

Cytosine methylation, methyltransferases
and flowering time in *Arabidopsis thaliana*

Ruth Kathleen Genger

A thesis submitted for the degree of Doctor of Philosophy
of The Australian National University

April 2000

Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this thesis contains no material previously published, or the result of any work by any other person, except where due reference is made in the text.

Ruth Kathleen Genger

Acknowledgements

First I would like to thank my CSIRO supervisors, Liz Dennis and Jean Finnegan, for their support, guidance and patience throughout my PhD studies. I especially appreciated Jean's enthusiasm, her advice on my experimental work, and her constructive comments during the writing process. Thanks to Liz for her constant encouragement and her helpful comments and insights. To my ANU supervisors, Dennis Bittisnich, Michael Udvardi and Kieran Kirk, thank you for maintaining my ANU contacts.

As a student in the CRC for Plant Science, I benefited from a stimulating intellectual environment and received generous financial support. I also gratefully acknowledge the financial support from CSIRO Plant Industry arranged by Jim Peacock, which came when it was most needed. I am glad to have had the opportunity to conduct research within the exciting scientific environment of CSIRO Plant Industry.

My colleagues in the laboratory have provided practical advice and lots of laughter over the years: Lyndall Thorpe, Lynn Hartweck, Leigh Farrell, Frank Hoeren, Marc Ellis, Kath Kovac and Xiaomei Wallace. Thank you Kath for your friendship, sense of humour and proof-reading skills. Julie Glover, Amy Chin-Atkins, Ian Watson and Jo Luck have given me so much support, as have many others at CSIRO Plant Industry – warm thanks to you all. Jen Price and the staff of the phytotron provided great technical assistance – thank you. My new colleagues in Program U are a wonderful group to work with – thank you for your friendship and encouragement. Many thanks also to Tony Brown and Jeremy Burdon, who have borne with patience the long period of thesis writing.

My housemates Michelle Buxton and Simon Gaul have helped create a little oasis of sanity and peace ... you're both wonderful!

Finally, thank you to my wonderful family for their constant love and support. For helping me to keep going; for always being there when I needed you; for sharing laughter and tears – much love and thanks.

Abstract

Environmental signals such as photoperiod and temperature provide plants with seasonal information, allowing them to time flowering to occur in favourable conditions. Most ecotypes of the model plant *Arabidopsis thaliana* flower earlier in long photoperiods and after prolonged exposure to cold (vernalization). The vernalized state is stable through mitosis, but is not transmitted to progeny, suggesting that the vernalization signal may be transmitted via a modification of DNA such as cytosine methylation. The role of methylation in the vernalization response is investigated in this thesis.

Arabidopsis plants transformed with an antisense construct to the cytosine methyltransferase *MET1* (AMT) showed significant decreases in methylation. AMT plants flowered significantly earlier than unvernalized wildtype plants, and the promotion of flowering correlated with the extent of demethylation. The flowering time of mutants with decreased DNA methylation (*ddm1*) was promoted only in growth conditions in which wildtype plants showed a vernalization response, suggesting that the early flowering response to demethylation operated specifically through the vernalization pathway.

The AMT construct was crossed into two late flowering mutants that differed in vernalization responsiveness. Demethylation promoted flowering of the vernalization responsive mutant *fca*, but not of the *fe* mutant, which has only a slight vernalization response. This supports the hypothesis that demethylation is a step in the vernalization pathway.

The role of gibberellic acid (GA) in the early flowering response to demethylation was investigated by observing the effect of the *gai* mutation, which disrupts the GA signal transduction pathway, on flowering time in plants with demethylated DNA. The presence of a single *gai* allele delayed flowering, suggesting that the early flowering response to demethylation requires a functional GA signal transduction pathway, and that demethylation increases GA levels or responses, directly or indirectly.

In most transgenic lines, AMT-mediated demethylation did not fully substitute for vernalization. This indicates that part of the response is not affected by *MET1*-mediated methylation, and may involve a second methyltransferase or a factor other than methylation. A second Arabidopsis methyltransferase, *METIIa*, was characterized and compared to *MET1*. The two genes are very similar throughout the coding region, and share the location of their eleven introns, indicating that they diverged relatively recently. Both are transcribed in all tissues and at all developmental stages assayed, but the level of expression of *MET1* is significantly higher than that of *METIIa*. The possible functions of *MET1*, *METIIa*, and other Arabidopsis cytosine methyltransferase genes recently identified are discussed.

