Flowering-time control and perennialism in *Arabis alpina*, a perennial relative of *Arabidopsis thaliana*

Inaugural-Dissertation

Zur

Erlangung des Doktorgrades

Der Mathematisch-Naturwissenschaftlichen Fakultät

Der Universität zu Köln

Vorgelegt von

Renhou Wang

Aus Changchun, China

Köln, April 2007

Die Vorliegende Arbeit wurde am Max-Planck Institute für Züchtungsforschung Köln, in der Abteilung für Entwicklungsbiologie der Pflanzen (Direktor Prof. Dr. George Coupland) angefertigt.

Berichterstatter: Prof. Dr. George Coupland
Prof. Dr. Ute Hoecker

Prüfungsvorsitz: Prof. Dr. Martin Hülskamp

Tag der mündlichen Prüfung: 02. Juli 2007

Acknowledgements

First of all, I would like to thank my supervisor Professor Dr. George Coupland for his great support, fruitful discussions and providing an excellent environment to do science. Thank you for teaching me to find my own ideas.

I am grateful to Professor Dr. Martin Hülskamp for being chair of the disputation and to Professor Dr. Ute Hoecker for being the second examiner.

Thanks to Dr. Jane Parker, my second supervisor, for her helpful discussions on my projects.

Special thanks to Dr. Nicolas Sauerbrunn and Jessica Adrian for help in editing this thesis and their helpful comments on the thesis.

Furthermore, I would like to thank the group of George Coupland at Max-Planck Institute for Plant Breeding Research, especially Dr. Maria Albani, Coral Vincent and Dr. Fabio Fornara for good collaboration and for providing a great working environment in every aspect. It was a pleasure to work with you and I enjoyed many interesting discussions.

I would like to thank Dr. Ralf Petri for his continuous kind help throughout my PhD study.

I would also like to thank IMPRS for the financial support for this project.

Finally, I would like to thank my parents for their constant help and support.

I would like to give my deepest thanks to my wife Haili Song for her love, support and trust.

Abstract

The life cycle of annual plants takes place within one year while perennials can live for many years and flower repeatedly. Juvenility and differential competence of meristems to flower are two prerequisites for a plant to be a perennial. To study the mechanisms underlying perenniality, we have developed *Arabis alpina* as a perennial model species. In *Arabis alpina Pajares* accession, vernalization treatment, a strong environmental floral inductive cue, can promote flowering in adult plants but not in juvenile plants. Furthermore, within a growing season, only some apical meristems flower while the rest remain vegetative. Homologs of several Arabidopsis flowering time genes, including *FLC*, *TFL1*, *FT* and *SOC1* were cloned from *Arabis alpina Pajares* and named *AaFLC*, *AaTFL1*, *AaFT* and *AaSOC1* respectively. High levels of sequence similarity between these *Arabis alpina* genes and their Arabidopsis counterparts, together with their functional conservation tested by expression in Arabidopsis suggest that *AaFLC*, *AaTFL1*, *AaFT* and *AaSOC1* are orthologs of *FLC*, *TFL1*, *FT* and *SOC1* respectively. During vernalization, *AaFLC* transcription was suppressed in both juvenile and adult *Arabis alpina* plants in similar patterns while *AaSOC1* expression increased in both cases, suggesting the existence of a repressor that acts after AaSOC1 to prevent flowering of juvenile plants. *AaTFL1* was expressed in inner cells of the whole main shoot apical meristem both before and during vernalization in juvenile plants. However, its expression in adult plants was restricted to much more localized regions during vernalization. Furthermore, an *AaTFL1* genomic fragment not only complemented *tfl1* mutant phenotypes, but also caused late flowering compared to wild-type plants. These results suggest that *AaTFL1* is repressed by veranlization in adult plants and lack of *AaTFL1* repression may play a role in juvenility in *Arabis alpina*. Diverse expression patterns of *AaTFL1* in side branches undergoing different fates suggest that *AaTFL1* may also be involved in the control of differential competence of these side branches to flower. The repression of *AaFLC* expression by vernalization treatment is not mitotically stable in all tissues since *AaFLC* expression increased after removal of the treatment in leaves and branches, which is in contras to the effect of vernalization on *FLC* in Arabidopsis. Correspondingly, *AaSOC1* expression decreased after vernalization. These results

imply a mechanism for maintaining seasonality in a perennial species. Moreover, suppression of *AaFLC* expression by RNAi removed the obligate requirement for vernalization to flower. However, *AaFLC* transcription levels do not mediate the difference in vernalization requirement among the accessions we tested.

Zusammenfassung

Der Lebenszyklus einer einjährigen („annual“) Pflanze vollzieht sich innerhalb eines Jahres, wohingegen mehrjähige Pflanzen („perennials“) über längere Zeiträume existieren und sich durch wiederholte Blüten- und Samenbildung auszeichnen. Die so genannte Juvenilität und die Existenz von Meristemen mit unterschiedlicher Kompetenz zur Blütenbildung stellen zwei wesentliche Vorrausetzungen für die Entwicklung zur mehrjährigen Pflanze dar.

Um die grundlegenden Mechanismen zu verstehen, die eine Pflanze zur Mehrjährigkeit befähigt, wurde die Pflanze *Arabis alpina* als Modelpflanze ausgewählt. Bei dem Ökotyp (Akzession) *Arabis alpina Pajares* kann die Blütenbildung durch eine längere Kälteperiode (= Vernalisierungsphase) bei adulten Pflanzen erheblich beschleunigt werden. Bei juvenilen Pflanzen hingegen führt eine Vernalisierung nicht zu einer Induktion der Blütenbildung. Außerdem entwickeln sich innerhalb einer Bühsaison immer nur einige Apikalmeristeme Blüten, wohingegen andere vegetativ bleiben und keine Blüten bilden.

Homologe einiger für die Regulation der Blütenbildung bedeutsamer Gene von *A. thaliana*, wie *FLC*, *TFL1*, *FT* and *SOC1,* wurden in *Arabis alpina Pajares* kloniert und entsprechend als *AaFLC*, *AaTFL1* etc. bezeichnet. Eine hohe Sequenzübereinstimmung zwischen den *A. alpina* Genen und ihren *A. thaliana* Homologen sowie eine funktional wirksame Expression dieser Homologe in *A. thaliana* legen nahe, dass *AaFLC*, *AaTFL1*, *Aa*FT und *AaSOC1* Orthologe der entsprechenden Gene in *A. thaliana* sind.

Bei *A. thaliana* ist *FLC* ein zentraler Repressor und *FT* sowie *SOC1* sind wichtige Positivregulatoren für die Blütenmeristementwicklung. Die Transkription von *AaFLC* war in juvenilen sowie in adulten *A. alpina* Pflanzen während der Vernalisierung reduziert wie es bei *FLC* in *A. thaliana* der Fall ist. Die Expression von *AaSOC*1 stieg sowohl in juvenilen wie adulten Pflanzen an, doch nur in adulten Pflanzen kommt es zur Blütenmeristementwicklung. Dies könnte darauf hindeuten, dass ein Repressor unterhalb von *AaSOC1* in juvenilen Pflanzen aktiv ist, der hier die Blüteninduktion verhindert.

Eine Expression von *AaTFL1* konnte in juvenilen Pflanzen in den inneren Zellen des gesamten apikalen Sproßmeristems, sowohl vor als auch während der Vernalisierung gezeigt werden. In adulten Pflanzen war die Expression von

AaTFL1 während der Vernalisierung auf vereinzelte Bereiche des apikalen Meristems beschränkt. Außerdem führte die Transformation des genomischen *AaTFL1*-Konstrukts in der *A. thaliana tfl1*-Mutante nicht nur zur Komplementation, sondern sogar zu einer im Vergleich zum Wildtyp verspäteten Blütenbildung. Diese Beobachtungen deutet darauf hin, dass *AaTFL1* bei der Kontrolle der juvenilen Phase in *A. alpina* beteiligt ist, da es in adulten Pflanzen zumindest in Teilbereichen des Meristems durch Vernalisierung reprimiert wird und diese Repression von *AaTFL1* in juvenilen Pflanzen ausbleibt. Weitere Untersuchungen zur *AaTFL1* Expression in verschiedenen Seitentrieben zeigten eine sehr differentielle Expression, was eine Rolle von *AaTFL1* bei der Kontrolle der Kompetenz zur Blütenbildung nahe legt. Die Repression von *AaFLC* ist im Kontrast zum *FLC* in *A. thaliana* nicht mitotisch stabil in allen Geweben und steigt nach der Kältebehandlung in den Blättern und Seitentrieben wieder an. Entsprechend konnte nach Beendigung der Vernalisierung eine Reduktion der *AaSOC1* Induktion festgestellt werden. Diese Daten implizieren einen Mechanismus in der die Saisonalität in der pereannualen Spezies *A.alpina* über die differentielle Regulation von *AaTFL1*, Aa*FLC* und Aa*SOC1* verschiedenen Meristemen gesteuert wird. Darüber hinaus führte eine *AaFLC*-Supression durch ein entsprechendes RNAi-Konstrukt dazu, dass *A. alpina Pajares* ohne die sonst obligate Vernalisierung zur Blütenbildung kommt.

Bei Expressions-Analysen in verschiedenen Akzessionen von *A.alpina* konnte allerdings kein Zusammenhang zwischen Blühkompetenz des Meristems und der Expression von Aa*FLC* festgestellt werden.

Table of contents

Acknowledgements .... **I**
Abstract .... **II**
Zusammenfassung .... **IV**
Table of contents .... **VI**
List of figures .... **IX**
List of abbreviations .... **XI**
1. General introduction **1**
1.1. Perennialism and Flowering in higher plants 1
1.2. Flowering-time control in *Arabidopsis thaliana* and other annual or biennial species 4
1.2.1. Gibberellin pathway 5
1.2.2. Photoperiod pathway 6
1.2.3. Autonomous pathway 8
1.2.4. Vernalization pathway 9
1.2.5. Integration of floral pathways and initiation of floral meristems 14
1.2.6. Other pathways involved in flowering-time control 16
1.3. Flowering control in polycarpic perennial plants 17
1.3.1. Perenniality in grasses 18
1.3.2. Perenniality and flowering control in woody perennials 18
1.4. *Arabis alpina*, a promising model species for studying perenniality and flowering-time control in perennials 20
1.5. Aims of the thesis 21
2. Flowering behavior of *Arabis alpina* **22**
2.1. Introduction 22
2.2. Juvenility in *Arabis alpina* 23
2.3. Polycarpic traits of *Arabis alpina* 26
2.3.1. Polycarpic traits of *Arabis alpina Pajares* 26
2.3.2. Polycarpic traits in *Arabis alpina Bonn* 32
2.4. Flowering time in response to day length and vernalization treatment in different *Arabis alpina* accessions 34
2.5. Effect of exogenous gibberellin treatment on flowering in *Arabis alpina* 35
2.6. Discussion 36
3. Molecular mechanisms underlying juvenility in *Arabis alpina* **41**
3.1. Introduction 41
3.2. *AaFLC* is an orthologue of the Arabidopsis *FLC* gene 43
3.3. *AaFLC* is expressed at similar levels in juvenile and adult *Arabis alpina* plants before and after vernalization 47
3.4. Reduction of *AaFLC* expression in transgenic *Arabis alpina Pajares* plants allowed flowering without vernalization 48
3.5. *AaFT* is an orthologue of Arabidopsis *FT* 51

3.6. During early stages of vernalization treatment, *AaFT* was expressed at low levels in both juvenile and adult plants 54
3.7. *AaSOC1* caused early flowering when ectopically expressed in Arabidopsis 56
3.8. *AaSOC1* expression increased during vernalization treatment in both juvenile and adult plants....58
3.9. *AaFT* and *AaSOC1* mRNA levels are increased in *AaFLC* dsRNAi lines....60
3.10. Cloning and sequence analysis of *AaTFL1*....61
3.11. Expression of *AaTFL1* from the CaMV35S promoter or its own promoter caused late flowering in Arabidopsis....63
3.12. In response to vernalization, *AaTFL1* shows different expression patterns in juvenile and adult plants....64
3.13. Discussion....65
3.13.1. Sequence and functional conservation of flowering time genes in *Arabis alpina* and *Arabidopsis thaliana*....65
3.13.2. Juvenility in vernalization response is not caused by a failure to repress *AaFLC* expression 69
3.13.3. Repression of floral transition occurs at a relatively late step in juvenile plants....70
3.13.4. *AaTFL1* expression pattern differs between *Arabis alpina* juvenile and adult plants 72
4. Roles of flowering time genes in polycarpic traits and seasonal flowering in *Arabis alpina Pajares* 77
4.1. Introduction 77
4.2. *AaTFL1* expression patterns differ in shoots showing different flowering behaviors 79
4.3. *AaSOC1* expression decreased after vernalization....81
4.4. The repression of *AaFLC* expression by vernalization was unstable after vernalization 82
4.5. Discussion....83
4.5.1. *AaTFL1* may be involved in differential competence of apical meristems to flower in *Arabis alpina* 83
4.5.2. Seasonal flowering is maintained in *Arabis alpina Pajares* by resetting expression of flowering-time genes after flowering 88
5. The correlation between expression of flowering-time genes and natural genetic variation in vernalization requirement 90
5.1. Introduction 90
5.2. In *Arabis alpina Bonn* and *Arabis alpina Pajares*, *AaFLC* mRNA levels were similar 91
5.3. Both *AaFT* and *AaSOC1* were expressed at higher levels in the *Arabis alpina Bonn* plants than in the *Arabis alpina Pajares* plants 92
5.4. *AaTFL1* expression patterns in *Arabis alpina Bonn* and *Arabis alpina Pajares* accessions during development 93
5.5. Discussion....94
6. General conclusions and perspectives 97
7. Materials and Methods 101
7.1. Materials 101
7.1.1. Chemicals....101
7.1.2. Enzymes 101
7.1.3. Kits 101

7.1.4. Vectors 102
7.1.5. Bacterial Strains 102
7.1.6. Oligonucleotides 102
7.1.7. Plant Materials 104
7.2. Methods 104
7.2.1. General molecular biological techniques 104
7.2.2. RNA isolation 105
7.2.3. Reverse transcription 105
7.2.4. Cloning of *AaFLC*, *AaSOC1* and *AaTFL1* 105
7.2.5. BAC library screening and sequencing of BAC DNA 106
7.2.6. Phylogenetic analysis 106
7.2.7. Southern blot 107
7.2.8. Construction of expression vectors 107
7.2.9. Agrobacterium-mediated transformation of Arabidopsis and *Arabis alpina* plants 108
7.2.10. *In situ* hybridization 109
7.2.11. Plant growth conditions and measurement of flowering time 109
7.2.12. GA treatment 109
7.2.13. Quantitative RT-PCR 109
8. References 111
9. Appendix 126
9.1. Alignment of AaFLC and AtFLC 126
9.2. CDS of *AaFLC* 126
9.3. Alignment of AaFT, AaTSF1, AaTSF2, AtFT, AtTSF, AaTFL1 and AtTFL1 127
9.4. CDS of *AaTFL1* 128
9.5. Alignment of AaSOC1 and AtSOC1 128
9.6. CDS of *AaSOC1* 129
Erklaerung 130
Lebenslauf 131

List of figures

Figure 1. The network underlying flowering-time control in *Arabidopsis thaliana*. 4
Figure 2. Effects of prolonged cold treatment on floral promotion in plants at different developmental stages. 24
Figure 3. Flowering time of *Arabis alpina Bonn* plants grown under different photoperiods. 25
Figure 4. Changes of axillary meristem identity in response to vernalization in adult *Arabis alpina Pajares* plants. 28
Figure 5. Flowering tendency of axillary shoots produced at nodes before No.16. 31
Figure 6. Localized senescence occurring after seed set. 32
Figure 7. Flowering and senescence in *Arabis alpina Bonn* plants. 33
Figure 8. The effect of vernalization on flowering time in *Arabis alpina Bonn* plants. 34
Figure 9. Effect of exogenous gibberellin treatment on growth and flowering of *Arabis alpina* plants. 36
Figure 10. Diagram of response to vernalization treatment in apical meristems of *Arabis alpina Pajares* plants. 38
Figure 11. *AaFLC* has a similar genomic organization as *AtFLC* and is a single copy gene. 44
Figure 12. Phylogenetic analysis of AaFLC and other FLC homologues. 45
Figure 13. Ectopic expression of *AaFLC* in Arabidopsis caused late flowering. 47
Figure 14. *AaFLC* expression in juvenile and adult *Arabis alpina Pajares* plants in response to vernalization treatment. 48
Figure 15. Phenotypes of *AaFLC* dsRNAi *Arabis alpina Pajares* lines. 50
Figure 16. *AaFLC* mRNA levels in *AaFLC* dsRNAi lines. 51
Figure 17. Phylogenetic analysis of AaFT, AaTSF1, AaTSF2, AaTFL1 and FT/TFL1 like proteins from other species. 52
Figure 18. Expression patterns and function of *AaFT*. 54
Figure 19. Expression of *AaFT* in juvenile and adult plants in response to vernalization treatment 56
Figure 20. Phylogenetic analysis of AaSOC1. 57
Figure 21. Ectopic expression of *AaSOC1* from the 35S promoter in Arabidopsis resulted in early flowering (right) compared to Col (left). 58
Figure 22. *AaSOC1* expression in juvenile and adult *Arabis alpina Pajares* plants in response to vernalization treatment. 59
Figure 23. Expression of *AaSOC1* and *AaFT* in *AaFLC* dsRNAi lines. 61
Figure 24. *AaTFL1* expression from either a 35S promoter or its native promoter delayed flowering in Arabidopsis. 62
Figure 25. *AaTFL1* expression tested by *in situ* hybridization in apices of juvenile and adult *Arabis alpina Pajares* plants in response to vernalization treatment. 65
Figure 26. *AaTFL1* expression in different types of axillary shoots of *Arabis alpina Pajares* plants. 80
Figure 27. *AaSOC1* mRNA levels in main shoot and axillary branch apices of *Arabis alpina*

Pajares plants in response to vernalization. 81
Figure 28. *AaFLC* expression apices and leaves of *Arabis alpina Pajares* plants in response to vernalization. 83
Figure 29. *AaFLC* expression in apices and leaves of *Arabis alpina Pajares* and *Arabis alpina Bonn* accessions. 92
Figure 30. *AaSOC1* and *AaFT* expression in *Arabis alpina Pajares* and *Arabis alpina Bonn* plants. 93
Figure 31. *AaTFL1* expression detected by *in situ* hybridization in apices of *Arabis alpina Bonn* and *Arabis alpina Pajares* accessions during development. 94

List of abbreviations

General abbreviations

°C	degree Celsius
3', 5'	3-prime, 5-prime
ABA	abscisic acid
bp	basepair
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
Col	Columbia
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
dsRNAi	doublestrand-RNA-interference
GA	gibberellic acid
h	hour
kb	kilobase
kV	kilovolt
l	liter
LB	Luria broth
LD	long day
Ler	Lansberg erecta
M	Mol
mg	milligram
miR	micro-RNA
ml	milliliter
mRNA	messenger RNA
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RS	reproductive shoot

RT	room temperature
SAM	shoot apical meristem
SD	short day
SDP	short day plant
TL	transitional leaf
UTR	untranslated region
UV	ultraviolet light
VL	vegetative leaf
VS	vegetative shoot
WT	wild type
µg	microgram

Amino acids

Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophane	Trp	W

Abbreviations of gene and protein names

AG	AGAMOUS
AP1	APETALA1
AP2	APETALA2
CAL	CAULIFLOWER

CCA1	CIRCADIAN CLOCK ASSOCIATED1
CDF1	CYCLIC DOF FACTOR1
CLF	CURLY LEAF
CO	CONSTANS
COP1	CONSTITUTIVE PHOTOMORPHOGENESIS1
CRY1	CRYPTOCHROME1
CRY2	CRYPTOCHROME2
EBS	EARLY BOLTING IN SHORT DAYS
EFS	EARLY FLOWERING IN SHORTD AYS
ELF3	EARLY FLOWERING3
ELF4	EARLY FLOWERING4
ELF7	EARLY FLOWERING7
ELF8	EARLY FLOWERING8
EMF"	EMBRYONIC FLOWER2
FCA	*
FD	*
FES1	FRIGIDA-ESSENTIAL 1
FKF1	FLAVIN-BINDING, KELCH REPEAT, F-BOX1
FLC	FLOWERING LOCUS C
FLD	FLOWERING LOCUS D
FLK	FLOWERING LATE WITH KH MOTIFS
FPA	*
FPF1	FLOWERING PROMOTING FACTOR1
FRI	FRIGIDA
FRL1	FRIGIDA-LIKE 1
FT	FLOWERING LOCUS T
FVE	*
FY	*
GAI	GA INSENSITIVE
GI	GIGANTEA
GID1	GA INSENSITIVE DWARF1
HAP	HEME ASSOCIATED PROTEIN
LD	LUMINIDEPENDENS
LFY	LEAFY
LHY	LONG ELONGATED HYPOCOTYL
miR159	microRNA159
miR172	microRNA172
PAF1	RNA POLII ASSOCIATED FACTOR1
PcG	Plycomb group gene
PHYA	PHYTOCHROME A
PHYB	PHYTOCHROME B

PHYC	PHYTOCHROME C
PHYD	PHYTOCHROME D
PHYE	PHYTOCHROME E
polII	RNA polymerase II
RGA	REPRESSOR OF ga1-3
RGL1	RGA-LIKE1
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1
SUF4	SUPPRESSOR OF FRIGIDA 4
TFL1	TERMINAL FLOWER1
TOC1	TIMING OF CAB EXPRESSION1
VIN3	VERNALIZATION INSENSITIVE3
VIP4	VERNALIZATION INDEPENDENT4
VRN1	VERNALIZATION1

1. General introduction

1.1. Perennialism and Flowering in higher plants

The life histories of higher plants can mainly be classified as annual, biennial and perennial. Under natural conditions, annual plants complete their life cycles (from seed germination to seed set and death of the mother plant) within one year (or one growing season). Biennial plants normally take between twelve and twenty-four months to complete their life cycles. In the first year, biennial plants grow vegetatively, and then they enter a period of dormancy during the cold winter. Many biennial plants have an obligate requirement for a long cold treatment to promote flowering, which is known as vernalization. The next spring or summer, the plants flower, producing fruits and seeds before the whole plant dies. Perennial plants, including monocarpic and polycarpic perennials, live for more than two years. Monocarpic perennials, such as some bamboo species, live for many years growing vegetatively, and then the subsequent floral transition occurs in all apical meristems, after which the seeds are formed and the whole plant dies (Battey and Tooke, 2002). Polycarpic perennial plants undergo vegetative growth for weeks to many years before flowering depending on the species. Different from annuals, biennials and monocarpic perennials, polycarpic perennials survive after the first flowering and flower annually (Thomas et al., 2000; Tan and Swain, 2006).

