Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.
Molecular studies of flowering in *Metrosideros excelsa*  
(*Myrtaceae*)

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

**Doctor of Philosophy**  
in  
**Plant Biology**  

at Massey University, Palmerston North,  
New Zealand

Lekha Sreekantan  
2002
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 *AP1* 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*. 
Acknowledgements

It is my greatest pleasure to place on record my thanks and gratitude to my supervisor and mentor, Professor Paula E. Jameson, who took me under her wings when I first approached Massey University to do doctoral research. She has been a friend and guide to me and a very venerable guru. She always had time to listen to my worries and was there to support me when I “stumbled”. It was her constant encouragement that helped me achieve all that is in this thesis and I am very much indebted to her for the careful corrections of this work.

Dr John Clemens, my co-supervisor was always helpful with suggestions on the research programme. I remember with gratitude, his help in setting up the glasshouse experiment and how he helped me in managing the plants. I am very thankful to him for the astute corrections of the drafts.

Dr Marian McKenzie, my other co-supervisor, introduced me to my first PCRs and gels. She had the answers to all my molecular biology doubts. I recollect with gratitude, those days we went “bud-harvesting” together. The tediousness of the job was never felt because Marian was there to help and give company.

Ivan Galis, post-doctoral fellow, was of immense help to me in discussing new ideas and techniques. I am greatly indebted to him for his unstinting support and help. He was there to give advice and company when I did the ‘Southerns’.

Liz Nickless introduced me to microscopy and wax embedding. Thank you Liz for all the time you spent in helping me view meristems under the confocal microscope and for recording the images with the digital camera. I am also thankful to Suzanne, Rob, Jason and all the other students in the molecular plant physiology lab. They were all good friends. I also am grateful to Geraldine and Charlotte, who were technicians in the lab.

Finally, I acknowledge Public Good Science Fund Native Ornamental Plants Programme for partly funding the project and Massey University for the Doctoral Scholarship.
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 37
2.1 Introduction 37
2.2 Microscopy techniques for viewing meristems of M. excelsa 37
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1 Plant material and study site</td>
<td>37</td>
</tr>
<tr>
<td>2.2.2 Techniques</td>
<td>38</td>
</tr>
<tr>
<td>2.2.3 Results</td>
<td>43</td>
</tr>
<tr>
<td>2.2.4 Discussion</td>
<td>47</td>
</tr>
<tr>
<td>2.3 Calendar of floral development in <em>Metrosideros excelsa</em></td>
<td>49</td>
</tr>
<tr>
<td>2.3.1 Materials and methods</td>
<td>49</td>
</tr>
<tr>
<td>2.3.2 Results</td>
<td>49</td>
</tr>
<tr>
<td>2.3.4 Discussion</td>
<td>55</td>
</tr>
<tr>
<td>Chapter 3. Isolation, cloning and Southern analysis of floral and inflorescence meristem identity genes from <em>Metrosideros</em> species.</td>
<td>58</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>58</td>
</tr>
<tr>
<td>3.2 Materials and Methods</td>
<td>59</td>
</tr>
<tr>
<td>3.2.1 Extraction and purification of DNA</td>
<td>59</td>
</tr>
<tr>
<td>3.2.2 Extraction of RNA</td>
<td>61</td>
</tr>
<tr>
<td>3.2.3 Polymerase chain reaction (PCR)</td>
<td>63</td>
</tr>
<tr>
<td>3.2.4 Reverse transcription-polymerase chain reaction (RT-PCR)</td>
<td>64</td>
</tr>
<tr>
<td>3.2.5 Isolation of the partial orthologue of <em>LEAFY (MEL)</em> from <em>M. excelsa</em></td>
<td>64</td>
</tr>
<tr>
<td>3.2.6 Isolation of the partial orthologues of <em>APETALA1</em> from <em>M. excelsa (MESAP1)</em> and <em>M. collina</em> ‘Tahiti’ (MTAP1)</td>
<td>65</td>
</tr>
<tr>
<td>3.2.7 Isolation of the partial orthologue of <em>TERMINAL FLOWER1</em> from <em>M. excelsa (METFL1)</em> and <em>M. collina</em> ‘Tahiti’ (MTTFL1)</td>
<td>65</td>
</tr>
<tr>
<td>3.2.8 Cloning of the gene fragments</td>
<td>66</td>
</tr>
<tr>
<td>3.2.9 DNA plasmid preparation</td>
<td>67</td>
</tr>
<tr>
<td>3.2.10 Sequencing</td>
<td>67</td>
</tr>
<tr>
<td>3.2.11 Southern Analysis</td>
<td>68</td>
</tr>
<tr>
<td>3.3 Results</td>
<td>70</td>
</tr>
<tr>
<td>3.3.1 Sequences of <em>MEL</em></td>
<td>70</td>
</tr>
</tbody>
</table>
3.3.2 Alignment of the amino acid sequence of MEL with other LFY-like amino acid sequences 72
3.3.3 Sequences of MESAPI and MTAPI 74
3.3.4 Alignment of the deduced amino acid sequences of MESAPI and MTAPI 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 TFLI-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, MESAPI, 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, MESAPI and METFLI 94
4.2.6 Restriction enzyme analysis of MESAPI 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
   4.3.3 PCR of RNA 99

4.3.4 Expression Southern 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 126

---

### 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

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix I</td>
<td>Recipes for common buffers and media</td>
<td>169</td>
</tr>
<tr>
<td>Appendix II</td>
<td>Vector circle maps of pGEM®-T and pGEM®-T Easy vectors</td>
<td>170</td>
</tr>
<tr>
<td>Appendix III</td>
<td>Preparation of competent cells</td>
<td>171</td>
</tr>
<tr>
<td>Appendix IV</td>
<td>DNA plasmid preparations</td>
<td>172</td>
</tr>
<tr>
<td>Appendix V</td>
<td>GAs in juvenile seedlings</td>
<td>173</td>
</tr>
<tr>
<td>Appendix VI</td>
<td>Endogenous gibberellins in <em>M. excelsa</em> in winter (June) and in summer (November)</td>
<td>174</td>
</tr>
<tr>
<td>Appendix VII</td>
<td>Primer Regions</td>
<td>175</td>
</tr>
</tbody>
</table>
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*.

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