Table of Contents

	page
Statement of Originality	ii
Acknowledgements	iii
Abstract	iv
Table of Contents	vi
List of Figures	xi
List of Tables	xiv
Chapter One General Introduction	1
1.1 The transition to flowering	1
1.2 The vernalization response	3
1.3 Natural variation in flowering time and vernalization response	5
1.4 The “classical” late flowering mutants	7
1.5 A model for the control of flowering time	8
1.5.1 Floral repressors	9
1.5.2 The constitutive pathway	13
1.5.3 The photoperiod pathway	15
1.5.4 The vernalization pathway	17
1.6 The role of gibberellic acid in the response to vernalization	20
1.7 Cytosine methylation	23
1.7.1 Cytosine methylation as genome defense	24
1.7.2 Cytosine methylation and eukaryotic development	25
1.7.3 Cytosine methylation and expression of tissue-specific genes	26
1.7.4 Cytosine methylation and transgene silencing	27
1.7.5 Cytosine methylation and genomic imprinting	32
1.7.6 Endogenous genes regulated by methylation	35
1.7.7 Cytosine methylation and the vernalization response	37
1.8 Cytosine methyltransferases	39
1.8.1 Prokaryote cytosine methyltransferases	39
1.8.2 Mammalian cytosine methyltransferases	41

1.8.3	Other eukaryote methyltransferases	42
1.8.4	Plant cytosine methyltransferases	43
1.8.5	Cytosine demethylation	45
1.9	Scope of thesis	48
Chapter Two Materials and Methods		51
2.1	Plant growth conditions for flowering time experiments	51
2.2	Genomic DNA extractions	51
2.2.1	CsCl gradient method of DNA purification	51
2.2.2	Dellaporta DNA mini-preparation	53
2.2.3	Modified Edwards DNA mini-preparation	54
2.2.4	Klimyuk DNA mini-preparation.....	55
2.3	Plant genotyping procedures	55
2.3.1	Southern analysis	55
2.3.2	PCR analysis for presence of <i>NptII</i> gene	56
2.3.3	PCR analysis for <i>nga8</i> and <i>ngaIII</i> microsatellite markers	57
2.3.4	PCR for GAPB CAPS marker	58
2.4	Estimation of methyl-cytosine levels by thin layer chromatography	58
2.4.1	Sample preparation	58
2.4.2	Thin layer chromatography	60
2.4.3	Analysis of thin layer chromatography	61
2.5	Cloning and characterization of <i>METI</i> and <i>METII</i> genomic clones	61
2.5.1	Screening cosmid library	61
2.5.2	Cloning and hybridization procedures	62
2.5.3	PCR for <i>METI</i> introns	63
2.5.4	Sequencing	63
2.6	RNA extractions and expression studies	64
2.6.1	Total RNA extractions	64
2.6.2	Screening expression libraries	65
2.6.3	Screening expression libraries by PCR for <i>METII</i>	67
2.6.4	RT-PCR for <i>METII</i> and <i>METI</i>	68
2.7	Plant transformation by vacuum infiltration	70

2.7.1	Cloning the <i>METI</i> antisense construct into a binary vector	70
2.7.2	Vacuum infiltration	71
2.7.3	Selection for transformants	72
Chapter Three The effect of reduced DNA methylation on the flowering time and vernalization response of <i>Arabidopsis thaliana</i>		74
3.1	Introduction	74
3.2	Materials and Methods	79
3.2.1	Plant lines	79
3.2.2	Assay for the presence of the transgene	80
3.2.3	Measurement of flowering time	80
3.2.4	Estimation of DNA methylation	81
3.3	Results	82
3.3.1	Plants with decreased DNA methylation flower early	82
3.3.2	Plants from 4 independent <i>METI</i> antisense families flower early ...	84
3.3.3	The promotion of flowering is correlated with the degree of demethylation	86
3.3.4	Effect of different light conditions and photoperiods on flowering time	90
3.3.5	Decreased methylation does not completely substitute for vernalization	92
3.3.6	Decreased DNA methylation (<i>ddm1</i>) mutant flowers early under short days	94
3.4	Discussion	96
Chapter Four Effect of demethylation on the flowering time of late flowering mutants of <i>Arabidopsis thaliana</i>		103
4.1	Introduction	103
4.2	Materials and Methods	107
4.2.1	Introduction of a <i>METI</i> antisense construct into late flowering mutant backgrounds	107
4.2.2	Experiments to measure flowering time	109
4.2.3	Analysis of F3 lines after measurement of flowering time	110