A higher plant initiates postembryonic development after seed germination. After germination, the seedling passes through a juvenile phase, during which it is not competent to flower even if exposed to normal inductive signals from the environment (Tan and Swain, 2006). This juvenile phase is followed by the adult phase which can be subdivided into vegetative and reproductive adult phases. During vegetative adult phase the plant is able to undergo the floral transition to the reproductive phase in response to appropriate environmental cues. During the reproductive phase, gametes are generated via meiosis. Pollination and fertilization cause fusion of the gametes which marks the start of embryonic development, and formation of seeds starts the next generation. Over millions of years plants have acquired the capacity to synchronise their developmental programmes with favorable environmental conditions, therefore to flower at the most appropriate

time, that maximizes reproductive success and consequently ensures their survivability through variable environments (Baurle and Dean, 2006).

The differences in their origins and the environments they have experienced during evolution influence the strategies that have been adopted by different species to ensure their reproductive success. Photoperiod and temperature are two of the most important environmental cues, while endogenous cues, such as the developmental age and hormone levels, also influence flowering time. Different plant species may exhibit different responses to a certain environmental factor in regulation of flowering. In terms of photoperiod response, plants can be classified into long-day plants (LDP), in which flowering is promoted when the day becomes longer than a critical length, short-day plants (SDP), in which flowering occurs when the day length becomes shorter, and day-neutral plants, in which flowering time is insensitive to day length (Salisbury, 1985). Furthermore, according to the extent to which the floral transition relies on photoperiod, SDP and LDP can again be classified into the ones with obligate or facultative requirements. Obligate long-day plants only flower in long days and never in short days. Facultative long-day plants flower in both long days and short days, but earlier in long days. Similarly, winter temperatures can act as an important environmental cue that promotes flowering in many plant species. The process that a long cold treatment promotes flowering is called vernalization. Different species may have different requirements for vernalization to promote flowering. Those with an obligate requirement will not flower until such a treatment is applied, and vernalization treatment can accelerate flowering of plants with a facultative vernalization requirement, while other plants do not show response to vernalization at all (Amasino, 2004).

Although favorable environmental factors play crucial roles in promoting flowering in most, if not all, plant species, they can not make a plant flower immediately after germination, suggesting endogenous floral repression exists in young seedlings. This repression, known as juvenility, is important for plants since it provides time to accumulate resources to produce more vegetative leaves, which facilitate photosynthesis and thereby ensure plants can support maximized amounts of seed development. The length of juvenility varies greatly in plants with different life cycles. In most annual plants, juvenility lasts for a short time of days or weeks. Correspondingly, these plants can flower within one year (or growing season) and therefore propagate rapidly. However, in most biennials, juvenility may last for several weeks or even longer, and together with other factors (e.g. those conferring

vernalization requirement), contribute to the repression of flowering in the first year, allowing plants to accumulate enough resources to make more flowers and seeds the next year. In perennials, juvenility has an even more dramatic influence on their life history. In monocarpic perennials, the juvenile phase may last for many years, making juvenility the major factor determining the length of vegetative growth. This long period of vegetative growth results in the production of numerous apical meristems. Once the plant matures, flowering occurs in response to environmental cues in all these meristems and consequently a large number of seeds are generated at one time. One advantage of this type of life history may be prevention of pests living on the seeds of the plants. Furthermore, the fast vegetative growth of some species like bamboo may help competition for resources with other species in the local area (Meilan, 1997). Juvenility in polycarpic perennials may last for several weeks to many years, depending on the species, and is a prerequisite for this type of life history in terms of its role in preventing precocious floral transition that would otherwise exhaust the plant's resources to carry out further vegetative growth.

Besides a longer juvenile phase, differential competence for floral induction in apical meristems within an individual plant is another prerequisite for polycarpic perennial life history (Thomas et al., 2000). Differential competence to flower allows some apical meristems to remain vegetative while others flower. Meristems that remain vegetative enable growth and flowering to occur in the following seasons. Thus juvenility and differential floral competence in apical meristems of a mature plant are the two major prerequisites for perenniality. On the one hand, these two aspects represent the major difference in flowering time control between annual and perennial species. On the other hand, once a perennial plant matures, floral transition occurs annually, at least in some apical meristems, in a similar way as that in an annual plant, suggesting that common mechnisms in flowering time control are shared in annuals and perennials (Tan and Swain, 2006). Furthermore, the incompetence to flowering in juvenile plants or some of the vegetative meristems of adult plants may be conferred by mechanisms that have some relation to those that repress flowering in annuals. Therefore, in this thesis, perenniality was investigated by studying flowering-time control in a polycarpic perennial species, *Arabis alpina*, and comparing the underlying mechanisms of flowering-time control in this species with its close annual relative *Arabidopsis thaliana*.

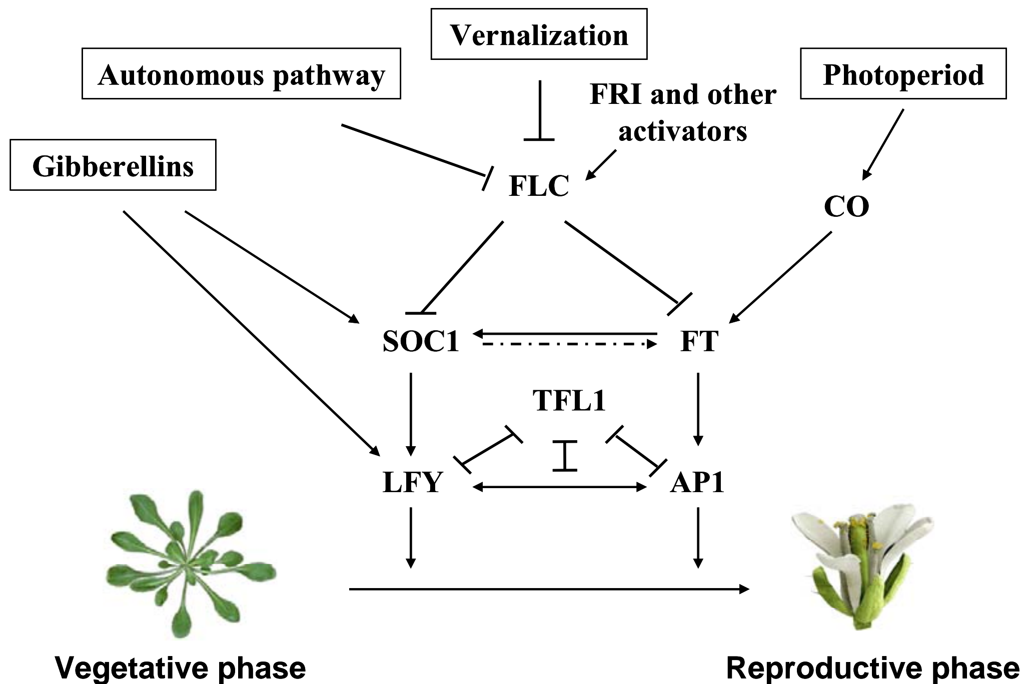


Figure 1. The network underlying flowering-time control in *Arabidopsis thaliana*. Only the four major pathways and some representative genes are shown.

1.2. Flowering-time control in *Arabidopsis thaliana* and other annual or biennial species

Recently, impressive progress has been made in understanding the molecular mechanisms controlling floral transition in annual species, especially in the model species *Arabidopsis thaliana*. Four major genetic pathways controlling flowering time have been identified: gibberellin, photoperiod, autonomous and vernalization pathways (Figure 1) (Mouradov et al., 2002; Boss et al., 2004). In addition to these major pathways, environmental factors such as light quality, light intensity, ambient temperature and nutrient state also affect flowering time (Mouradov et al., 2002; Boss et al., 2004). Crosstalk and interconnections have been identified among flowering-control pathways, demonstrating that a complex network exists to control the floral transition. Signals from different floral pathways converge to regulate the transcription of floral pathway integrator genes, which in turn induce the expression of floral meristem identity genes that confer floral identity on lateral primordia to establish the floral meristem. Studies on flowering time control in

other plant species suggest that both conservation and diversity exist in molecular mechanisms controlling floral transition in different species, possibly reflecting the various processes of adaptive evolution (Roux et al., 2006).

1.2.1. Gibberellin pathway

The flowering-promotion effect of the phytohormone gibberellins (GAs) was first demonstrated by exogenous application of GAs (Lang, 1957; Langridge, 1957). Recent molecular genetic studies have confirmed these physiological findings through analysis of mutants that disrupt either GA biosythesis or signaling, or through manipulating the expression of genes involved in these processes (Wilson et al., 1992; Huang et al., 1998; Coles et al., 1999; Olszewski et al., 2002).
Bioactive forms of GA are synthesized through the activities of a series of enzymes and GA is perceived by a soluble receptor, GID1 (Olszewski et al., 2002; Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006). GA signaling is negatively regulated by members of the DELLA proteins, including GAI, RGA, RGL1, RGL2 and RGL3, which compose a subfamily of the GRAS family of putative transcriptional regulators (Peng et al., 1997; Silverstone et al., 2001; Lee et al., 2002; Wen and Chang, 2002). The binding of GA to GID1 promotes an interaction between GID1 and DELLA proteins and this interaction promotes DELLA proteins to be ubiquitinated and subsequently degraded (Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006).

A strong mutation in the Arabidopsis gene *GA1*, which encode an enzyme that carries out the first committed step in GA biosynthesis, caused Arabidopsis to never flower under SDs and flower later in LDs compared to wild type, suggesting that GA plays an essential role in promoting flowering in SDs in Arabidopsis (Blazquez et al., 1998). Overexpression of GA-20 oxidase, an enzyme late in the GA biosynthesis pathway, caused early flowering in both LDs and SDs (Huang et al., 1998; Coles et al., 1999). Overexpression of *FLOWERING PROMOTING FACTOR1* (*FPF1*), a gene proposed to be involved in the GA signaling pathway, also accelerates the floral transition (Kania et al., 1997). Furthermore, deletion of the DELLA domain of GAI stabilized the protein, resulting in late flowering (Wilson et al., 1992; Willige et al., 2007), whereas loss-of-function mutations in

both GAI and RGA partially repress the late flowering phenotype of *ga1* mutants (King et al., 2001). Triple mutants carrying mutations in the three GID1 homologues of Arabidopsis (*gid1a*, *gid1b*, *gid1c*) never flower even when grown in continuous light conditions or treated with GA (Willige et al., 2007). Recent results suggested GA4 is the active endogenous gibberellin in mediating floral transition in Arabidopsis although exogenous application of both GA3 and GA4 can dramatically accelerate flowering in SDs (Eriksson et al., 2006). In contrast, it has been shown in monocot *Lolium temulentum*, GA5 and GA6 are the active GAs in the induction of flowering (King et al., 2001b; King et al., 2003), suggesting that the active forms of GA in flowering control may differ in different species.

GA acts to promote flowering, at least in part, by upregulating *LEAFY* (*LFY*) expression. In *ga1-3* mutants, *LFY* expression was strongly reduced and exogenous GA application increased *LFY* transcript levels in both the wild type and *ga1-3* mutant (Blazquez et al., 1998). Moreover, in *ga1-3*, constitutive expression of *LFY* accelerated flowering although it did not fully complement the flowering time effect of the mutant (Blazquez et al., 1998). The effect of GA on *LFY* expression was found to act through a cis-element in the *LFY* promoter which can be bound by a MYB-transcription factor, AtMYB33, in vitro (Blazquez and Weigel, 2000; Gocal et al., 2001). *AtMYB33* mRNA can be targeted by micro-RNA miR159 for degradation, and overexpression of miR159 reduced *LFY* transcription and delayed flowering (Achard et al., 2004). miR159 expression was found to be regulated by GA and abscisic acid (ABA) (Achard et al., 2004; Reyes and Chua, 2007). In addition, GA were shown to regulate expression of *SOC1* (Moon et al., 2003), and the study on a mutation in *EARLY BOLTING IN SHORT DAYS* (*EBS*) suggested that GA might also regulate *FT* expression (Gomez-Mena et al., 2001; Pineiro et al., 2003).

1.2.2. Photoperiod pathway

Photoperiod is one of the most important environmental factors that affect the floral transition. Classical physiological experiments demonstrated that plants perceive photoperiod mainly in the leaf, and transmit the information in the form of an elusive signal named florigen to the apical meristem to promote flowering (Knott,

1934; Zeevaart, 1976; Lang et al., 1977; Zeevaart, 1982; Corbesier and Coupland, 2006; Imaizumi and Kay, 2006). Recent advances in studying the facultative LDP Arabidopsis and plants of other photoperiodic-response types indicate that the core of the day-length measurement mechanism lies in the circadian regulation of *CONSTANS* (*CO*) expression and the subsequent photoperiodic induction of the transcription of the *FLOWERING LOCUS T* (*FT*) gene (Hayama and Coupland, 2004; Corbesier and Coupland, 2006; Imaizumi and Kay, 2006).

In Arabidopsis, light signals are perceived by different photoreceptors, at least including phytochromes (PHYA, PHYB, PHYC, PHYD and PHYE), which perceive red and far-red light, and cryptochromes (CRY1 AND CRY2), which perceive blue/UV-A light, as well as the ZEITLUPE (ZTL)/FLAVIN-BINDING, KELCH REPEAT, AND F-BOX 1 (FKF1)/LOV KELCH PROTEIN2 (LKP2) family (Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001; Imaizumi et al., 2003; Balasubramanian et al., 2006b). Light input resets the circadian clock in plants (Somers et al., 1998). The core oscillator of the circadian clock consists of a negative feed-back loop comprising the MYB transcription factors, CCA1 and LHY, and the CCT-domain protein TOC1 (Schaffer et al., 1998; Wang and Tobin, 1998; Strayer et al., 2000; Alabadi et al., 2001). Light promotes transcription of *CCA1* and *LHY* in the morning as well as the translation of LHY protein, and CCA1 and LHY in turn suppress the expression of *TOC1* (Martinez-Garcia et al., 2000; Alabadi et al., 2001; Kim et al., 2003). Since TOC1 is involved in activation of *LHY* and *CCA1* transcription, the reduction in TOC1 is followed by a decrease in *LHY* and *CCA1* expression. By the evening, LHY and CCA1 protein levels have decreased, which allows an increase in *TOC1* expression. High levels of TOC1 in turn upregulate *LHY* and *CCA1* expression to peak the next morning.

This circadian feedback loop generates a series of rhythmic outputs including the circadian-regulated flowering-time genes *FKF1*, *GIGANTEA* (*GI*), and *CO* (Fowler et al., 1999; Suarez-Lopez et al., 2001; Imaizumi et al., 2003). In addition to their roles in the circadian clock, FKF1 and GI also act as the clock output to activate *CO* expression. FKF1 is an F-box protein and acts in a light-dependent manner to upregulate the transcription of *CO* by degrading CYCLING DOF FACTOR 1 (CDF1) in the late afternoon. CDF1 is a Dof transcription factor which represses *CO* transcription by directly binding to the promoter in the morning in the absence of high levels of FKF1 (Imaizumi et al., 2005). *GI*, which encodes a large nuclear protein with unknown function, is necessary for *CO* expression regardless of

photoperiod since in *gi* mutant *CO* expression is depressed in both LD and SD (Fowler et al., 1999; Suarez-Lopez et al., 2001; Mizoguchi et al., 2005). In addition to regulation of *CO* transcription, light also regulates the stability of CO protein, PHYA and CRY2 stabilize CO in the late afternoon of a LD treatment while PHYB promotes CO degradation in the morning (Valverde et al., 2004). Furthermore, recent results suggest that SPA1, 3 and 4, and COP1 are also involved in regulation of CO stability and activity at least under SD conditions (Ishikawa et al., 2006; Laubinger et al., 2006).

Therefore, in the facultative LDP Arabidopsis, *CO* transcription shows a rhythmic pattern, peaking both in the late afternoon and at night under LDs and only at night under SDs. However, CO protein accumulates above a threshold level to activate the downstream floral promoter gene *FT* only under LDs as CO is only stabilized in the light.

Since CO does not contain a typical DNA binding domain, it was proposed to induce *FT* expression through interacting with other transcription factors that directly bind to the *FT* promoter. Recent results suggest that CO is recruited to the *FT* promoter by interacting with subunits of the HAP protein complex, including HAP3 and HAP5 (Ben-Naim et al., 2006; Wenkel et al., 2006). Furthermore, accumulating results indicate that FT acts as a component of the floral signal which moves from the leaves to the shoot apical meristem (See Section 1.2.5. for the detailed introduction)

In rice, a SDP, a conserved circadian regulation mechanism was suggested to regulate the expression of *Hd1*, a rice *CO* orthologue (Yano et al., 2000). However, different from the relationship between *CO* and *FT*, in LD conditions Hd1 represses the expression of *Hd3a*, the rice orthologue of *FT* (Lin et al., 2000; Izawa et al., 2002; Hayama et al., 2003). Conversely, Hd1 upregulates *Hd3a* transcription during the night in SDs and thereby promotes flowering in SDs (Hayama et al., 2003).

1.2.3. Autonomous pathway

So far, seven loss-of-function autonomous pathway mutants, *fca*, *fy*, *fpa*, *flk*, *fld*, *fve* and *ld* have been isolated (Koornneef et al., 1991; Lee et al., 1994; Chou and Yang,

1998; Lim et al., 2004). These mutants flower late but retain a photoperiodic response. Then late flowering can be overcome by vernalization and the mRNA levels of *FLOWERING LOCUS C* (*FLC*), which encodes a potent MADS box floral repressor (Michaels and Amasino, 1999; Sheldon et al., 1999), is elevated in these mutants (Michaels and Amasino, 2001; Lim et al., 2004). Moreover, mutations in *FLC* are epistatic to mutations in the autonomous pathway genes, suggesting that the main function of autonomous pathway genes is to repress *FLC* expression, thereby promoting flowering (Michaels and Amasino, 2001; Lim et al., 2004).

