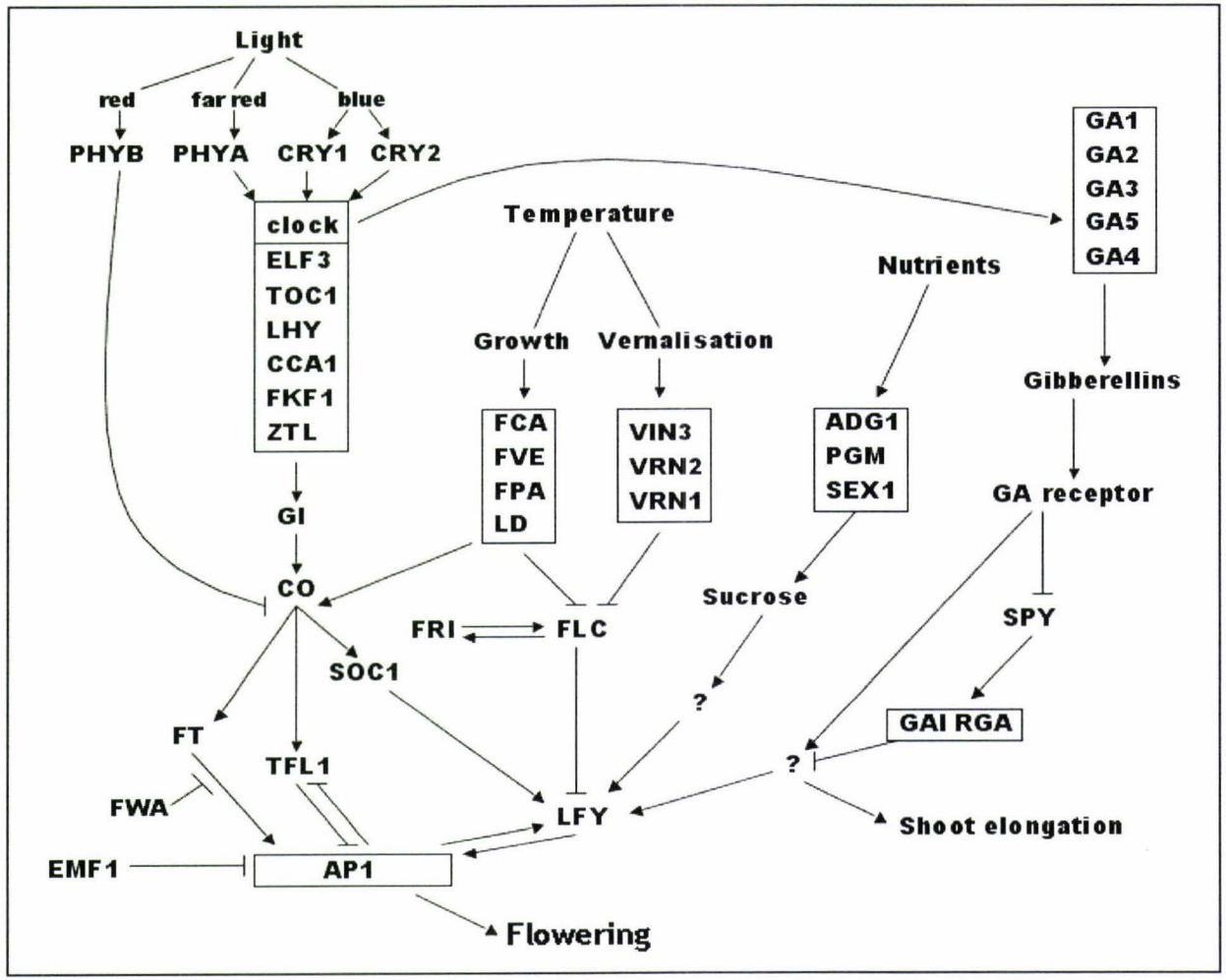



**Figure 1.7** The interactions between flowering time gene products, inflorescence meristem identity genes, and floral meristem identity genes in *Arabidopsis* to promote and repress flowering. Modified from Blazquez (2000).

## 1.7 Flowering in *Metrosideros*

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

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

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

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

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

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

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

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

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

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

## 1.8 Rationale and aims of this research

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

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

### 1.8.1 Specific objectives

Specific objectives of this work were:

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

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

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

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

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

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

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

*collina*.

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

### 1.8.2 Presentation of results

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

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