4.3 Results	111
4.3.1 Selection of <i>METI</i> antisense-containing lines for the late flowering mutants	111
4.3.2 Vernalization response of late flowering mutant lines	113
4.3.3 Demethylation caused by <i>METI</i> antisense expression	115
4.3.4 Effect of demethylation on flowering time of late flowering mutants	115
4.3.5 Effect of <i>FRI</i> on flowering time	119
4.4 Discussion	120
Chapter Five The role of gibberellic acid in the early flowering response of <i>METI</i> antisense plants	126
5.1 Introduction	126
5.2 Materials and Methods	131
5.2.1 Effect of GA ₃ on flowering time of <i>METI</i> antisense plants	131
5.2.2 Effect of <i>gai</i> mutation on flowering time of <i>METI</i> antisense plants	132
5.3 Results	134
5.3.1 Effect of GA on flowering time of <i>METI</i> antisense plants	134
5.3.2 Effect of reduced methylation on flowering time of <i>gai</i> mutants ...	136
5.4 Discussion	137
Chapter Six Multiple cytosine methyltransferase genes in <i>Arabidopsis thaliana</i>	142
6.1 Introduction	142
6.2 Materials and Methods	146
6.2.1 Isolation of <i>METII</i> genomic clones	146
6.2.2 Restriction and hybridization analysis of Ac71 and COS5K	147
6.2.3 DNA sequencing and sequence analysis	147
6.2.4 PCR to identify <i>METI</i> intron positions	147
6.2.5 Protein modeling	148
6.2.6 Expression studies	148
6.2.7 Mapping the chromosomal location of <i>METII</i>	150
6.3 Results	150
6.3.1 Sequence analysis of a methyltransferase-like ORF	150

6.3.2	Arabidopsis has a second DNA methyltransferase gene	151
6.3.3	Methyltransferase domain	153
6.3.4	Amino terminal domain	154
6.3.5	Chromosomal location of <i>METIIa</i>	157
6.3.6	Expression pattern of <i>METI</i> and <i>METIIa</i>	157
6.4	Discussion	160
Chapter Seven General Discussion		167
7.1	The role of methylation in the vernalization response	167
7.2	Future work	170
7.2.1	Down-regulation of <i>FLC</i> by vernalization and demethylation	170
7.2.2	The role of GA in the vernalization response	175
7.2.3	Other Arabidopsis cytosine methyltransferases	177
7.3	Final conclusions	180
References		181

List of Figures

		following page
Figure 1.1	A model of pathways to flowering in Arabidopsis	9
Figure 3.1	Spectra of three fluorescent light sources used in flowering time experiments	81
Figure 3.2	Promotion of flowering in plants from T2 and T3 generations of <i>METI</i> antisense transgenic family #10	82
Figure 3.3	Flowering time of vernalized and unvernallized T3 plants of <i>METI</i> antisense line 10.1	83
Figure 3.4	Correlation of methylation level with promotion of flowering by demethylation in lines from families 4, 10, 22.6 and 39	88
Figure 3.5	Flowering time of unvernallized plants from lines 10.1 and 10.4	89
Figure 3.6	Relationship between the C24 vernalization response and the proportion of that response for which the early flowering response to demethylation substitutes	92
Figure 3.7	A model for passive demethylation through disruption of maintenance methylation during DNA replication	98
Figure 4.1	A model for the transition from vegetative growth to flowering, showing the role of the floral repressor <i>FLC</i> in the autonomous and vernalization pathways	104
Figure 4.2	Crossing and selection procedures	107
Figure 4.3	Polymorphism between C24 and <i>Ler</i> using the GAPB marker	108
Figure 4.4	Identification of F2 plants from crosses between <i>fe</i> or <i>fca</i> , and C24 or <i>METI</i> antisense line T3#10.5, homozygous for the mutant locus	108