Although all members of the autonomous pathway act to repress *FLC* expression, the regulation occurs at different levels. FCA, an RNA binding protein, interacts via its WW domain with FY, a 3'-end-processing and poly-adenylation factor, (Macknight et al., 1997; Simpson et al., 2003). Although FCA negatively regulates its own expression in an FY-dependent manner by promoting the use of an internal polyadenylation site in the *FCA* pre-mRNA, the mechanism by which it regulates *FLC* expression remains unknown (Macknight et al., 1997; Simpson et al., 2003). Both *FPA* and *FLOWERING LATE WITH KH MOTIFS* (*FLK*) also encode proteins carrying RNA binding domains (Schomburg et al., 2001; Lim et al., 2004), but as for FCA, no evidence has been shown that they directly regulate *FLC* mRNA processing. *FLOWERING LOCUS D (FLD)* encodes a protein homologous to a human protein that functions in the histone deacetylase complex (He et al., 2003), and *FVE* codes for the nuclear WD-repeat protein, MSI4 (Ausin et al., 2004). Both FLD and FVE were proposed to modulate chromatin status through a deacetylase complex which deacetylates histone residues of *FLC* chromatin, leading to the suppression of its expression (He et al., 2003; Ausin et al., 2004). Finally, *LUMINIDEPENDENS* (*LD*) encodes a homeodomain protein with unknown function (Lee et al., 1994).

1.2.4. Vernalization pathway

Many plants, including winter annuals, most (if not all) biennials and many perennials, acquire competence to flower in spring by responding to exposure to winter temperatures. This vernalization requirement helps to ensure that flowering

does not occur in the fall before the onset of unfavorable conditions in the following winter, but rather that flowering occurs rapidly in the favorable conditions during spring. Classical physiological experiments showed that vernalization treatment can be “remembered” by plants: the vernalized state can persist through many mitotic cell divisions even after the plants are returned to optimal temperatures for growth, but vernalization requirement is reset after sexual reproduction (Wellensiek, 1962, , 1964; Lang, 1965). These results suggest that vernalization is an epigenetic response (Sung and Amasino, 2005).

In the model plant *Arabidopsis thaliana*, the first locus that was shown to confer a vernalization requirement was *FRIGIDA* (*FRI*) (Napp-Zinn, 1987). Further studies indicated that *FRI* plays a major role in vernalization requirement in a large number of accessions (Burn et al., 1993; Lee and Amasino, 1993; Clarke and Dean, 1994). Recent advances in genetic studies revealed that *FRI* acts by upregulating the expression of *FLC*, which encodes a MADS box protein that represses flowering (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). *FLC* is expressed at high levels in most late flowering accessions that show a vernalization response and at lower levels in rapid cycling accessions. Loss-of-function mutations in *FLC* convert a winter annual into a summer annual. In wild type plants, vernalization quantitatively represses *FLC* expression and after vernalization *FLC* expression is maintained at low levels until the next generation (Michaels and Amasino, 1999; Sheldon et al., 1999).

Before winter, in vernalization-requiring accessions, *FLC* is expressed at high levels due to the activities of activator genes. The first group of these genes includes *FRI*, *FRIGIDA-LIKE 1* (*FRL1*), *FRIGIDA-ESSENTIAL 1* (*FES1*) and *SUPPRESSOR OF FRIGIDA 4* (*SUF4*) (Johanson et al., 2000; Michaels et al., 2004; Schmitz et al., 2005; Kim et al., 2006b; Kim and Michaels, 2006). Both *FRI* and *FRL1* encode plant specific proteins containing a coiled-coil domain. *FES1* encodes a CCCH zinc finger protein and *SUF4* encodes a C2H2-type zinc finger protein. These proteins are specifically required for activation of *FLC* expression and *FLC* transcription is suppressed in mutants of these genes. Epistasis analysis indicates that these genes act cooperatively to promote the expression of *FLC* (Schmitz et al., 2005). Protein-protein interaction assays suggest that they may act in the same protein complex (Kim et al., 2006b). Although the mechanism by

which this group of genes promotes *FLC* expression remains unknown, histone H3 lysine 4 trimethylation levels.(H3K4 trimethylation, which is a mark of active genes) in *FLC* chromatin are much higher in wild-type plants carrying dominant alleles of these genes than in mutants (He et al., 2004a; Kim and Michaels, 2006). This suggests that regulation of chromatin structure may be important in the activation of *FLC* by FRI and related proteins.

A second group of genes required for *FLC* activation includes *ACTIN RELATED PROTEIN 6* (*ARP6*, also called *EARLY IN SHORT DAYS 1* (*ESD1*) and *SUPPRESSOR OF FRIGIDA 3* (*SUF3*)) and *PHOTOPERIOD INDEPENDENCE 1* (*PIE1*), which encode homologs of components of the yeast SWR1 complex (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006). SWR1 is an ATP-dependent remodeling complex involved in histone H2A variant deposition (Kobor et al., 2004). Indeed, recent results indicate that ARP6 and PIE1 are required for deposition of H2A.Z at multiple loci including *FLC* and its homologous genes *MADS AFFECTING FLOWERING 4* (*MAF4*) and *MAF5* to maintain high-level expression of these genes (Deal et al., 2007). Other genes involved in activating *FLC* expression encode components of the yeast transcriptional activating PAF1 (RNA polymerase II associated factor 1) complex (He et al., 2004a; Oh et al., 2004). In yeast, this complex activates gene expression by recruiting a histone H3K4 methyltransferase-containing complex to the chromatin of target genes through interacting with the methyltransferase SET1 (Krogan et al., 2003; Ng et al., 2003). A similar mechanism may exist in Arabidopsis for PAF1 complex to promote *FLC* expression since lesions in genes encoding both components of PAF1 complex and EARLY FLOWERING IN SHORT DAYS (EFS), a homolog of SET1, result in the loss of trimethylation of H3K4 in *FLC* chromatin and the loss of the ability to activate *FLC* transcription (He et al., 2004a; Oh et al., 2004; Kim et al., 2005).

Even in the presence of the activator genes described above, vernalization can repress *FLC* expression through regulating *FLC* chromatin structure. Before vernalization, *FLC* chromatin is in an active conformation, enriched in covalent modifications on histones such as acetylation of lysines 9 and 14 of histone 3 (H3K9 and H3K14) and methylation of H3K4, which are thought to be the marks of active chromatin (Sung and Amasino, 2004). During vernalization, these

active-chromatin-related modifications are reduced whereas other modifications including methylation of H3K9 and H3K27 increase (Bastow et al., 2004; Sung and Amasino, 2004; Mylne et al., 2006; Sung et al., 2006b; Sung et al., 2006a; Greb et al., 2007). In other eukaryotic organisms, methylation of H3K9 and H3K27 result in mitotically stably repressed states of target genes through the recruitment of transcriptional repressor complexes (Ringrose and Paro, 2004; Fuchs et al., 2006). Genetic analysis of Arabidopsis mutants showing impaired vernalization response identified a series of genes, including *VERNALIZATION INSENSITIVE 3* (*VIN3*), *VIN3-LIKE 1* (*VIL1,* also called *VERNALIZATION 5* (*VRN5*)), *VERNALIZATION 1* (*VRN1*) and *VERNALIZATION 2* (*VRN2*), homologs of which have been suggested to be involved in chromatin remodeling in other organisms (Gendall et al., 2001; Levy et al., 2002; Bastow et al., 2004; Sung and Amasino, 2004; Mylne et al., 2006; Sung et al., 2006b; Sung et al., 2006a; Greb et al., 2007). Lesions of these genes result in failure to repress *FLC* expression after vernalization and a reduction in repressive modifications (e.g. methylation of H3K9) in *FLC* chromatin. Therefore, these genes function to establish and/or maintain the effect of vernalization on *FLC* expression through changing chromatin structure. *VIN3*, which encodes a PHD-finger protein, is only expressed after exposure to a prolonged period of cold and its expression is switched off quickly after return to warm temperatures, suggesting that it is an important early component of vernalization response (Sung and Amasino, 2004). In addition, *LIKE HETEROCHROMATIN PROTEIN 1* (*LHP1*) has been suggested to function in maintenance of the repression of *FLC* expression caused by vernalization treatment (Mylne et al., 2006; Sung et al., 2006b).

Although *FLC* is the major gene responsible for the generation of vernalization requirement, *flc* null mutants still weakly respond to vernalization (Michaels and Amasino, 2001), suggesting that other genes contribute to the vernalization requirement. In the Arabidopsis genome, there are five additional *FLC* homologs, *MADS AFFECTING FLOWERING* (*MAF1-5*; *MAF1* is also known as *FLM*), and they have been shown to act as floral repressors when ectopically expressed (Ratcliffe et al., 2001; Ratcliffe et al., 2003; Scortecci et al., 2003). Mutations in *MAF1*/*FLM* and *MAF2* cause earlier flowering. Therefore, these genes may play a role in vernalization requirement. In particular, *MAF2* is required for preventing premature vernalization in response to brief cold spells (Ratcliffe et al., 2003). Also

VIL1/*VRN5* and *VIN3* are required for establishing vernalization-mediated repressed state of both *FLC* and *FLM*/*MAF1*, suggesting that similar mechanisms may be used to silence both *FLC* and *FLM*/*MAF1* expression during vernalization (Sung et al., 2006a). In addition, upregulation of some floral promoter genes, such as *AGL24* and *AGL19*, have also been suggested to be involved in acceleration of flowering in response to vernalization in *FLC* independent pathways (Yu et al., 2002; Michaels et al., 2003b; Schonrock et al., 2006; Sung et al., 2006a).

Genetic analysis indicates that the involvement of *FLC* in the establishment of vernalization requirement seems to be conserved in other members of the *Brassicaceae* family (Osborn et al., 1997; Schranz et al., 2002; Kim et al., 2006a; Okazaki et al., 2007). Recently, by analyzing the EST databases, Reeves et al. predicted that vernalization-responsive *FLC* homologs exist in at least half of eudicot species (Reeves et al., 2006). However, the mechanisms underlying vernalization requirement in angiosperms are not as conserved as those that mediate photoperiod response: in cereals no *FLC* homolog has been found and the winter annual behavior is conferred by other genes. For instance, in wheat and barley dominant alleles of *VERNALIZATION 1* (*VRN1*), which encodes a MADS box floral activator, cause a spring growth habit, whereas dominant alleles of *VERNALIZATION 2* (*VRN2*), which encodes a ZCCT family zinc-finger protein, are necessary for a winter growth habit (*VRN1* and *VRN2* in cereals have no relationship with Arabidopsis *VRN1* and *VRN2*) (Tranquilli and Dubcovsky, 2000; Yan et al., 2003; Yan et al., 2004). In many winter varieties of wheat, *VRN2* is expressed at high levels, thereby repressing *VRN1* expression. Prolonged cold exposure promotes flowering by shutting off *VRN2* and relieving the repression of *VRN1* (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Yan et al., 2004). In cereals, spring growth habit has been shown to arise mainly from the failure of *VRN2* to repress *VRN1* either because of loss-of-function mutations in *VRN2* or because of deletions of regions of *VRN1* that confer *VRN2*-mediated repression (Yan et al., 2003; Yan et al., 2004; Fu et al., 2005).

1.2.5. Integration of floral pathways and initiation of floral meristems

The multiple floral induction pathways quantitatively regulate a common set of targets. These genes, the floral pathway integrators, at least include *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) and *LEAFY* (*LFY*), which activate the program for initiation of floral meristems and the subsequent specification of floral organs (Mouradov et al., 2002; Simpson and Dean, 2002). *FT* encodes a 20 kDa protein homologous to the phosphatidylethanolamine-binding or Raf kinase inhibitor proteins (Kardailsky et al., 1999; Kobayashi et al., 1999) and is expressed in the vasculature of leaves (Takada and Goto, 2003; An et al., 2004; Wigge et al., 2005). *ft* mutants flower late in LD but not SD, whereas constitutive expression of *FT* causes extremely early flowering and a terminal flower under both conditions. *SOC1* encodes a MADS-box transcription factor and is expressed in the whole plant, while its expression increases rapidly in the apex during floral transition (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). *soc1* mutants flower late in both LD and SD and overexpression of *SOC1* causes early flowering (Borner et al., 2000; Lee et al., 2000). *LFY* encodes a new type of plant specific transcription factor (Weigel et al., 1992; Parcy et al., 1998). It is expressed weakly in young leaf primordia during vegetative growth and increases to high levels in floral meristems (Blazquez et al., 1997; Blazquez et al., 1998). Overexpression of *LFY* from a constitutive promoter or introduction of an additional copy of *LFY* including its own promoter accelerates flowering, while mutations in *LFY* delay flowering (Weigel and Nilsson, 1995).

CO is the major output of the photoperiod pathway and it promotes flowering through activating the expression of *FT* and *SOC1*. In *co* mutants, the expression of all of these genes is reduced to different extents (Nilsson et al., 1998; Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2000; Suarez-Lopez et al., 2001), and constitutive activity of *CO* induces early flowering and increases the expression of all integrator genes (Simon et al., 1996; Samach et al., 2000). Furthermore, overexpression of *FT*, *SOC1* or *LFY* can complement, to different degrees, the late flowering phenotype of *co* mutants (Nilsson et al., 1998; Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000). However, recent microarray data

suggest that *FT* is the major direct target of CO (Wigge et al., 2005). Although CO acts to promote *FT* expression in leaf vasculature, FT functions in apical meristems to activate expression of *AP1*, a floral meristem identity gene, through the interaction with a bZIP transcription factor FD (An et al., 2004; Abe et al., 2005; Wigge et al., 2005), suggesting that *FT* mRNA, FT protein or some resulting products may translocate from the vasculature in the leaf to the apical meristem. Grafting tomato plants overexpressing *SFT*, a tomato orthologue of *FT*, to non-induced tomato plants resulted in early flowering in these plants, but no *SFT* mRNA was detected to transfer through the graft junction (Lifschitz et al., 2006). This result indicates that FT protein or some downstream product may comprise the transmissible signal. Recently, the observation that the phloem sap exuded from *Brassica napus* plants grown in LDs contained proteins homologous to FT and TSF (a floral activator with very high homology to FT) further suggests possible movement of FT protein along the vasculature tissues and its possible role as a component of the florigen signal (Giavalisco et al., 2006). Latest reports indicate that FT and Hd3a proteins are components of the signals that move from leaves to apical meristems to activate flowering in Arabidopsis and rice, respectively (Corbesier et al., 2007; Tamaki et al., 2007). In addition to its role in activating *AP1* expression, FT can also promote the expression of *SOC1* (Yoo et al., 2005). Furthermore, it is suggested that the activation of *LFY* by CO is through the activity of *SOC1* (Lee et al., 2000; Mouradov et al., 2002; Jack, 2004), but more evidence is required to confirm this notion. As described above, *LFY*, and possibly *SOC1* also integrate floral signals from the GA pathway (Section 1.2.1).

The signals from the autonomous and the vernalization pathways are integrated at the level of regulation of *FLC* expression (Boss et al., 2004). Expressing *FLC* from either an apical meristem- or a phloem- specific promoter established that FLC acts in both tissues to achieve the full repression of flowering (Searle et al., 2006). Detailed analysis suggests that FLC represses *FD* and *SOC1* expression in apical meristems and *FT* and *SOC1* in the phloem, and that repression of *FLC* by autonomous pathway genes and vernalization relieves the expression of *FT*, *SOC1* and *FD*, consequently promoting floral transition (Searle et al., 2006).

Activation of the floral pathway integrator genes induces the expression of a spectrum of downstream genes to establish the identity of floral meristems and the

subsequent specification of floral organs. LFY activate expression of *AP1* and *CAULIFLOWER* (*CAL*), which encodes a MADS box transcription factor highly homologous to AP1, by directly binding to their promoters (Parcy et al., 1998; Wagner et al., 1999; William et al., 2004). Expression of *AP1* in turn positively regulates *LFY* expression in the floral meristems (Liljegren et al., 1999). Moreover, LFY and AP1 repress the expression of *TERMINAL FLOWER 1* (*TFL1*) to assure the establishment of floral meristems on the flanks of apical meristems. On the other hand, TFL1 suppresses both the upregulation and the activities of *LFY* and *AP1* to maintain apical meristems from being converted to floral meristems (Weigel et al., 1992; Bowman et al., 1993; Gustafson-Brown et al., 1994; Bradley et al., 1997). Although TFL1 and FT are homologs, they exhibit antagonistic functions in the regulation of flowering time. The fact that all developmental phases (vegetative, early inflorescence and late inflorescence) of the shoot apical meristem are prolonged in TFL1-overexpression lines and that all these phases are shortened in *tfl1* mutants indicate that TFL1 is a floral repressor that regulates floral transition through all the major developmental stages (Ratcliffe et al., 1998; Ratcliffe et al., 1999). During the vegetative stage, *TFL1* is only weakly expressed in the inner cells of the shoot apical meristem, whereas after floral transition it is highly expressed in the sub-dome domain of the inflorescence apex as well as in axillary meristems (Bradley et al., 1997; Ratcliffe et al., 1998). Consistent with the opposite functions of FT and TFL1, *tfl1* mutants flower early as well as forming a terminal flower, which resembles the phenotypes caused by overexpression of *FT* (Shannon and Meeks-Wagner, 1991; Bradley et al., 1997; Kardailsky et al., 1999; Kobayashi et al., 1999).

1.2.6. Other pathways involved in flowering-time control

In addition to the regulation from the four major pathways described above, flowering time is also influenced by other factors (many of them are related to biotic or abiotic stress), such as light quality (Cerdan and Chory, 2003), ambient temperature (Blazquez et al., 2003; Balasubramanian et al., 2006; Lee et al., 2007), nitrogen oxide (NO) (He et al., 2004b), abscisic acid (ABA) (Razem et al., 2006), ethylene (Achard et al., 2007) and salicylic acid (Martinez et al., 2004). Flowering

regulatory signals arising from most of these factors have already been shown to be integrated into or interlinked with the flowering-time-control network described above. Signals generated by growing plants under canopy shade conditions ultimately promote flowering by activation of *FT* expression (Cerdan and Chory, 2003). Elevated ambient temperatures also increase *FT* expression to accelerate flowering (Blazquez et al., 2003; Balasubramanian et al., 2006; Lee et al., 2007). NO delays flowering through suppressing the expression of *CO* and *GI*, and increasing the expression of *FLC* (He et al., 2004b). ABA is suggested to bind to FCA, which is thought to be an ABA receptor, and the binding abolishes the function of FCA-FY complex, which in turn causes accumulation of FLC and consequent late flowering (Razem et al., 2006). Ethylene delays flowering possibly by modulating the activity of DELLA proteins and decreasing the levels of bioactive GAs (Achard et al., 2007). Although the detailed mechanism by which salicylic acid regulates flowering remains unknown, it was suggested that photoperiod and autonomous pathways are involved (Martinez et al., 2004).

1.3. Flowering control in polycarpic perennial plants

Tremendous effort has also been expended to study flowering-time control in perennials because of their important roles in both ecology and agriculture (Thomas et al., 2000). However, smaller progress has been made in understanding the molecular mechanisms underlying flowering in perennials than in the annual model species Arabidopsis. In many cases (especially, for the woody perennials whose juvenility usually persist for years), the length of the juvenile phase and the large size of the plant constitute major hindrances for genetic analysis with perennial species. The limitation in availability of varieties that can be crossed and relatively difficult transformation process also contribute to difficulties in studying these species. Although not as fast as in Arabidopsis, important progress has been made recently in understanding the mechanisms controlling flowering time in perennial species. Some of this progress (especially in perennial grasses and woody perennials) is introduced in the following sections.

1.3.1. Perenniality in grasses

Most crops, such as wheat, rice, and maize are annual members of the grass family while their wild relatives are perennials. Important turf grasses are also perennials. In many grass species, vegetative meristems are maintained as vegetative tillers or rhizomes while floral transition in other apical meristems occurs (Hu et al., 2003; Townsend, 2006). Mapping the major QTLs (*Rhz2* and *Rhz3*) linked to perennial traits (rhizomatousness) using populations generated by crossing perennial and annual rice species revealed that the QTLs from the perennial parent were dominant, implying that perenniality might be ancestral to annuality (Hu et al., 2003). A weak perennial habit was conferred on annual wheat by a single chromosome addition from its wild relative *Thinopyrum elongatum*, suggesting that a small number of genes might be enough to exert the perennial life cycle (Lammer et al., 2004).

1.3.2. Perenniality and flowering control in woody perennials

Woody perennials form the forests on earth, and also provide the fruits and other resources (e.g. wood) for human beings. Therefore, efforts to understand the mechanisms regulating growth and development, especially the floral transition, of woody perennials have long been made (Longman, 1976; Zimmerman, 1985b; Meilan, 1997). Knowledge on flowering-time control can suggest practical ways to manipulate the development of woody perennials by for example preventing flowering to improve the quality of wood or promoting flowering to accelerate the breeding process and to make genetic analysis easier. Early physiological experiments revealed a series of factors that affect flowering of woody perennials: grafting, culture conditions such as temperature, photoperiod and mineral nutrition, physical constraints (root restriction, shoot training and girdling), phytohormones and growth retardants (Longman, 1976; Zimmerman, 1985b; Meilan, 1997). Recent transgenic studies in woody perennial species have also begun to reveal the molecular mechanisms underlying flowering-time control in these plants.

In poplar, overexpression of the Arabidopsis floral meristem identity gene *LFY* caused extremely early flowering (Weigel and Nilsson, 1995), suggesting that the

mechanisms controlling flowering time may be conserved between herbaceous annuals and woody perennials. In addition to early flowering, overexpression of Arabidopsis *LFY* in poplar caused the conversion of all vegetative meristems to reproductive meristems, leading to premature cessation of flower development and plant death (Meilan, 1997). This observation is particularly interesting because it might imply a close relationship between flowering, polycarpy and senescence, even although the induction of flowering is artificial. Recent results showed that overexpression of *PtFT1* and *PdFT2*, two *FT* orthologs in poplar, caused very early flowering of transgenic poplar plants (Bohlenius et al., 2006; Hsu et al., 2006). Furthermore, in non-transgenic plants expression of both *PtFT1* and *PdFT2* increased with development from juvenile to adult phase. Therefore, poplar *FT* orthologs may play a role in juvenility. Short-day treatment, which normally induces growth cessation in poplar, failed to exert its effect on transgenic poplar plants overexpressing *PtFT1*, whereas *PtFT*-knockdown poplar plants were hypersensitive to short days in the induction of growth cessation (Bohlenius et al., 2006). Together with the finding that the critical daylengths required for inducing growth cessation in poplar varieties from different habitats positively correlates with the latitudes of their habitats, these results indicate that *FT* orthologs play an important role in mediating short-day-induced growth cessation (Bohlenius et al., 2006) and suggest that orthologs of Arabidopsis flowering-time genes may also play a role in regulating perennial-life-cycle specific characters. The specific upregulation of PdFT2 expression observed in axillary buds with a reproductive fate but not those with a vegetative fate implies that *FT* orthologs from poplar may contribute to the diverse responses to floral induction in different apical mersitems (Hsu et al., 2006).

Similarly, in citrus plants, overexpression of either Arabidopsis floral meristem identity genes *LFY* and *AP1* or its endogenous *FT* homolog caused very early flowering (Pena et al., 2001; Endo et al., 2005). This observation further supports the notion that the fundamental mechanisms controlling floral transition in higher plants are conserved and that juvenility is regulated by genes controlling flowering time (Tan and Swain, 2006). Isolation and analysis of expression patterns and function of a homolog of Arabidopsis *TFL1* from apple trees, *MdTFL1*, explored the contribution of floral repressors to juvenility in woody perennials (Kotoda and Wada, 2005; Kotoda et al., 2006). Apple trees in which MdTFL1 expression was reduced by DsRNAi flowered within the first year rather than 5 years, strongly

suggesting that *MdTFL1* contributes to juvenility in apple trees (Kotoda and Wada, 2005; Kotoda et al., 2006).

1.4. *Arabis alpina*, a promising model species for studying perenniality and flowering-time control in perennials

Arabis alpina (Alpine rock cress), which is widely used to decorate gardens, belongs to the *Brassicaceae* family that is currently estimated to comprise about 338 genera and 3,700 species (Koch et al., 1999; Warwick et al., 2006). In addition to sexual reproduction, *Arabis alpina* propagates through vegetative colonization via forming adventitious roots on the vegetative part of some axillary branches. The adventitious roots are produced from lower nodes of the stem and fall onto the soil allowing an *Arabis alpina* plant to live for many years as an expanding colony (Whittaker, 1993). The characteristics of *Arabis alpina* make it a promising perennial model species for studying many biological issues, especially flowering time control and perenniality. First, as a member of the *Brassicacae* family, *Arabis alpina* is closely related to other members, importantly including *Arabidopsis thaliana*, which is the most popular model for studying modern plant biology, and Brassica species, many of which are important crops (Koch et al., 1999; Koch et al., 2000; Koch et al., 2001; Bailey et al., 2006; Clauss and Koch, 2006). *Arabis alpina* diverged from *Arabidoposi*s *thaliana* around 20 million years ago. On one hand, the increasing knowledge from the studies of Arabidopsis can be easily transferred to studies of *Arabis alpina*. On the other hand, the knowledge acquired from *Arabis alpina* will be very helpful for studies of Brassica species, which are more closely related to *Arabis alpina* than Arabidopsis is (Bailey et al., 2006). Furthermore, like *Arabidopsis thaliana*, *Arabis alpina* is diploid (2n = 16) and is self compatible (Koch et al., 1999; Koch et al., 2000), which makes genetic analysis and application of many tools (e.g., mutagenesis) more straightforward. Another advantage of *Arabis alpina* is that more than 140 accessions have been collected from a wide range of habitats and the phylogeography of *Arabis alpina* has been reported (Koch et al., 2006). By analyzing synonymous mutations rates of some marker genes of accessions collected from different regions, Koch et al. suggested that less than 2 million years ago *Arabis alpina* originated in Asia minor and

migrated three times from there into different regions in Asia, Europe and east Africa, and finally formed the current distribution pattern (Koch et al., 2006). The existence of genetic divergence in different accessions of this diploid species makes map-based cloning strategy feasible. Moreover, the successful growth of different accessions in diverse environments makes *Arabis alpina* a promising candidate for studying mechanisms underlying adaptive evolution. Compared with Brassica species the small genome size of *Arabis alpina* (Genome sizes: *Arabis alpina*, 392 Mb; Arabidopsis thaliana, 157 Mb; Brassica rapa, 529 Mb.) and the absence of the genome triplication that occurred in the Brassica species implies that less redundant sequences exist in its genome, which will make investigation of the function of individual genes easier (Johnston et al., 2005; Lysak and Lexer, 2006). The relatively smaller size (up to 40 cm in height) and faster growing cycles (Seed-to-seed time for most accessions grown in favorable experimental conditions is 6 months) of *Arabis alpina* make it possible to grow them and carry out genetic analysis in large scale in the laboratory (our unpublished observation), which is impossible for most woody perennials. Recently, other collaborators and we have developed powerful tools (e.g., BAC library) and built a genetic linkage map of *Arabis alpina*, facilitating further investigations on multiple biological issues with this species.

1.5. Aims of the thesis

In this thesis I initiate a comparative analysis of flowering time control in *Arabis alpina* and Arabidopsis. I describe the vernalization response of *Arabis alpina* and establish a system that can be used to study juvenility. I clone *Arabis alpina* homologues of *FLC*, *SOC1* and *TFL1* and test their expression patterns in response to vernalization and their correlation with juvenility, seasonal flowering and differential competence of meristems to flower. I also develop a transformation system for *Arabis alpina* that allows the involvement of *Arabis alpina* genes in flowering time control to be functionally tested.

2. Flowering behavior of *Arabis alpina*

2.1. Introduction

Most relevant information on flowering of *Arabis alpina* was introduced in Chapter I, so here only a brief introduction will be presented covering the origin of axillary shoot apical meristems.

The basic body plan of a seed plant is created during embryogenesis through the establishment of an apical-basal axis. The axis is defined by a shoot apical meristem (SAM) at one end and the root apical meristem at the other end (McSteen and Leyser, 2005). During the postembryonic phase, the SAM produces a series of lateral organs on its flanks. During the vegetative phase, the lateral organs produced by the SAM include leaves and the axillary meristems in the axils of leaves. Axillary branches are then generated from the axillary meristems. After the transition of the SAM from the vegetative phase to the reproductive phase, the organs produced from the SAM are leaves and axillary meristems, from which inflorescence branches grow out later. But later on, floral meristems arise from the SAM and flowers are the prominent (or only) forms of lateral organs (Schmitz and Theres, 2005).

The origin of axillary meristems has long been debated. Based on morphological observations of different species, two opposing hypotheses were proposed concerning their origin. Some researchers insist that axillary meristems initiate *de novo* in leaf axils (Snow and Snow, 1942), whereas others propose they derive from a group of stem cells that detach from the SAM at the same time as leaf initiation and never lose their meristematic identity (Garrison, 1955). Recent studies provide molecular markers to investigate the origin of axillary meristems. Study of the function and expression patterns of the *LATERAL SUPPRESSOR* genes from tomato and Arabidopsis (*LS* and *LAS* respectively) were particularly instructive (Schumacher et al., 1999; Greb et al., 2003). *LS* and *LAS* are orthologs. Loss-of-function mutations of *LAS* have reduced branching from nodes carrying rosette leaves due to failure in the development of axillary meristems in these nodes (Greb et al., 2003). A similar reduced branching phenotype due to failure in the development of axillary meristems was observed in vegetative nodes of tomato

(Schumacher et al., 1999). In Arabidopsis, *LAS* transcripts are expressed at the axils of all leaf primordia derived from the primary SAM. *LAS* expression originally begins to accumulate early during leaf initiation on the SAM just as the primordium starts to form a bulge (Greb et al., 2003). Further studies examining *LAS* expression together with other meristem identity genes indicate that axillary meristem initiation starts at the time of leaf initiation with the definition of an adaxial zone of high meristematic potential. The cells in this zone can either rapidly develop into a fully developed axillary meristem or can remain in an undifferentiated state if *LAS* is present (McSteen and Leyser, 2005).
In this chapter, the flowering behavior of *Arabis alpina Pajares* and *Arabis alpina Bonn* as well as its correlation with juvenility and differential competence of meristems in different branches to flower will be described.

2.2. Juvenility in *Arabis alpina*

Arabis alpina Pajares has an obligate requirement for vernalization treatment to induce flowering. Previous work in our laboratory indicated it has a 3-4 week long juvenile phase in vernalization response (called juvenility in vernalization response). For example, when a twelve-week cold treatment was applied to plants that had been grown under long-day conditions at normal temperatures for more than 4 weeks, the main shoot apices underwent the floral transition during the late stages of the treatment. However, when the same cold treatment was applied to seeds or plants that had been grown at normal temperatures for less than 4 weeks, no floral transition occurred during the cold treatment or even after plants were moved back to warm temperatures. Therefore, plants younger than 4 weeks old could not respond to vernalization. To test whether longer cold treatment can promotes flowering in young plants, two week old *Arabis alpina Pajares* plants were exposed to 4 °C for more than 12 months. No flowering occurred during the treatment or even after they were moved back to a warm environment (Figure 2A-C) although at the end of vernalization the plants had already produced many more leaves and were much larger than 4-week-old plants grown at normal temperatures (Figure 2B and 2D). However, floral buds could be seen clearly at the end of the cold treatment of plants that had been grown under LDs for 4 weeks and then given

a ten-month cold treatment (Figure 2E). These observations not only confirmed the existence of juvenility in vernalization response in *Arabis alpina* but also suggested that juvenility can be maintained or prolonged during vernalization treatment of plants that are younger than 4 weeks old when first exposed to cold. Furthermore, the delay in flowering caused by vernalization of plants younger than 4 weeks old was also observed in *Arabis alpina Bonn* accession, which does not show a requirement for vernalization to flower (Figure 2F and 2G; See Section 2.4 for more detailed information about the Bonn accession).

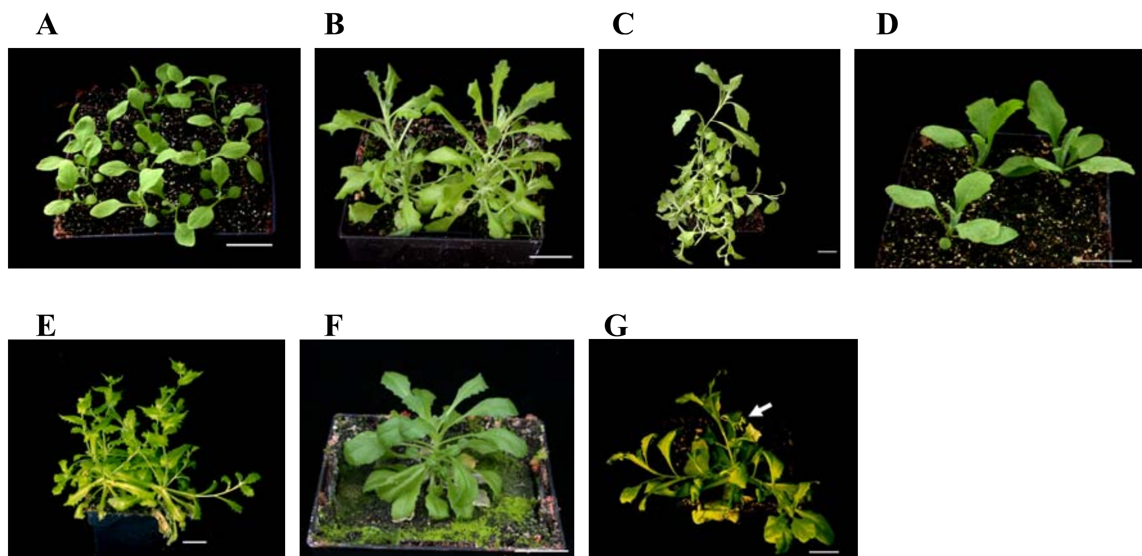


Figure 2. Effects of prolonged cold treatment on floral promotion in plants at different developmental stages.
(A) 2 week old *Arabis alpina Pajares* plants grown under LDs. **(B)** Plants from **(A)** after exposure to a 12 month cold treatment. No flowering was observed. **(C)** Plants from **(B)** after 4 weeks grown in LDs. No flowering was detected. **(D)** 4 week old *Arabis alpina Pajares* plants grown under LDs. **(E)** Floral buds were already visible after an 10 month cold treatment was applied to plants shown in **(D)**. **(F)** An *Arabis alpina Bonn* plant which was first grown in LDs for 2 weeks and then treated with cold for 13 months. No flowering was observed. **(G)** An *Arabis alpina Bonn* plant which was first grown in LDs for 4 weeks and then treated with cold for 8 months. Flowering was observed. The arrow was used to highlight the floral buds. Bars, 2 cm.

To test whether the juvenility in flowering of *Arabis alpina* occurs more generally than in vernalization, the response to a floral promoting photoperiod (see Section 2.4) was tested in the *Arabis alpina Bonn* accession. Juvenility in photoperiod response was assayed by testing sensitivity to long-day treatments in accelerating the floral transition in young and old plants. Plants were firstly grown in short days

for 1 week or 5 weeks and then moved into long days. After being exposed to long days for one more week, the plants were moved back to short days and flowering time was compared in groups exposed to different treatments. Flowering time was expressed in terms of the numbers of total leaves produced on the primary stems upon flowering. As shown in Figure 3, although exposure to the long day photoperiod dramatically accelerated flowering in 5 week old plants, the same long-day treatment failed to significantly accelerate flowering in 1 week old plants, indicating that juvenility in photoperiod response exists in *Arabis alpina Bonn* plants.

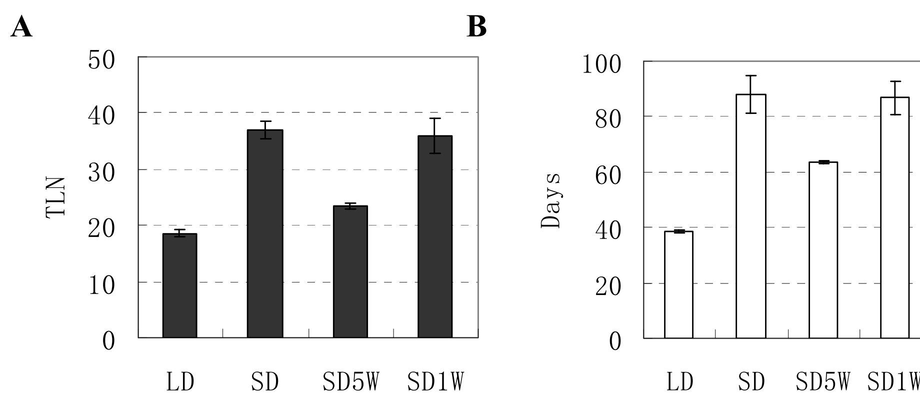


Figure 3. Flowering time of *Arabis alpina Bonn* plants grown under different photoperiods.
(A) Flowering time measured in terms of total leaves formed. **(B)** Flowering time in terms of days between germination and the emergence of the first floral buds. LD, continuous long day conditions. SD, continuous short day conditions. SD1W, one week of short days followed by 1 week of long days and then continuous short days. SD5W, 5 weeks of SDs followed by 1 week of LDs and then continuous short days.

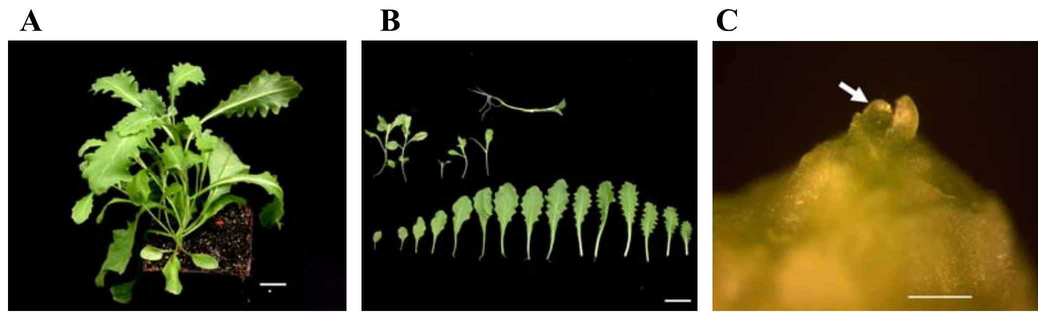
In summary, *Arabis alpina* has an approximately three-week-long juvenile phase and during this phase, plants do not flower although they are exposed to strong environmental inductive signals, such as those from vernalization treatment and inductive photoperiod. Various lengths of juvenility have been described in a spectrum of perennial plant species, especially in tree plants, whose juvenility normally lasts for many years (Longman, 1976; Zimmerman, 1985b; Meilan, 1997).

2.3. Polycarpic traits of *Arabis alpina*

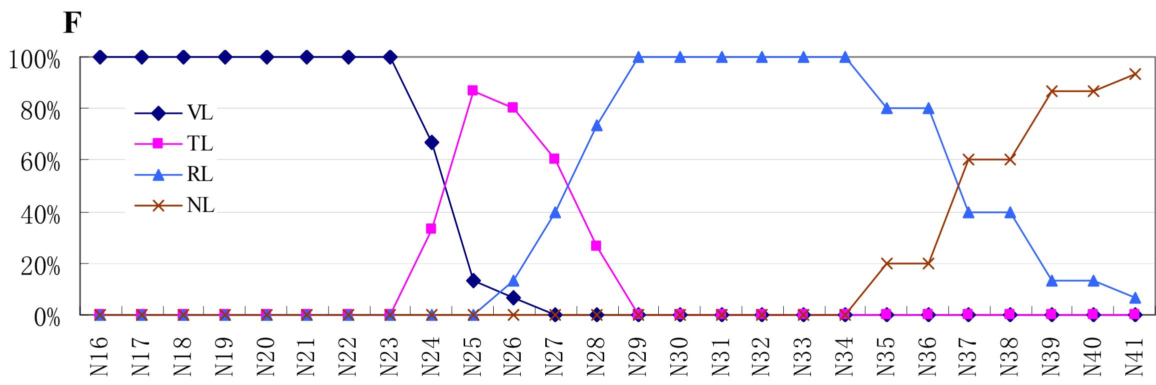
Even when exposed to the same floral inductive environmental cues, flowering in different apical meristems of the same *Arabis alpina* plant occurs very asynchronously, spanning many months and in most cases even different growing seasons. After flowering and seed set, senescence only occurs in the flowering shoots and therefore the plant can survive for many years. These characteristics indicate that *Arabis alpina* is a polycarpic perennial species. The diverse responses to the same environmental cues suggest different apical meristems vary in their competence to flower.