Figure 4.5	Days from germination to elongation of primary inflorescence for F3 lines from crosses between <i>fe</i> and either the T3 <i>METI</i> antisense line 10.5 (AMT) or C24	114
Figure 4.6	Days from germination to elongation of primary inflorescences for F3 lines <i>fca</i> -AMT #21 and <i>fca</i> -C24 #1	114
Figure 4.7	Days from germination to elongation of primary inflorescences for F3 lines <i>fca</i> -AMT #21 and <i>fca</i> -C24 #1, showing different <i>FRI</i> genotype classes	118
Figure 5.1	Gibberellin biosynthesis pathways	126
Figure 5.2	Vernalization dependent and independent pathways to flowering	129
Figure 5.3	Effect of vernalization and/or GA ₃ addition on flowering time of <i>METI</i> antisense and C24 plants	134
Figure 5.4	Days from germination to elongation of primary inflorescence for F1 plants from crosses between <i>Ler</i> and AMT, <i>gai</i> and AMT, and <i>gai</i> and C24	136
Figure 6.1	Structure of the <i>METII</i> coding region	146
Figure 6.2	Structure of <i>METII</i> and <i>METI</i> genes, showing the identical positions of introns II to XI	149
Figure 6.3	Southern analysis of Ac71 clone, showing fragments which hybridize to regions of the <i>METII</i> cDNA clone	150
Figure 6.4	Comparison of the <i>METII</i> RT-PCR product, spanning the predicted position of introns III and IV, with the genomic sequence in this region	152
Figure 6.5	Comparison of the conserved methyltransferase motifs in <i>METI</i> and <i>METII</i>	153
Figure 6.6	Structures of <i>METI</i> and <i>METII</i> predicted proteins, modelled on the structure of M.HhaI	154
Figure 6.7	Alignment of methyltransferase domains of twelve eukaryotic cytosine methyltransferases	154

Figure 6.8	Alignment of amino-terminal domains of eight plant cytosine methyltransferases	155
Figure 6.9	Dotplot comparison of the mouse Dnmt1 and Arabidopsis METIIa amino terminal domains	157
Figure 6.10	Detection of an RFLP for Ac71	157
Figure 6.11	Linkage map of Arabidopsis chromosome 4	157
Figure 6.12	DNA sequence downstream of the <i>METIIa</i> gene, containing a second open reading frame	158
Figure 6.13	Products of RT-PCR with primers specific to <i>METII</i>	158
Figure 6.14	RT-PCR products for <i>METI</i> and <i>METII</i>	159
Figure 6.15	Southern analysis of <i>METI</i> and <i>METII</i> RT-PCR gels	159
Figure 7.1	Alternative models of pathways to flowering, focusing on the mode of regulation of <i>FLC</i> through the vernalization and autonomous pathways	172

List of Tables

		following page
Table 2.1	Primers flanking putative introns in <i>METI</i>	63
Table 3.1	Plant lines and growth conditions for experiments to measure flowering time	80
Table 3.2	Days from germination to elongation of primary inflorescence for <i>METI</i> antisense plants grown in Experiment One	82
Table 3.3	Days from germination to elongation of primary inflorescence for <i>METI</i> antisense plants grown in Experiment Two	84
Table 3.4	Days from germination to elongation of primary inflorescence for <i>METI</i> antisense plants grown in Experiment Three	84
Table 3.5	Days from germination to elongation of primary inflorescence for <i>METI</i> antisense plants grown in Experiment Four	86
Table 3.6	Days from germination to elongation of primary inflorescence for <i>METI</i> antisense plants grown in Experiment Five	86
Table 3.7	Days from germination to elongation of primary inflorescence for <i>METI</i> antisense plants grown in Experiment Six	90
Table 3.8	Days from germination to elongation of primary inflorescence for <i>METI</i> antisense plants grown in Experiment Seven	90
Table 3.9	Promotion of flowering in <i>METI</i> antisense transgenic lines as a proportion of the C24 vernalization response under different light spectra	92

Table 3.10	Days from germination to elongation of primary inflorescence for <i>ddm1</i> plants grown in long or short photoperiods	95
Table 4.1	F2 plants generated from crosses of late flowering mutants <i>fe</i> and <i>fca</i> with <i>MET1</i> antisense line T3#10.5 (AMT) and C24 wildtype	112
Table 4.2	5-methylcytosine levels in F3 plants from crosses of late flowering mutants <i>fe</i> and <i>fca</i> with <i>MET1</i> antisense line T3#10.5	115
Table 4.3	Flowering behaviour of unvernallized and vernalized F3 plants of four lines from crosses of <i>fe</i> with AMT or C24	116
Table 4.4	Flowering behaviour of unvernallized and vernalized F3 plants of two lines from crosses of <i>fca</i> with AMT or C24	118
Table 5.1	Analysis of flowering time of F1 plants from crosses between <i>gai</i> and either AMT or C24, and between <i>Ler</i> and AMT	136

Dedicated to my dear mother

Kathleen Elizabeth Genger

1929 - 1994

and my loving father

Johannes Jacobus Genger