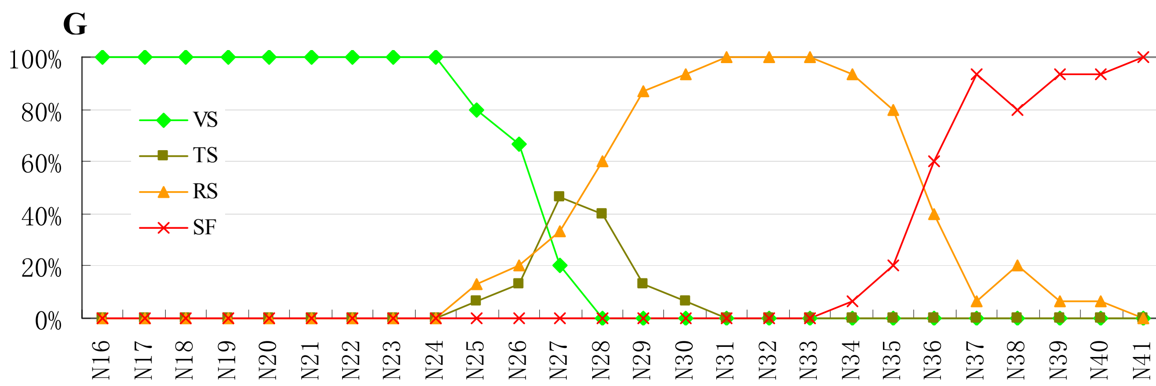
2.3.1. Polycarpic traits of *Arabis alpina Pajares*

To further understand polycarpy in *Arabis alpina*, flowering behaviors of apical meristems in different shoots were described in response to vernalization treatment in *Arabis alpina Pajares* accession, which has an obligate requirement for vernalization to flower. *Arabis alpina Pajares* plants were first grown in long days for 8 weeks, when the plants had already produced a spectrum of side branches of different sizes (Figure 4A and 4B), and then a twelve week cold treatment was applied to these plants. After the cold treatment, the plants were moved back to long days at normal temperatures, and the behavior of each branch was recorded.
The number of nodes and the organs produced at each node before vernalization was scored. Figure 4B illustrates the leaves present at each of the first 15 nodes (with cotyledon nodes not included). Upper nodes were counted by micro-dissecting the apex of the plant (Figure 4A) and it was found the smallest leaf primordium produced by 8 week old plants before vernalization was that from the No.26 or No. 27 node (Figure 4C). The leaf at No. 16 node was marked in each plant to allow the position of each node after vernalization to be defined. No. 16 node (or leaf) was chosen because in these plants no axillary branch primordium was observed at the leaf axils of nodes above node 16. Therefore, after vernalization, if any axillary branch grew out of a leaf axil above node 16, then most possibly, it was produced during or after the treatment. Furthermore, No. 16 leaf was the smallest leaf that could be easily marked before vernalization.


A
B
C

D
E


F
100%
80%
60%
40%
20%
0%
VL
TL
RL
NL
N16
N17
N18
N19
N20
N21
N22
N23
N24
N25
N26
N27
N28
N29
N30
N31
N32
N33
N34
N35
N36
N37
N38
N39
N40
N41


G
100%
80%
60%
40%
20%
0%
VS
TS
RS
SF
N16
N17
N18
N19
N20
N21
N22
N23
N24
N25
N26
N27
N28
N29
N30
N31
N32
N33
N34
N35
N36
N37
N38
N39
N40
N41

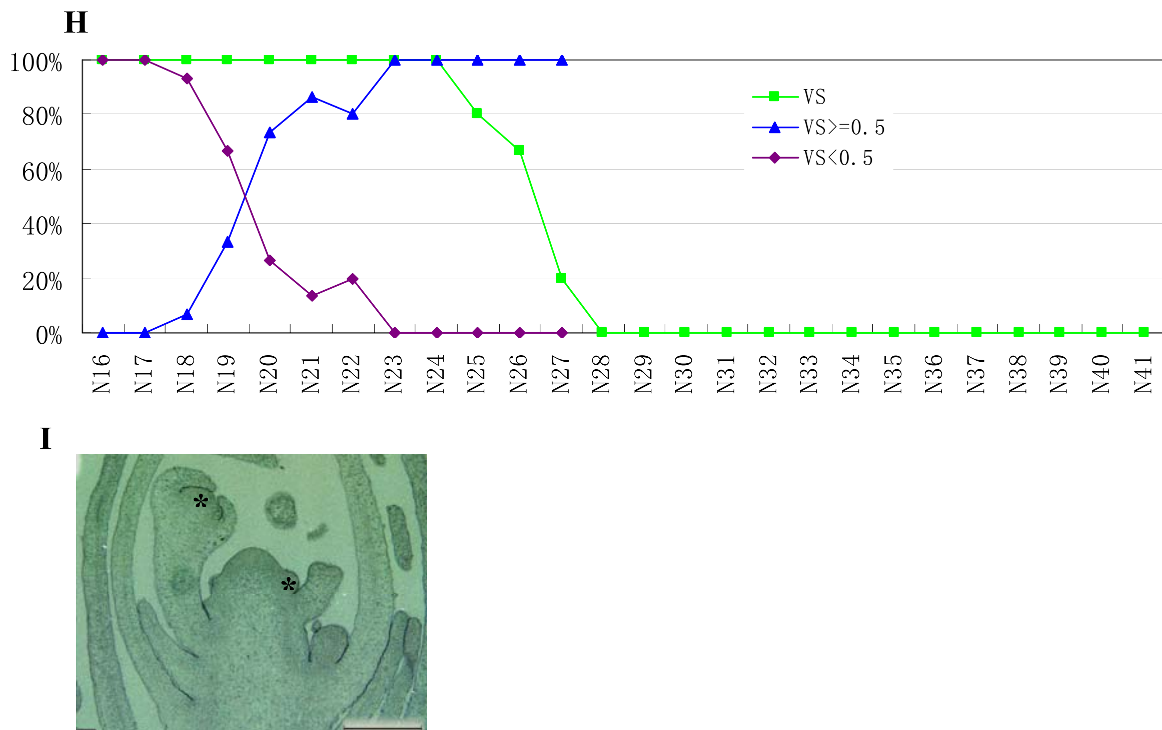


Figure 4. Changes of axillary meristem identity in response to vernalization in adult *Arabis alpina Pajares* plants.
(A) An *Arabis alpina Pajares* plant which had been grown in LDs for 8 weeks. Bar, 2 cm. **(B)** The dissected plant in **(A)**, showing the structure present at each node. Bar, 4 cm. **(C)** The micro-dissected apex of a representative 8 week old *Arabis alpina Pajares* plant as in **(A)**. The arrow labels the primordium of the No. 27 true leaf. Bar, 250 µm. **(D)** A flowering plant 4 weeks after a 12 week vernalization treatment. Bar, 10 cm. **(E)** The dissected parts of the plant in **(D)**. Note that not all organs produced from the early node were shown. The arrow represents the No.16 node. Bar, 5 cm. **(F)**. Distribution of different types of primary leaves along the primary stem from node 16 to node 41. VL, vegetative leaves; TL, transitional leaves; RL, reproductive leaves; NL, no subtending leaf existed **(G)** Distribution of different types of axillary branches (or flowers) along the primary stem from node 16 to node 41. VS, vegetative axillary shoots; TS, transitional axillary shoots; RS, reproductive axillary shoots; SF, solitary flowers. **(H)**. Distribution of vegetative axillary shoots with arrested vs. released growth after vernalization. VS<0.5, vegetative axillary shoots smaller than 0.5 cm. **(I)**. At the end of a 12 week cold treatment and before moving to higher temperatures, floral buds (asterisks) with or without subtending leaves already appeared on the flank of the SAM. Bar, 250µm. 15 plants were used in **(F)-(H)**

Morphological changes of leaves on the shoot often reflect programmed changes in identity of the shoot meristem from which the leaves were produced (Poethig, 2003). For example, changes in morphology of primary leaves closely correlate with phase transition of the Arabidopsis main shoot apical meristem. A similar

correlation is also found in *Arabis alpina Pajares* plants (Figure 4A and 4B). Before vernalization, *Arabis alpina Pajares* continuously produces leaves with a long and clear petiole and the distal part of the leaf blade is much broader than its proximal part (Figure 4A and 4B). These leaves are designated as vegetative leaves (VLs) considering the vegetative identity of the meristem from which they were initiated. After vernalization, heart-shaped leaves lacking a petiole appeared at nodes where flowers or flowering shoots were generated. Since these leaves subtended organs which were in reproductive phase, we named them reproductive leaves (RLs). As shown in Figure 4E, the transition from vegetative leaves to reproductive leaves was a gradual and continuous process. The leaves produced at nodes between the vegetative and the reproductive leaves were called transitional leaves (TLs). The petioles of TLs are flanked or completely substituted by lamina, so that the shapes are between those of the VLs and RLs. Furthermore, TLs from the lower nodes are more similar to VLs and those from the upper nodes more like RLs (Figure 4D and 4E). Interestingly, TLs mainly included those produced from nodes 25 to 27, which were the last nodes at which leaf primordia were produced before vernalization treatment (Figure 4C and 4F).

The identities of the primary lateral organs that grew out during and after vernalization treatment from the axils of leaves were also scored. Four types of organs were found: vegetative branches (VSs), partially flowering branches or transitional shoots (TSs), fully flowering branches or reproductive shoots (RSs) and solitary flowers (Figure 4D and 4E). Furthermore, a clear acropetal pattern of the arrangement of the primary lateral organs was found along the primary stems from the labeled node 16 up to the inflorescence meristem. VSs with subtending VLs were followed by VSs, TSs or RSs with TLs, and then by RSs with RLs, and then by solitary flowers with RLs and finally solitary flowers without any subtending leaf (Figure 4E, 4F and 4G). Although in most individuals, very small RLs subtending solitary flowers reappeared at several nodes formed later on, the organ produced finally by each of the upper nodes was a single flower (Figure 4E, 4F and 4G). Interestingly, the TSs mainly arose around N27, which were the last nodes formed before the vernalization treatment started (Figure 4C and 4G). After vernalization, side branches from upper nodes (mainly from node N20 on) tended to grow out and become larger (longer than 0.5 cm) while those from lower nodes

(mainly from nodes N16 to N19) tended to remain at a smaller size (Figure 4H). Furthermore, if the main shoot apices were removed after vernalization treatment, the arrest of the growth of the side branches at lower nodes was released, and vegetative side branches grew out at these nodes (data not shown). These observations demonstrated that after vernalization, the growth of side branches was regulated by apical dominance mediated by the main shoot apex and that this suppressed most strongly the growth of the earlier side branches. At the end of a 12-week vernalization treatment, solitary floral primordia without any subtending leaves appeared on the flank of the primary shoot apical meristems (Figure 4I), indicating that the major process of transition from vegetative to reproductive phase had already been completed before the plants were moved back to normal growth temperatures.

A correlation between the location of a node on the shoot and its tendency to produce flowers was also tested on the primary shoot for nodes produced before node 16. Before vernalization, axillary shoots were already visible in the axils of leaves formed in nodes between NC1 and N12 while in most cases no shoot primordia were visible between N13 and N15. As shown in Figure 5A, side shoots from some nodes, such as N1, N2, N7, N8 and N9, tended to flower after vernalization whereas shoots from other nodes, such as CN1 (cotyledon node 1) and N11 to N15 tended to maintain vegetative. However, the observation that the majority of the flowering side branches were much larger than the vegetative ones before the treatment, suggested that there might be a correlation between the size of side branches before vernalization and their fate after it (Figure 5A). A close correlation between the size of side branches and flowering was observed (Figure 5B). Almost 95% of the side branches longer than 2 cm before vernalization flowered after the treatment while more than 96% of branches smaller than 0.1 cm remained vegetative. Therefore, longer side branches tended to flower and smaller ones tended to stay vegetative after vernalization, and this was a stronger correlation than the location where the branch occurred. The size of the side branch may be an indication of the developmental stage of the axillary meristem and the data indicate that this is the key factor that determines the fate of the meristem after vernalization (Figure 5).

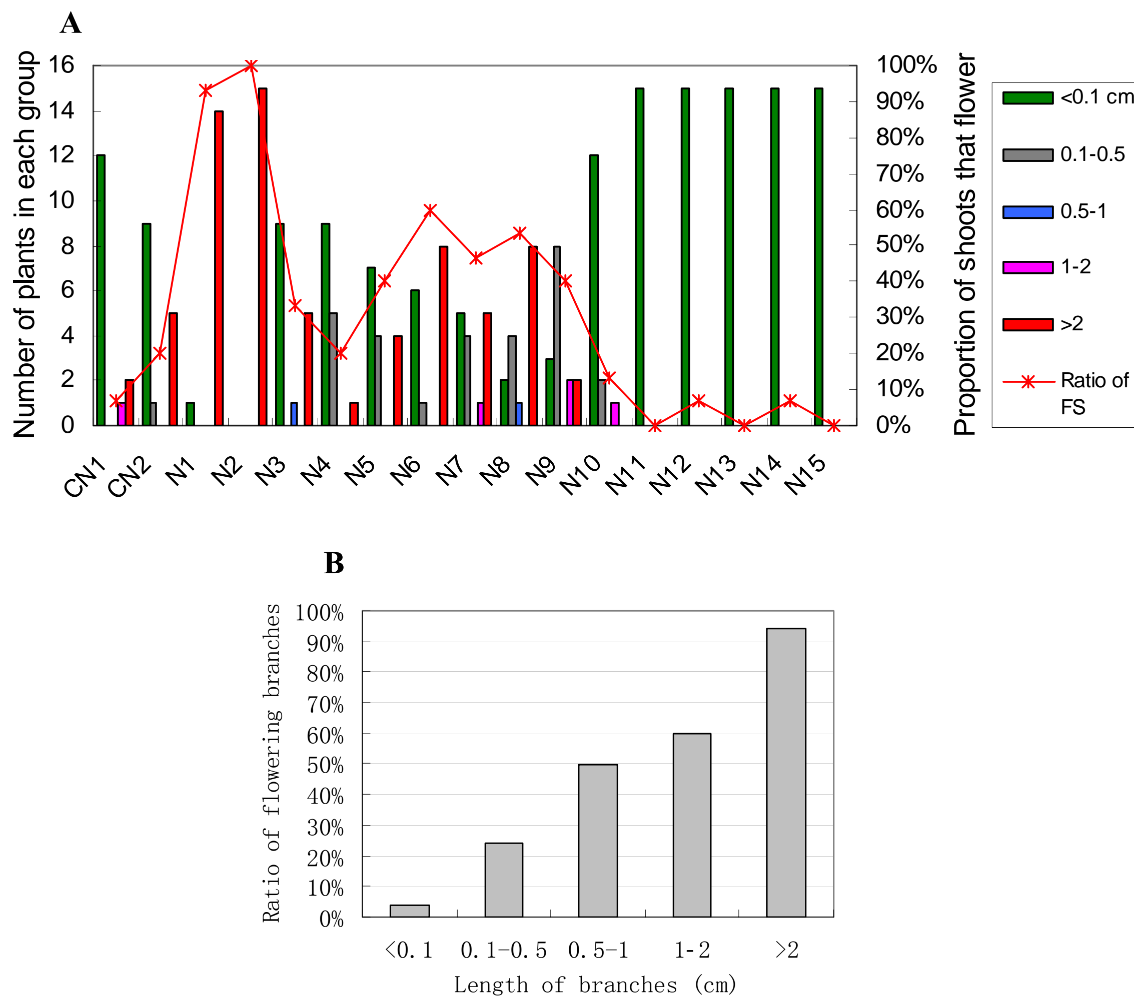


Figure 5. Flowering tendency of axillary shoots produced at nodes before No.16.
(A) Distribution of primary axillary branches of various sizes before vernalization (the rectangle bars that correspond to the left Y axis) and distribution of flowering primary axillary branches after vernalization (the read stars which correspond to the right Y axis) The color of the rectangular bars indicates their length before vernalization (see the legend). The X axis represents the position of each node along the primary stem. CN, cotyledon node; N1 the first cotyledon-adjacent node bearing a true leaf; FS, flowering primary axillary side branches. **(B)** Relationship between size of side branches before vernalization and flowering after vernalization. 15 plants were used.

After flowering and seed set, the flowering shoots gradually underwent basipetal senescence and finally died. These parts include the flowering upper part of the side branches produced before vernalization and the upper part of the main shoot stems until the nodes where the TSs were produced (Figure 6). The senescence of the TSs is delayed and in some cases the basal part of these branches will survive

by a reversion and generate vegetative secondary branches from the nodes in their basal part (Figure 6). The localized senescence which only happened in the flowering part suggests a close correlation between the reproductive phase and subsequent senescence. As shown in Figure 6, the vegetative side branches (type II in Figure 10) and the vegetative basal part of the flowering big side branches (type I) produced before vernalization survive and maintain vegetative growth, continuously producing new vegetative axillary apical meristems.

In response to a second vernalization treatment, similar events will happen to the branches formed from meristems that were maintained vegetative in the first vernalization treatment. In this way, although a plant repeats flowering after each vernalization treatment (or winter), the plant can always maintain some meristems in the vegetative phase, which allows it to survive for many years. Furthermore, *Arabis alpina* plants can also produce adventitious roots from their vegetative stems, changing a single plant into many colonies after a period of growth in the field.

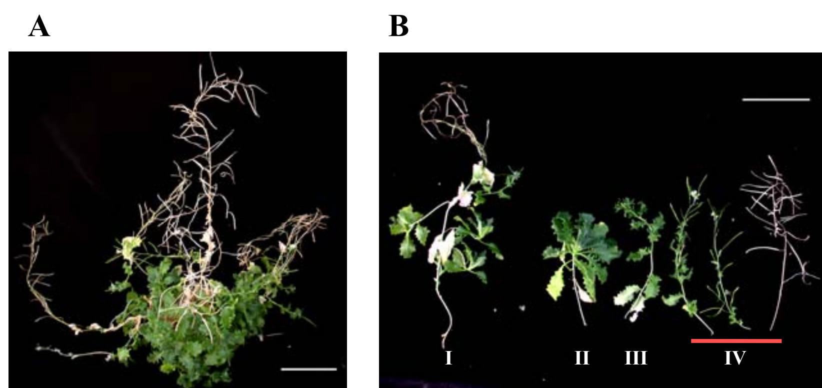


Figure 6. Localized senescence occurring after seed set.
(A) Senescence was occurring in an *Arabis alpina Pajares* plant which had been treated with cold for 12 weeks and then grown in LDs for about 14 weeks. **(B)** Different types of side branches. Senescence had already occureded in the flowering part of type I and some type IV branches. Senescence in other type IV branches would occurred later

2.3.2. Polycarpic traits in *Arabis alpina Bonn*

In *Arabis alpina Bonn*, flowering can occur without vernalization treatment (See Section 2.4). As shown in Figure 7, changes in the shape of leaves along the main

shoot implied a continuous change in the identity of the SAM during shoot growth. Correspondingly, the number of secondary leaves produced on each primary branch decreased acropetally. This co-relation suggested that the nature of side branches might be influenced by the activity or the identity of the SAM in an "origin determined" way, or a "neighborhood determined" way or a combination of both (See Section 2.6 for more details). This influence, together with the apical dominance effect, suggests that flowering occurs in the Bonn accession in the following way. Floral transition occurs firstly in the SAM and the adjacent branches, and with a short delay flowering happens in the apices of large side branches which were produced early from the basal nodes of the primary stems. Later on, floral transition continued in the apical meristems of branches produced along the primary stem from both ends to the middle part. Among these branches, if growth was arrested, floral transition in those branches would also be delayed compared with that in the adjacent branches. After seed set, senescence occurs in the flowering part while branches of higher order were generated continuously from the axillary meristems in the basal vegetative part, thereby allowing continuation of the perennial growth habit. Floral transition would occur in these newly grown out branches under favorable conditions (Figure 7C).

Figure 7. Flowering and senescence in *Arabis alpina Bonn* plants.
(A) An *Arabis alpina Bonn* plant which had been grown in LDs for 8 weeks. Bar, 10 cm. **(B)** A dissected 8 week old plant. Bar, 10 cm. **(C)** An 11 month old plant. The flowering part of the main shoot and the primary branches had senesced and flowering was happening in the apical meristems of the secondary branches. Bar, 5cm.

2.4. Flowering time in response to day length and vernalization treatment in different *Arabis alpina* accessions

In different accessions of *Arabidopsis thaliana*, the effect of vernalization in accelerating flowering can differ tremendously. In those late flowering winter annual accessions, such as Stockholm, vernalization dramatically accelerates flowering while the accelerating effect of vernalization in quick cycling accessions like Columbia-0 is very limited. As described above, the previous work in our lab identified both the *Arabis alpina Pajares* accession, which has an obligate requirement for vernalition to flower and another accession, *Arabis alpina Bonn*, which can flower without vernalization. To better understand the variation in response to the same environmental floral inductive cues in different *Arabis alpina* accessions, the effect of vernalization on flowering time in the Bonn accession was also tested. As described in Section 2.2, vernalization could not accelerate flowering in juvenile *Arabis alpina Bonn* plants. When older plants that had been grown under LDs for 4 weeks before a twelve week cold treatment was applied, were tested, no significant effect was found in regulation of flowering time (Figure 8).

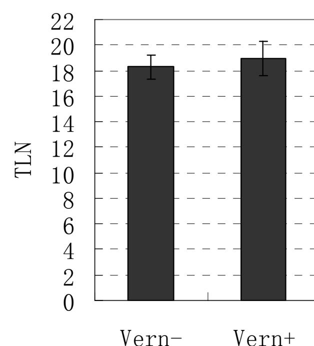


Figure 8. The effect of vernalization on flowering time in *Arabis alpina Bonn* plants.
The plants were grown in LDs before and after vernalization treatment. TLN, total primary leaf number.

To test effect of day length in accelerating flowering of *Arabis alpina Bonn*, flowering time of plants grown in LDs and SDs was compared. As seen in *Arabidopsis thaliana*, long photoperiods accelerated flowering in *Arabis alpina Bonn* (Figure 3). *Arabis alpina Bonn* plants grown in SDs flowered, but much later

than those in LDs (Figure 3). This result, together with the observation that, most if not all *Arabis alpina* plants flower in spring in natural conditions, suggests that *Arabis alpina* is a facultative long day species.

2.5. Effect of exogenous gibberellin treatment on flowering in *Arabis alpina*

Since GA plays an important role in promoting flowering in a variety of plant species, especially Arabidopsis, a close relative of *Arabis alpina*, evaluating the effect of GAs on floral promotion, especially the obligate requirement for vernalization in the Pajares accession and juvenility, would be helpful in understanding flowering time control networks in *Arabis alpina*. Firstly, the effect of exogenous application of GAs on flowering time under SDs was tested in the *Arabis alpina Pajares* and the *Arabis alpina Bonn* accessions. In the Bonn accession, exogenous application of GA3 or GA4 only slightly accelerated flowering although the effect on stem elongation in both cases was dramatic (Figure 9A and 9B). In *Arabis alpina Pajares* a similar effect on stem elongation was observed with both GA3 and GA4 treated plants as seen in the above experiment with the Bonn accession, but none of the Pajares plants flowered after 3 months of GA treatment. Similar GA3 and GA4 treatments were carried out in both accessions in LDs. Neither GA3 nor GA4 application accelerated flowering in the *Arabis alpina Bonn* accession. Similarly, the obligate requirement for vernalization for flowering was not overcome when either gibberrelin was used (Data not shown).

Whether exogenous GA treatment can overcome juvenility in young plants was also tested. *Arabis alpina Pajares* plants were grown in LDs for two weeks, and then transferred to 4 °C and grown there for 7 months. After the cold treatment, plants were moved back to normal temperatures. During the pre-cold LDs and the whole period of the cold treatment, GA3 was applied to the plant once or twice a week. As shown in Figure 9C and 9D, no flowering occurred in plants sprayed either with GA3 or the mock solution although again more dramatic stem elongation was observed in plants treated with GA3.

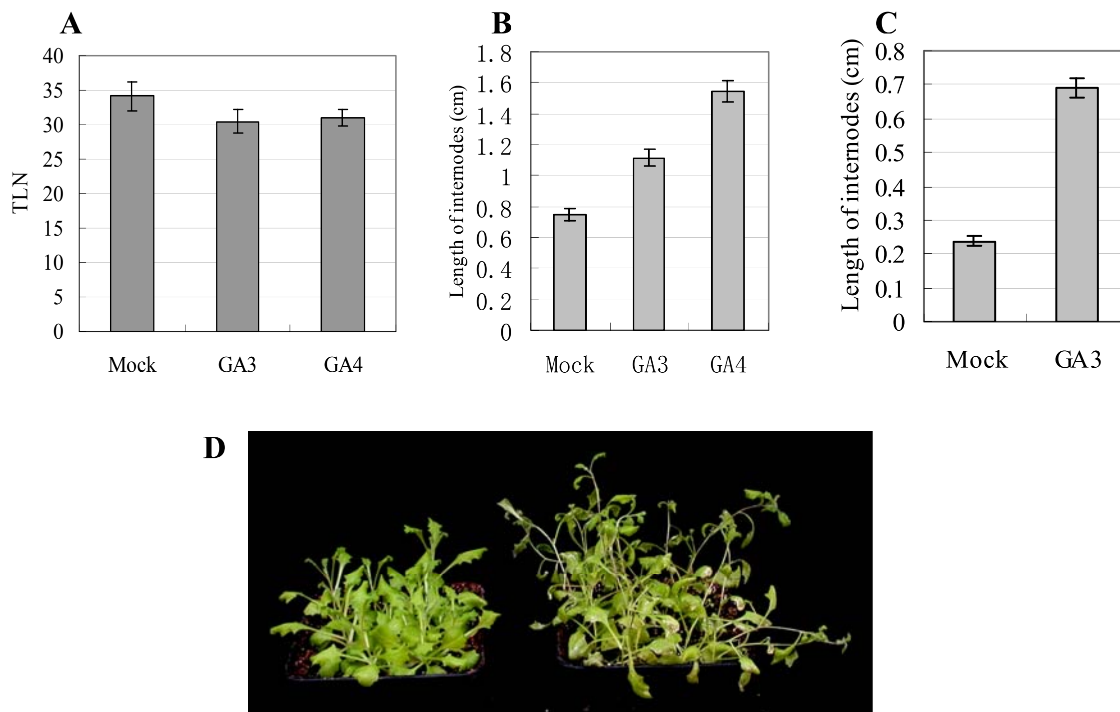


Figure 9. Effect of exogenous gibberellin treatment on growth and flowering of *Arabis alpina* plants.
(A and B) Effects of gibberellin treatment on flowering **(A)** and stem elongation **(B)** in *Arabis alpina Bonn* plants. Plants were grown under SDs. **(C and D)** GA3 treatment before and during vernalization promoted stem elongation but not flowering in juvenile *Arabis alpina Pajares* plants. The average length of the internodes was measured in **(C)** and **(D)**.

2.6. Discussion

In Arabidopsis and other species, axillary meristem initiation starts at the same time as leaf initiation with the definition of an adaxial zone of high meristematic potential (McSteen and Leyser, 2005). Therefore, a similar process for the establishment of axillary meristems may also exist in *Arabis alpina*.
The changes in morphology and identity of leaves and side branches in *Arabis alpina Pajares* in response to vernalization treatment allowed the types of side branches formed on the shoot to be classified (Figure 10). At normal temperatures, leaves were continuously generated on the flank of the primary apical meristem of the Pajares plants. Together with the initiation of leaves on the flanks of the SAM, axillary meristems were also formed at the axils of the leaves. Because of their

origin and/or proximity to the SAM, the characteristics and identity of the axillary meristmes were influenced by the SAM, especially in the early stages. During the process of establishment, axillary meritems acquired relatively independent identity, which made them less sensitive or insensitive at all to floral inductive signals. This process may be similar to juvenile SAMs or may be a distinct mechanism. When side branches produced from these axillary meristems grew longer their sensitivity to floral induction signals or competence to flower increased. In largest side branches (type I branches in Figure 10) the apical meristems were as sensitive to floral induction as the SAM. Actually, the apical meristems of these axillary branches (AMs) recapitulated the developmental program of the primary apical meristem. The out growth rate and therefore, the degree of development of axillary shoots and their meristems was regulated by the SAM, presumably through auxin-mediated apical dominance. Therefore, when plants were transferred to cold temperatures for vernalization, the meristems at axils of different leaves had developed into different stages, including undifferentiated axillary meristems, shoot primordia and mature shoots of different sizes (Figure 4A and 4B). These axillary meristems at different developmental stages exhibited different sensitivity to floral inductive signals generated from the vernalization treatment. The earlier their stage of development the less sensitive to floral induction they were. As a result, among the axillary meristems produced before vernalization flowering only occurred in those large side branches (the type I branches in Figure 10) and not on the smaller ones (type II branches in Figure 10).

In contrast to the axillary meristems produced before vernalization treatment, the flowering response of those initiated during the treatment showed a strong locational effect. A gradual change was seen in organs produced from the main shoot apical meristem during vernalization treatment: from vegetative shoots to shoots showing progressively stronger reproductive traits, and finally to solitary flowers (Figure 4G). This transition in axillary shoots co-occurred with that of the morphology of primary leaves: from VLs to TLs, then to RLs, and finally to the disappearance of leaves (Figure 4F). This co-occurrence suggests a correlation exists between SAM identity and the identity of organs arising at the node. One hypothesis to explain this phenomenon can be named “origin determined”. The location of the transitional axillary shoots (TSs, or type III side branches, see

Figure 10) at the nodes which were being initiated when vernalization treatment started suggests that at the beginning of vernalization these axillary meristems were in the process of being formed. Vernalization may therefore have caused a change in the identity of the SAM, and the primordia formed from the meristem, which in turn influence the identity of the axillary meristem. An alternative hypothesis, which can be called "neighborhood-determined", is that some floral signals were generated from the vernalized SAM and these signals could be transmitted along the primary stem for a short distance and became weaker gradually during transmission downward. With the SAM being more vernalized, stronger signals were emitted from it. This signal antagonized or influenced the establishment of the insensitivity of the axillary meristems to floral induction signals. As a result, the axillary meristems near the SAM acquired more reproductive identity while those far from the SAM were more vegetative. Of course, a combination of the "origin-determined" and the "neighborhood-determined" hypotheses may also be possible.

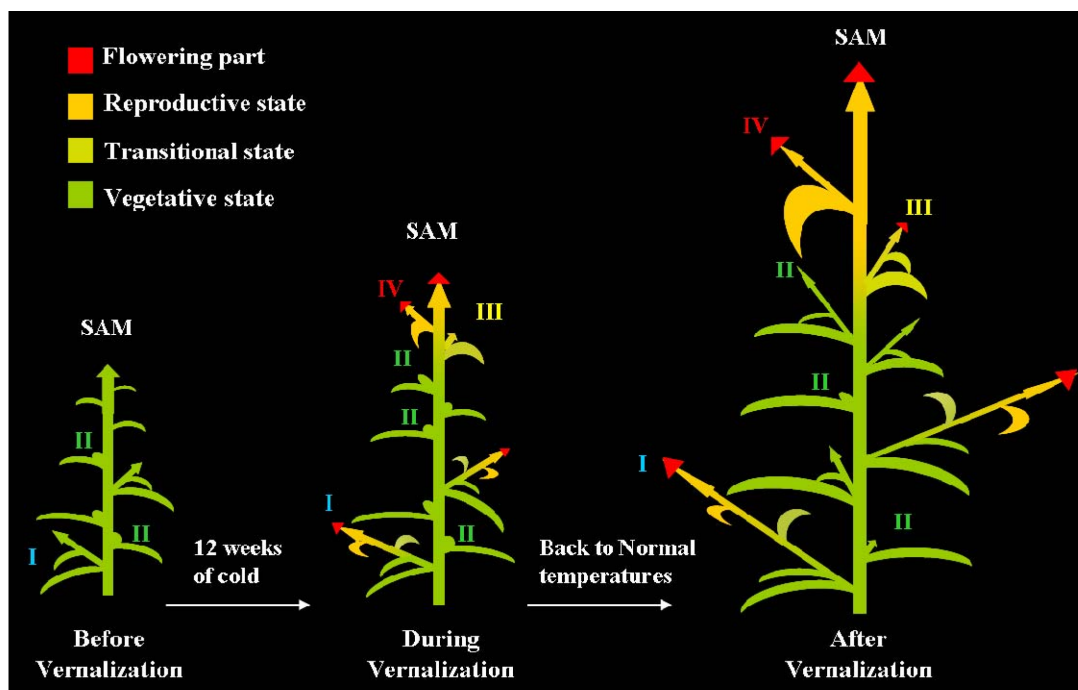


Figure 10. Diagram of response to vernalization treatment in apical meristems of *Arabis alpina Pajares* plants.
SAM, main shoot apical meristem; I, II, III and IV represent four different types of axillary branches.

As described in Section 2.3, in *Arabis alpina*, there is a close correlation between flowering and localized senescence. One possible mechanism underlying the correlation is that floral transition induces the local expression of senescence promoting genes. Another possibility is that flowering and seed formation induced the production of senescence-related signaling molecules, such as abscisic acid, accumulation of which causes senescence. Although these molecules may also be transported to the vegetative part, the accumulation of other molecules, a good candidate of which is cytokinin which is massively produced in the root and is transported to the aerial tissues (Sakakibara, 2006), can antagonize the function of the senescence promoting molecules.

Therefore, according to the flowering behaviors in *Arabis alpina Pajares* in response to vernalization, the side branches can be put into four types (Figure 10). The first class consists of the large side branches produced before vernalization. They had become relatively independent of the SAM before vernalization and during vernalization treatment the apical meristems of these shoots behaved similarly to the primary shoot apical meristem and finally flowered. The second class of axillary meristems are those small or undeveloped shoots or axillary meristems at nodes produced before vernalization treatment. At the end of or after the treatment, the axillary meristems at these nodes either developed into vegetative side branches or their growth was arrested to some degree, and as a result they were maintained as small vegetative branches or even shoot primordia. Transitional side shoots are the type III side shoots. These branches originated from the axillary meristems produced around the time that vernalization treatment started. At that time, the primary meristem had not been fully vernalized. Although the morphology of leaves at these nodes changed, flowering was variable in these side branches. The meristems generating the type IV side branches were initiated from the SAM during vernalization treatment. Flowering happened in these branches as in the main shoot apex. The developmental stage in which an individual axillary meristem was at the time when the vernalization treatment started was therefore a major factor in determining the fate of the axillary meristem after vernalization. The activity and state of the SAM may also play a role in determining the fates of axillary meristems, especially the fates of the type III and type IV branches.

Similar to the differential floral transition in different meristems of *Arabis alpina*

Pajares plants, the flowering behaviors in different meristems of *Arabis alpina Bonn* plants may also be illustrated with the "origin determined" or the "neighborhood-determined" hypothesis. When *Arabis alpina Bonn* plants are grown under floral inductive conditions such as LD photoperiods, the SAM retains vegetative during juvenile phase and continuously produces vegetative axillary meristems on its flanks. Furthermore, during the process of formation, the axillary meristems may also acquire an independent identity that makes them less sensitve to floral induction than the SAM from where they were established. With development, the SAM becomes more and more competent to flower (or more and more reproductive), and therefore the newly-initiated axillary meristems also become more and more competent to flower. In addition to the influence from the main shoot SAM, during the process of growing-out, the apical meristems of axillary shoots also independently become more and more competent to flower by a similar mechanism as in the SAM of the main shoot.

As in *Arabidopsis thaliana*, vernalization and LD potoperiods induce flowering in *Arabis alpina*. However, exogenous application of GAs can only slightly accelerate flowering in *Arabis alpina* although it can efficiently accelerate flowering in *Arabidopsis thaliana* in SDs (Eriksson et al., 2006). These data indicate that both similarities and differences exist in the mechanisms controlling flowering time in *Arabis alpina* and *Arabidopsis thaliana*.

3. Molecular mechanisms underlying juvenility in *Arabis alpina*

3.1. Introduction

Perennial plant species, including monocarpic and polycarpic perennials, have a prolonged juvenile phase while annuals exhibit a very short juvenile phase. Juvenility is defined here as a lack of competence to flower in young seedlings even when they are exposed to environments that induce flowering in older plants. In many species, juvenility is coupled with suites of specific morphological, physiological and biochemical characteristics, such as leaf shape and size, trichome distribution on leaf surfaces, leaf retention, photosynthetic efficiency and rooting capacity of cuttings (Poethig, 1990; Jones, 1999). However, the association of these characteristics with juvenility varies from species to species and in many cases no clear association exists at all (Jones, 1999; Brunner and Nilsson, 2004). Therefore, in this thesis the competence to flower is the major criterion to distinguish juvenile and adult plants.

Although the molecular mechanisms underlying juvenility remain unclear, physiological studies have shown that phase change from juvenility to maturity is a gradual and continuous process (Zimmerman, 1985b; Meilan, 1997). The incompetence of juvenile plants to flower has led to the speculation that the underlying mechanism of juvenility may involve activities of floral regulators (Brunner and Nilsson, 2004). Juvenility may result from the presence of strong floral repressors or the incapability to upregulate floral promoters in young plants. Consistent with this idea, manipulation of the expression levels of exogenous or endogenous floral activators or repressors induced precocious flowering in some woody perennial plants that exhibit juvenile phases lasting for several years (Weigel and Nilsson, 1995; Pena et al., 2001; Endo et al., 2005; Kotoda and Wada, 2005; Bohlenius et al., 2006; Hsu et al., 2006; Kotoda et al., 2006).

Constitutive expression of the Arabidopsis floral meristem identity gene *LFY* was shown to cause very early flowering in transgenic aspen trees (Weigel and Nilsson, 1995). A similar precocious-flowering phenotype was observed when *LFY* or *AP1*

was overexpressed in citrus trees (Pena et al., 2001). However, these findings may not provide information about the natural regulation of juvenility considering that in Arabidopsis *LFY* and *AP1* are both floral meristem identity genes downstream of the network regulating flowering time control (Figure 1).

In Arabidopsis, *FT* is an important floral activator, integrating floral signals from multiple pathways (Boss et al., 2004). Recently, *FT* homologs (*PtFT1* and *PdFT2*) were cloned from poplar trees (Bohlenius et al., 2006; Hsu et al., 2006). Overexpression of either of these genes in poplar caused precocious flowering. Furthermore, the expression levels of these genes in adult poplar plants are 2-3 times of those in juvenile plants (Bohlenius et al., 2006; Hsu et al., 2006). These results suggested that regulation of expression of poplar *FT* homologs might be involved in juvenility. A similar suggestion came from the precocious flowering phenotype observed in transgenic citrus plants overexpressing a citrus *FT* homolog (Endo et al., 2005).

Based on the observation that *CsTFL1*, a citrus *TFL1* homolog, was expressed at much higher levels in juvenile than adult plants, Pillitteri et al. suggested that *CsTFL1* may play a role in juvenility (Pillitteri et al., 2004). Stronger evidence for a role of *TFL1* homologs in juvenility in perennials came from analyzing the expression and function of *MdTFL1s*, a homolog of *TFL1* from apple trees. *MdTFL1* was preferentially expressed in vegetative tissues in apple trees and reduction of its expression in juvenile plants by RNA interference (RNAi) caused extremely precocious flowering (Kotoda and Wada, 2005; Kotoda et al., 2006).

Given the possibility that genes involved in flowering-time control also play a role in juvenility, we started to investigate juvenility in *Arabis alpina* by analysis of homologs of Arabidopsis flowering-time genes. Considering the close relationship of *Arabis alpina* and *Arabidopsis thaliana* and that, in Arabidopsis, *FLC* (or called *AtFLC*) plays a key role in regulation of flowering time through integrating both endogenous and environmental cues, we tested whether an *FLC* counterpart exists in *Arabis alpina* and plays an important role in flowering time control. As stated above, vernalization can efficiently promote flowering in adult but not juvenile *Arabis alpina Pajares* plants. A possible cause for the failure in the promotion of flowering by vernalization in juvenile plants might be the failure to repress an *FLC* ortholog by vernalization treatment in these plants. Alternatively, although

expression of the putative *FLC* ortholog was repressed by vernalization, another floral repressor (or repressors) might block floral transition in juvenile plants, thereby possibly functioning downstream of FLC. To reveal the molecular mechanism underlying juvenility, an *FLC* ortholog from *Arabis alpina* (named *AaFLC*) was cloned. Although *AaFLC* encoded as strong a floral repressor as *AtFLC* does, the similar patterns of its expression in juvenile and adult plants during vernalization treatment suggested that the failure to flower in juvenile plants was not caused by unsuccessful repression of expression of this gene. To further understand juvenility, orthologs of other flowering control genes, *SOC1 (AtSOC1)*, *FT (AtFT)* and *TFL1 (AtTFL1)* were cloned and their roles were investigated. It was found that *AaTFL1*, an ortholog of *AtTFL1*, might play an important role in juvenility in *Arabis alpina*.

3.2. *AaFLC* is an orthologue of the Arabidopsis *FLC* gene

The *FLC* homolog cloned from *Arabis alpina Pajares* (see Materials and Methods for the details of cloning), named *AaFLC*, has a similar genomic organization as Arabidopsis *FLC* (*AtFLC*) (Figure 11A). *AaFLC* comprises seven exons and introns. In *Arabidopsis thaliana*, *AtFLC* is a single copy gene, but in Brassica species there are usually several copies of *FL*C homologs (Tadege et al., 2001; Schranz et al., 2002). To check how many *FLC* homologs exist in the *Arabis alpina* genome, a Southern blot was carried out using genomic DNA from *Arabis alpina Pajares* plants. High stringency hybridization and a probe including the genomic sequence from exon 2 to exon 4 of *AaFLC* were used. As shown in Figure 11, when restriction enzymes (BamHI and BglII) that have two recognition sites within the probe sequence were used, three bands appeared for each digestion reaction. HidIII, which has one recognition site in the probe sequence, produced two detectable bands, while EcoRI and SpeI, of which no recognition site was found in the probe sequence, generated only one visible band. These results suggest that there is only one *AaFLC* gene in the *Arabis alpina* genome (Figure 11B).

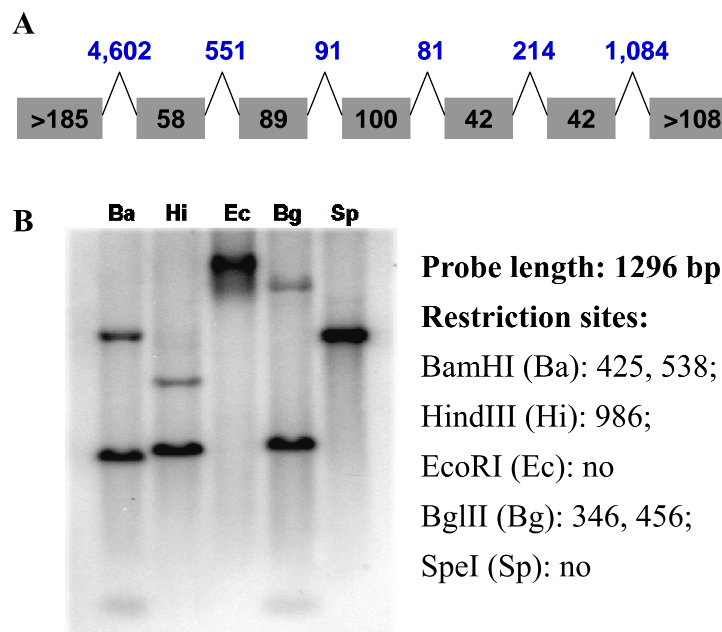


Figure 11. *AaFLC* has a similar genomic organization as *AtFLC* and is a single copy gene. **(A)** Genomic organization of *AaFLC* gene. Rectangles represent exons. Curved lines represent introns. Numbers indicate the length of exons and introns. **(B)** Southern blot to test copy number of *AaFLC*. Letters above each track indicate the restriction enzymes used. The text on the right indicates the enzyme cleavage sites in the 1296 bp long probe (see Materials and Methods).

The deduced AaFLC protein, consisting of 207 amino acids, shows 85% sequence identity with AtFLC and is also an MIKC type MADS box protein (Michaels and Amasino, 1999; Sheldon et al., 1999). Phylogenetic analysis of the deduced amino acid sequence suggested that AaFLC resides in the same sub-clade as AtFLC and other FLC homologues from the *Brassicaceae* family (Figure 12). AaFLC is in a different sub-clade from other closely related members of the Arabidopsis FLC/MAF protein family (including AtMAF1-5). The result of sequencing a BAC carrying the *AaFLC* revealed that the synteny between the chromatin around *AaFLC* and that around *AtFLC* was conserved (Data not shown). The homologs of genes both upstream and downstream of *AtFLC*, such as genes *At5g10130* and *At5g10150*, were also present in the BAC where *AaFLC* was.

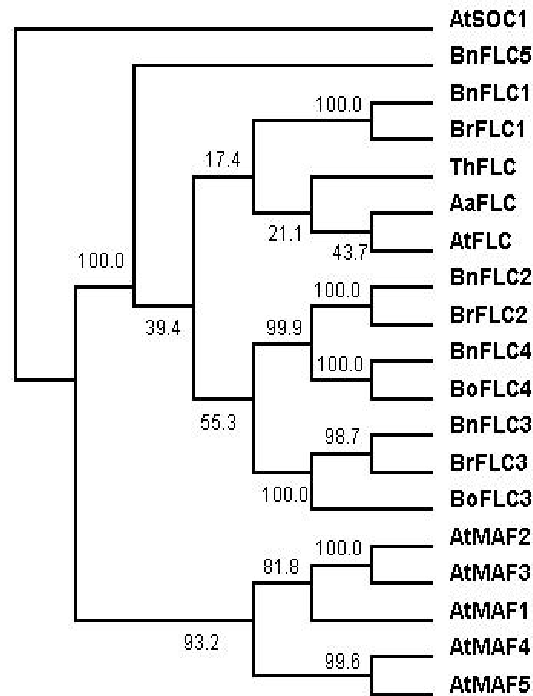


Figure 12. Phylogenetic analysis of AaFLC and other FLC homologues.
Aa, *Arabis alpina*. At, *Arabidopsis thaliana*. Bn, *Brassica napus*. Br, *Brassica rapa*. Bo, *Brassica oleracea*. Th, *Thellungiella halophila*. Bootstrap numbers shown at nodes are percentage of 1000 replicates.

To test functional conservation of AaFLC, the full length coding sequence was expressed in wild type Columbia-0 plants under the control of a CaMV35S promoter (*35S:AaFLC*). Although Arabidopsis FLC acts as a strong floral repressor, overexpression of *FLC* in Col-0 wild type plants under the control of the 35S promoter generated, in addition to late flowering plants, a high proportion of early-flowering transgenic plants (Sheldon et al., 1999; Ratcliffe et al., 2003). Similar phenotypes were also observed when overexpressing other *FLC*/*MAF* family genes (Sheldon et al., 1999; Ratcliffe et al., 2001; Ratcliffe et al., 2003). Therefore, a *35S:FLC* construct was also made and used to transform Col-0 plants as a control for the *35S:AaFLC* construct (See Materials and Methods for details). As shown in Figure 13A, when overexpressed in Col under the 35S promoter, the majority of T1 *35S:AaFLC* or *35S:FLC* plants flowered slightly earlier to or at a similar time as non-transgenic Col plants. However, in both cases, a small proportion of transgenic plants flowered dramatically late, and some of them did not flower before they died four to five months after germination. Therefore,

AaFLC and *FLC* modified flowering time in a comparable way, suggesting that like Arabidopsis *FLC*, *AaFLC* also encodes a floral repressor. Further analysis was performed with two late flowering *35S:AaFLC* lines in the T3 generation. As shown in Figure 13 (B and C) for one representative line, flowering was strongly delayed in these lines compared to the Col-0 control plants. In the late flowering *35S:AaFLC* plants tested, expression of *AaFLC* mRNA was detected (data not shown). In Arabidopsis FLC represses *FT* and *SOC1* mRNA expression. Expression of these endogenous floral pathway integrator genes was tested in *35S:AaFLC* plants, and the levels of *FT* and *SOC1* were dramatically reduced (Figure 13D and 13E).

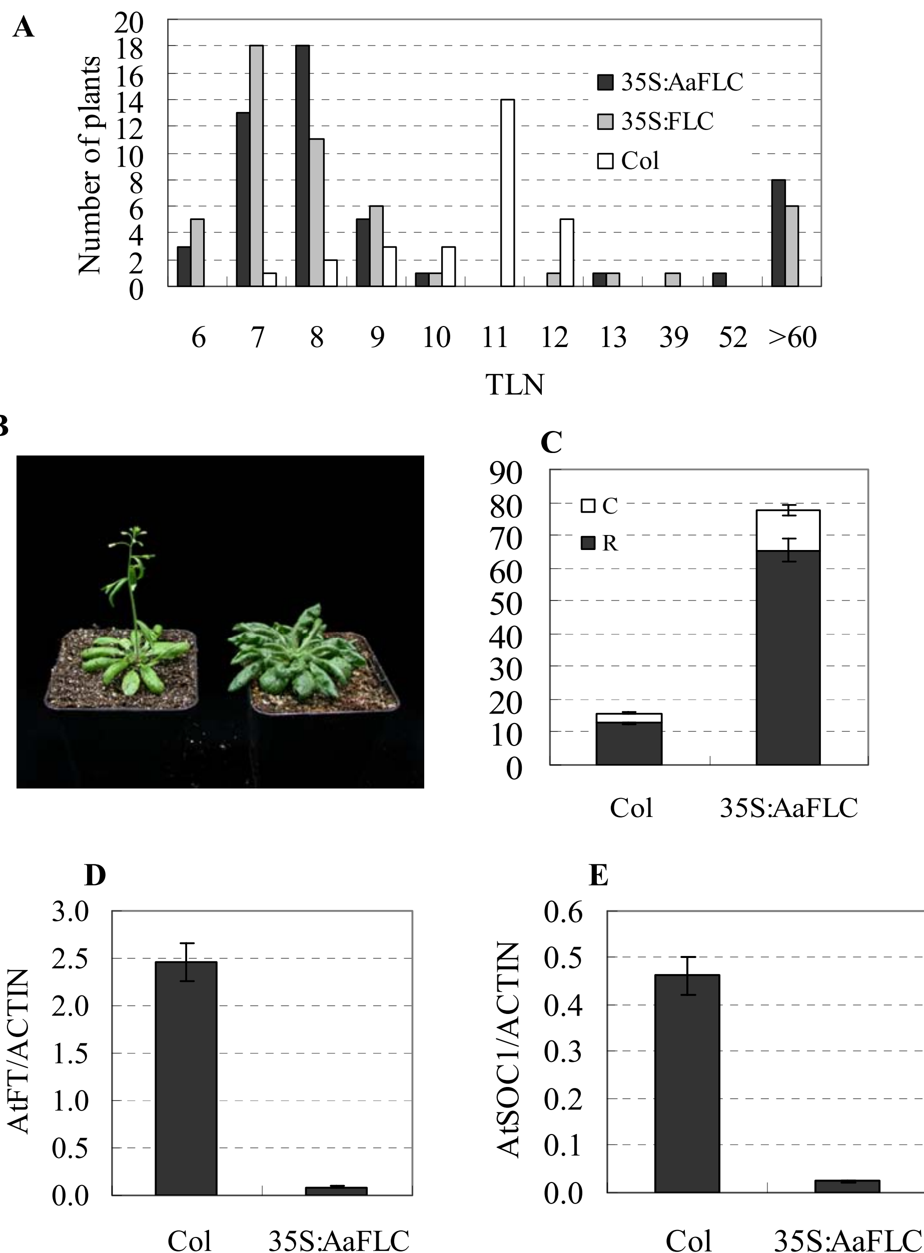


Figure 13. Ectopic expression of *AaFLC* in Arabidopsis caused late flowering.
(A) Flowering time of T1 *35S:AaFLC* (n=50), *35:FLC* (n=50) and Col-0 (n=28) plants. **(B)** Comparison of flowering time in a Col-0 (left) plant and a representative *35S:AaFLC* plant (right). **(C)** Number of rosette and cauline leaves (R and C respectively) produced by Col-0 and *35S:AaFLC* plants when they flowered. Data for 24 T3 plants from a representative T1 line. Plants were grown under LDs. **(D)** *AtFT* mRNA levels in *35S:AaFLC* plants and Col-0 controls. **(E)** *AtSOC1* mRNA levels in plants as in **(D)**.

In summary, the results of sequence analysis and functional tests in transgenic Arabidopsis indicated that *AaFLC* can function as a floral repressor by reducing *SOC1* and *FT* transcription and is an ortholog of *AtFLC*.

3.3. *AaFLC* is expressed at similar levels in juvenile and adult *Arabis alpina* plants before and after vernalization

One possible mechanism underlying the juvenility in vernalization response in *Arabis alpina* is that vernalization can repress *AaFLC* expression in adult but not juvenile plants. To check whether this is the case, *AaFLC* expression was tested using quantitative RT-PCR. Since in Arabidopsis, *AtFLC* functions in both apices and leaves (Searle et al., 2006), it would not be surprising that *AaFLC* also works in both tissues. Therefore, the expression of *AaFLC* in both leaves and apices was tested. Firstly, plants were grown under LDs for 2 weeks and 8 weeks providing juvenile and adult plants, respectively. Then a twelve week cold treatment was applied to both types of plants. Apices and expanded leaves were harvested before and during the vernalization treatment. In apices, before vernalization, *AaFLC* mRNA was expressed at slightly higher levels in adult than in juvenile plants (Figure 14A and 14B). During vernalization treatment, *AaFLC* mRNA levels gradually decreased in both juvenile and adult plants (Figure 14A), and in both cases had fallen markedly after 3 week vernalization. Decrease of *AaFLC* mRNA was also observed in leaves of both juvenile and adult plants during vernalization, and the decrease in leaves was even more dramatic than that in apices (Figure 14B). The similar expression pattern of *AaFLC* in both juvenile and adult plants during vernalization suggested that the different floral response in juvenile and adult plants was not caused by different regulation of *AaFLC* transcription.

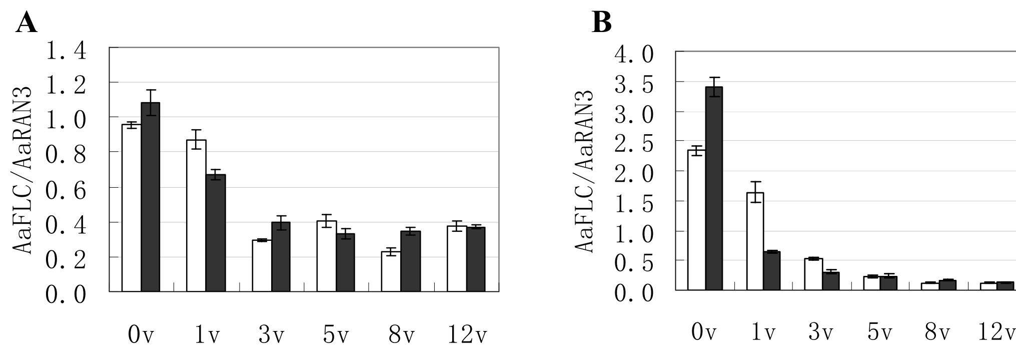


Figure 14. *AaFLC* expression in juvenile and adult *Arabis alpina Pajares* plants in response to vernalization treatment.
(A) Relative *AaFLC* mRNA levels in apices. **(B)** Relative *AaFLC* mRNA levels in leaves. X axis, weeks in vernalization treatment. 0v, before vernalization treatment. White bars, juvenile plants. Black bars, adult plants.

3.4. Reduction of *AaFLC* expression in transgenic *Arabis alpina Pajares* plants allowed flowering without vernalization

To confirm the role of AaFLC as a floral repressor, a double strand *AaFLC* RNAi construct was made, in which two copies of an *AaFLC* cDNA fragment in reverse orientation were ligated to each end of an intron (See Figure 15A for the diagram of the organization) of the RNA interference vector pJawohl8-RNAi (NCBI TaxID 188084). The *AaFLC* cDNA fragment used in this construct included the 3'-UTR and the C-terminal coding region. The construct was named pJawohl8-RH63-64 (See Materials and Methods for more details). A floral dipping method similar to that used for transformation of Arabidopsis was developted to transform *Arabis alpina Pajares* plants (Clough and Bent, 1998). Although the transformation efficiency was quite low, in total 7 independent BASTA resistant transformants were acquired (Table 1). None of the transformants flowered very early. However, flowering gradually happened in both the main shoot apex and apices of axillary shoots in one line (L3) about 4 months after sowing, and one month later in apices of several axillary shoots in another line (L5) (Table 1 and Figure 15F and 15G). In another line, L1, although no flower appeared, the floral transition did happen to some degree in some branches, as suggested by the morphological changes in the leaves produced more than 5 months after sowing. The newly produced leaves in

this line exhibited strong characteristics of the reproductive leaves, the lamina getting broader and the petiole getting shorter and disappeared gradually (Figure 15C, 15D and 15E). No morphological change in leaves or flowering has been observed so far in any of the other lines, including L2, L4, L6 and L7. Another noticeable phenotype exhibited in the transgenic lines except L4 was that the transgenic plants were taller than the non-transgenic control plants (Figure 15H). When the rate of total leaf production on the primary stems was checked 70 days after sowing, it was found that both transgenic and non-transgenic plants produced similar number of leaves at that time (Data not shown). Therefore the difference in height of transgenic and non-transgenic plants resulted from stem elongation in transgenic plants. As shown in Figure 15I, the internodes of the primary stem of transgenic lines (except L4) were significantly longer than those of the control plants. Furthermore, the extent of stem elongation coincided with the early flowering phenotype (Table 1 and Figure 15I).

Table1: Flowering time and leaf morphology of *AaFLC* dsRNAi T1 plants

Transgenic lines	§RLs	$Flowering	Stem elongation	Transition happened in
*L1	165	NA	Yes	Apices of several branches
L2	NA	NA	Yes	No
L3	105	120	Yes	Apices of the primary shoot and most axillary branches
L4	NA	NA	No	No
L5	128	149	Yes	Apices of several branches
L6	NA	NA	Yes	No
L7	NA	NA	Yes	No

* *35S:AaFLC* dsRNAi lines 1 to 7; § Days from germination to the emergence of reproductive related leaves; NA, The relevant events did not happened at all; $ Days from germination to the emergence of the first floral bud. Wild type plants produce no reproductive leaves (RLs) and do not flower under these conditions.

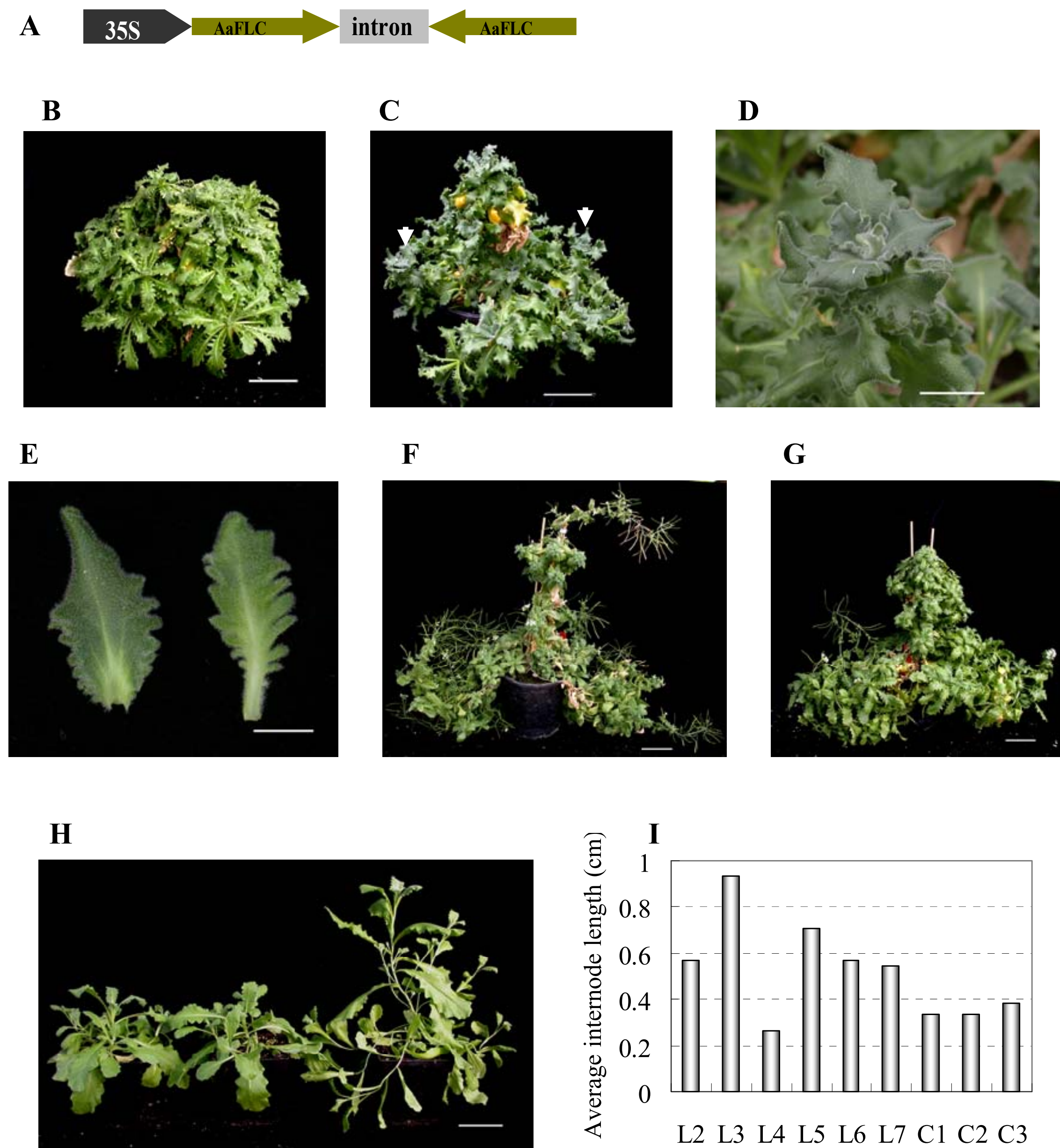


Figure 15. Phenotypes of *AaFLC* dsRNAi *Arabis alpina Pajares* lines.
(A) Diagram of the *AaFLC* dsRNAi construct. **(B)** A non-transgenic control plant. Bar, 5 cm. **(C)** Transgenic line L1 exhibited reproductive traits (the arrows). Bar, 5 cm. **(D)** A higher magnification image of a branch from the plant shown in **(C)**. Bar, 2 cm. **(E)** A young leaf lacking a petiole produced by L1 (left) and a leaf at a similar developmental stage from a non-transgenic control plant (right). Bar, 1 cm. **(F)** Flowering and seed setting occurred in a transgenic line, L3, without vernalization. Bar, 5 cm. **(G)** Flowering and seed setting only occurred in a few branches of L5. Bar, 5 cm. **(H)** Comparison of a nontransgenic plant (left) with L4 (middle) and L3 (right). Bar, 5cm. **(I)** Average length of internodes of transgenic plants (L2-L7) and 3 non-transgenic controls (C1-C3). Plants in **(B-G)** were 6.5 months old while those shown in **(H)** and **(I)** were 2 months old.

Quantitative real-time PCR was performed to check the efficiency of reduction of *AaFLC* mRNA expression in these RNAi lines. In all transgenic lines except line 4 *AaFLC* mRNA levels in expanding leaves were dramatically reduced between 7 to 15 fold (Figure 16). These data suggested RNAi worked efficiently in most of these transgenic lines. Coincidently, transgenic line L3, in which flowering occurred the earliest and in both the main and the axillary shoots, showed the strongest reduction in *AaFLC* mRNA levels (about 15 fold reduction). A 12 fold reduction in *AaFLC* mRNA levels was detected in line 5, in which flowering occurred only in several axillary shoots and later than in L3. Whereas in line 4, which was not phenotypically distinguishable from the non-transgenic control plant (Figure 15H), *AaFLC* mRNA level was not reduced at all. These results support the idea that AaFLC functions as a floral repressor in *Arabis alpina*.

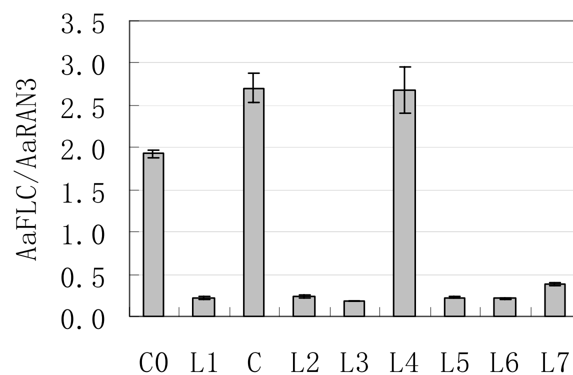


Figure 16. *AaFLC* mRNA levels in *AaFLC* dsRNAi lines.
L1-L7, *AaFLC* dsRNAi lines; C0, wild type control plants for L1; C, wild type control plants for L2-L7. RNA was extracted from expanding leaves of 6-week old plants.

3.5. *AaFT* is an orthologue of Arabidopsis *FT*

In both juvenile and adult plants, *AaFLC* expression decreases with vernalization treatment in similar patterns, but flowering does not occur in juvenile plants. This result suggests that juvenility is caused by events happening downstream of *AaFLC* transcription, so that even in the absence of AaFLC flowering cannot occur in juvenile plants. In Arabidopsis, *FT* (*AtFT*) is a direct target of AtFLC (Searle et al., 2006) and, as a floral pathway integrator gene, plays an important role in control of

flowering time. Investigating the function of *AtFT* ortholog(s) would be helpful to understand flowering-time control and juvenility in *Arabis alpina*.

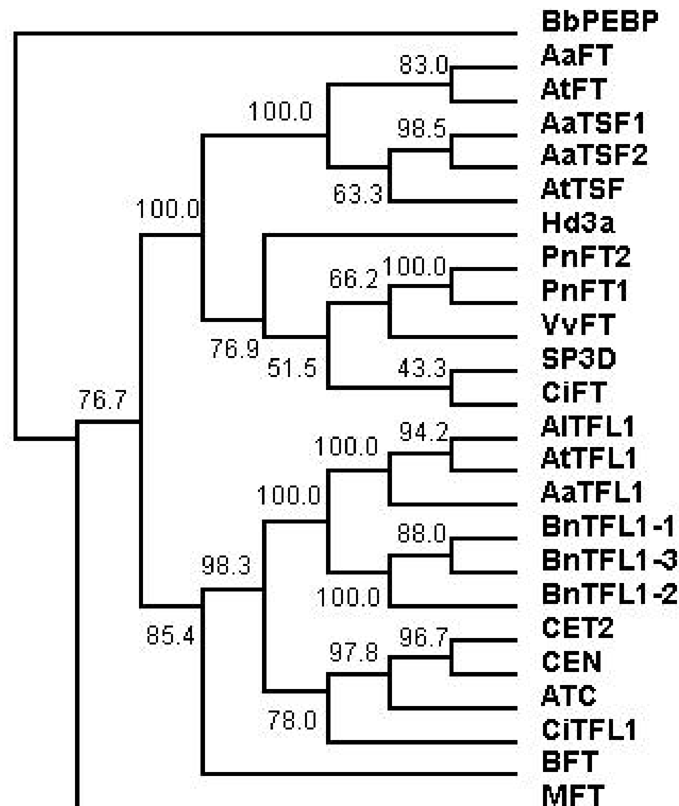


Figure 17. Phylogenetic analysis of AaFT, AaTSF1, AaTSF2, AaTFL1 and FT/TFL1 like proteins from other species.
Aa, *Arabis alpina*. At, *Arabidopsis thaliana*. Al, *Arabidopsis lyrata*. Bn, *Brassica napus*. Pn, *Populus nigra*. Ci, *Citrus sinensis*. Vv, *Vitis vinifera*. Bb, *Branchiostoma belcheri*. SP3D is from *Lycopersicon esculentum*. ATC, BFT and MFT are from *Arabidopsis thaliana*. CEN is from *Antirrhinum majus*. CET is from *Nicotiana tabacum*. Hd3a is from *Oryza sativa*. Bootstrap numbers shown at nodes are percentage of 1000 replicates.

Three *FT/TSF* like genes were cloned in our laboratory and all of them showed a similar genomic organization to *AtFT* or *AtTSF*, consisting of 4 exons and 3 introns (Plogander, 2005). To reveal whether these genes are *AtFT* orthologs a phylogenetic analysis was performed to compare the deduced amino acid sequences with FT/TFL1 like proteins from a variety of other species (Figure 17). One of these sequences had higher similarity with AtFT than any other FT/TFL like proteins from Arabidopsis or other species, and therefore this sequence was named

AaFT. Predicted AaFT consists of 175 amino acids, which is the same as AtFT. AaFT shows 90 percent sequence identity with AtFT (See the Appendix), and the amino acids (Tyr85 and Gln140) that were proven to be critical for AtFT functionality are conserved in AaFT (Hanzawa et al., 2005; Ahn et al., 2006). The other two proteins showed higher similarity with AtTSF than other proteins and were named AaTSF1 and AaTSF2, respectively. AaTSF1 encodes a 176 amino acid protein and AaTSF2 encodes a 175 amino acid protein. Expression analysis suggested that *AaTSF2* expression was not detectable with RT-PCR in various tissues while *AaTSF1* was expressed at very low levels and was quite difficult to detect with quantitative real-time PCR (data not shown). In contrast, expression of *AaFT* was easily detected with both semi-quantitative PCR and quantitative real-time PCR in leaves of plants grown under LDs (Figure 18A and Figure 19). Like *AtFT*, *AaFT* also showed a diurnal expression pattern under LDs with a peak in the evening, and was only expressed at very low levels under SDs (Figure 18A).

AaFT function was investigated by overexpression from a 35S promoter in transgenic *Arabidopsis thaliana* Columbia-0 plants. Similar to overexpression of *AtFT*, more than 2/3 of the independent T1 *35S:AaFT* plants (in total more than 50 lines) flowered very early and produced terminal flower (one representative line was shown in Figure 18B). To further confirm this result, two representative independent lines were selected to be analyzed in the T2 generation. Under both LDs and SDs, *35S:AaFT* plants flowered early and the inflorescences of the transgenic plants terminated as single flowers (Data from one line is shown in Figure 18C and 18D) (Kardailsky et al., 1999; Kobayashi et al., 1999). Moreover, expression of *AtSOC1* mRNA was increased in transgenic Arabidopsis plants overexpressing AaFT (Figure 18E) as observed for *35S:AtFT* transgenic plants (Yoo et al., 2005). Therefore, all the data described above suggest that *AaFT* promotes flowering and is an *Arabis alpina* ortholog of *AtFT*.

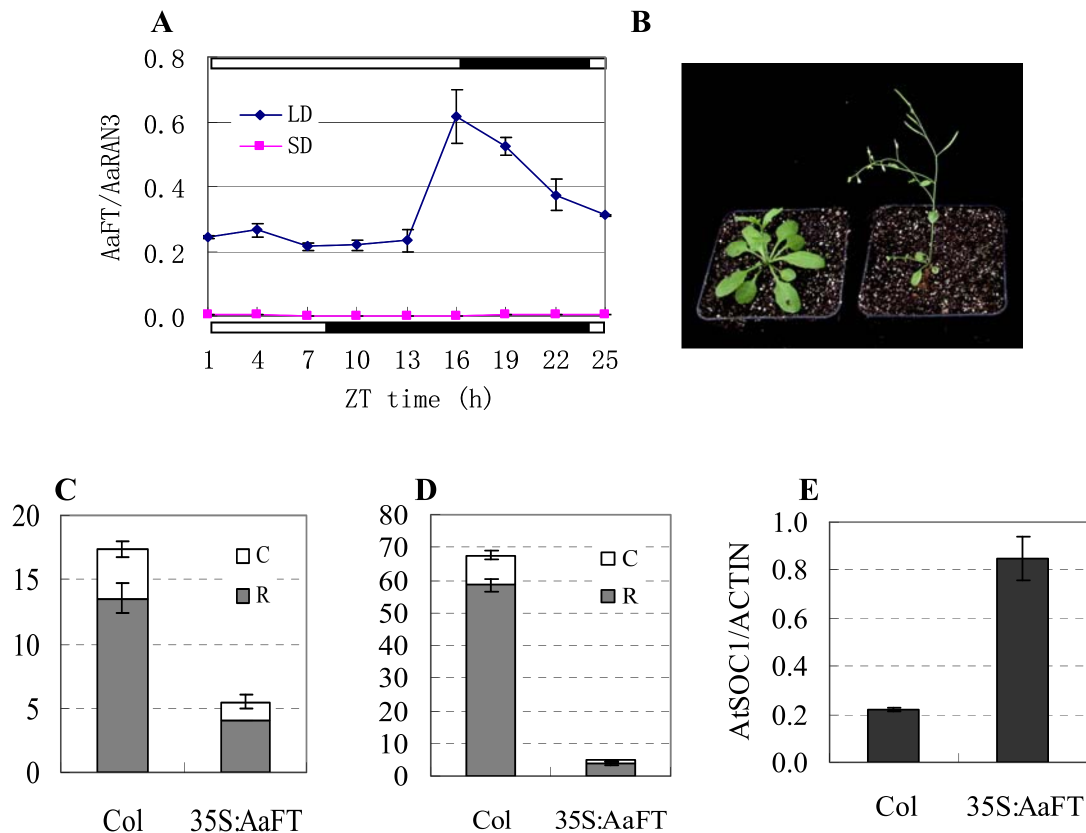


Figure 18. Expression patterns and function of *AaFT*.
(A) Daily expression patterns of *AaFT* mRNA under LDs and SDs. Black and white bars on the top and the bottom indicate the time points to switch between light and dark under LDs and SDs, respectively. **(B)** *35S:AaFT* caused early flowering in Arabidopsis. Left, a representative Col-0 plant. Right, a representative *35S:AaFT* plant. Plants were grown under LDs. **(C and D)** Comparison of flowering time in terms of numbers of rosette leaves (R) and cauline leaves (C) produced when flowering occurred in *35S:AaFT* plants and wild type *Arabidopsis thaliana Columbia* controls under LDs **(C)** and SDs **(D)**. **(E)** Expression levels of *AtSOC1* in *35S:AaFT* plants and wild type Col plants. The *35S:AaFT* plants shown in **(A)** was a representative T1 plant, and plants used in **(C)** and **(D)** were T2 plants originally from a representative T1 line.

3.6. During early stages of vernalization treatment, *AaFT* was expressed at low levels in both juvenile and adult plants

In poplar, *PnFT2*, a poplar ortholog of *AtFT*, was proposed to play an important role in controlling juvenility (Bohlenius et al., 2006; Hsu et al., 2006). To reveal the possible role of *AaFT* in juvenility in *Arabis alpina*, its expression patterns in

juvenile and adult plants were compared in response to vernalization treatment. *AaFT* expression was tested in total RNA used to test *AaFLC* expression in Section 3.3. Before vernalization, *AaFT* mRNA level in leaves of adult plants was about 2.5 fold higher than in leaves of juvenile plants (Figure 19A). In leaves of both adult and juvenile plants, *AaFT* mRNA expression rapidly decreased to background levels soon after the cold treatment started, and was maintained at low levels throughout the rest of the vernalization treatment, in which SDs was used since it was thought to be similar to the winter (Figure 19A)(Sung et al., 2006a). Before vernalization, in apices of both juvenile and adult plants, *AaFT* mRNA was expressed at very low levels, although it was slightly higher in those of adult plants. In the early stages of vernalization treatment (at least the first 5 weeks), *AaFT* expression in apices of both juvenile and adult plants was maintained at background levels. However, 8 weeks in vernalization treatment, *AaFT* mRNA levels increased dramatically in apices of adult plants and later on (at the end of vernalization) *AaFT* transcript levels began to decrease. Based on longitudinal section, no floral primordia were present 8 weeks in vernalization (data not shown). In contrast, increase of *AaFT* expression was not detected in apices of juvenile plants throughout the cold treatment (Figure 19A). Although *AaFT* mRNA levels were not increased in apices of the adult plants that had been treated with cold for 5 weeks, reproductive leaves and, in some cases, flowers were produced if the plants were moved back to normal temperatures after a 5-week-cold treatment (Figure 19B and 19C). These floral transition-related phenotypes had never been seen in juvenile plants with the same treatment. Therefore, although *AaFT* expression was upregulated in late stages of vernalization treatment, this upregulation could not account for the floral transition that occurred in the early stages of the treatment.

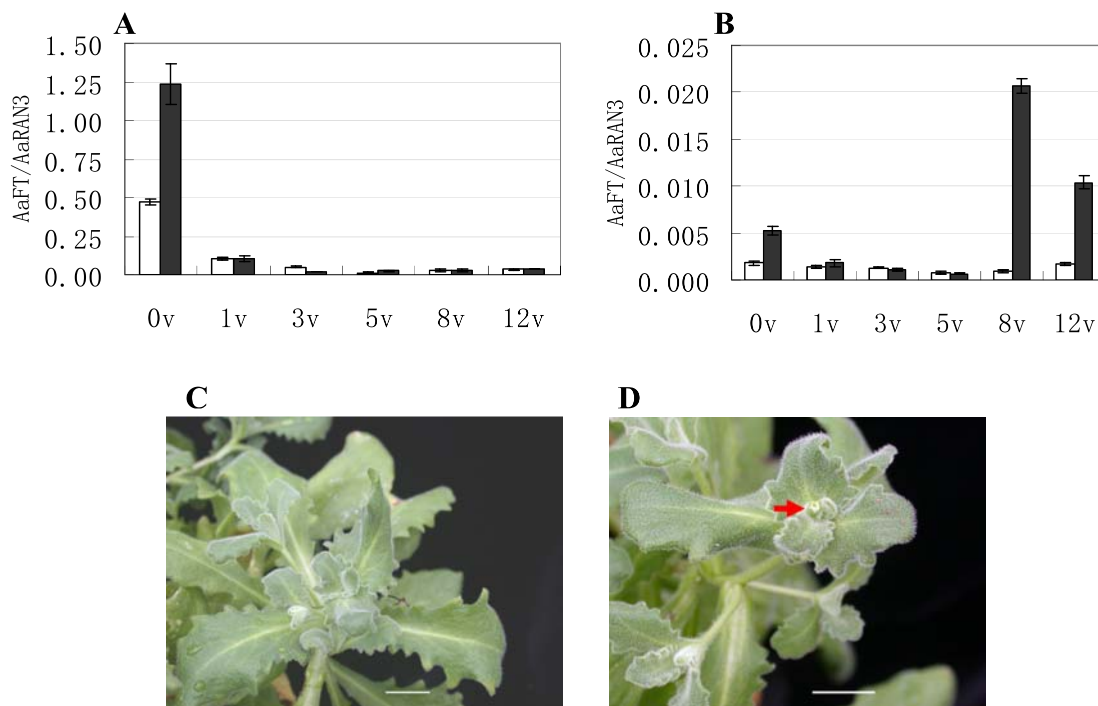


Figure 19. Expression of *AaFT* in juvenile and adult plants in response to vernalization treatment.
(A and B) *AaFT* expression in leaves **(A)** and apices **(B)**. Open bars represent juvenile plants and filled bars adult plants. X axis indicates weeks in vernalization treatment. **(C and D)** In adult plants which were treated with cold for 5 weeks and then grown at normal temperatures, floral transition occurred as indicated by production of reproductive leaves **(C and D)** and in some cases one or two flowers (the arrow in **(D)**) from apices.

3.7. *AaSOC1* caused early flowering when ectopically expressed in Arabidopsis

In Arabidopsis, *SOC1* (*AtSOC1*) is a floral pathway integrator gene that promotes flowering and is negatively regulated by AtFLC. During vernalization treatment, *AtFLC* expression is down-regulated and as a result expression of *AtSOC1* increases. In juvenile *Arabis alpina* plants, although vernalization treatment efficiently reduced *AaFLC* expression levels, flowering did not occur. This could be explained if genes downstream of *FLC* are repressed in additional ways in juvenile plants. To test this, attempts were made to clone a *SOC1* ortholog from *Arabis alpina*. A similar strategy (See Materials and Methods for details) as used for cloning *AaFLC* was adopted to clone putative ortholog(s) of *AtSOC1*. A gene

showing high homology to *AtSOC1* was cloned and was named *AaSOC1*. *AaSOC1* encodes a 216 amino acid MADS box protein which shows 93 percent sequence identity with AtSOC1. Phylogenetic analysis indicated that AaSOC1 resided in a clade of MADS box proteins that only included SOC1 like proteins from different species and AaSOC1 had higher sequence similarity to AtSOC1 than to any other Arabidopsis protein (Figure 20A).The function of AaSOC1 was tested by ectopic expression in Arabidopsis under the control of a CaMV35S promoter. Full length CDS was used to make the construct. Among 53 primary transformants, clearly early flowering phenotypes were observed in 26 plants, of which 18 plants flowered extremely early (Figure 20B), and the rest flowered at similar time as the wild type plants, suggesting that, like AtSOC1, AaSOC1 can also function as a potent promoter of the floral transition.

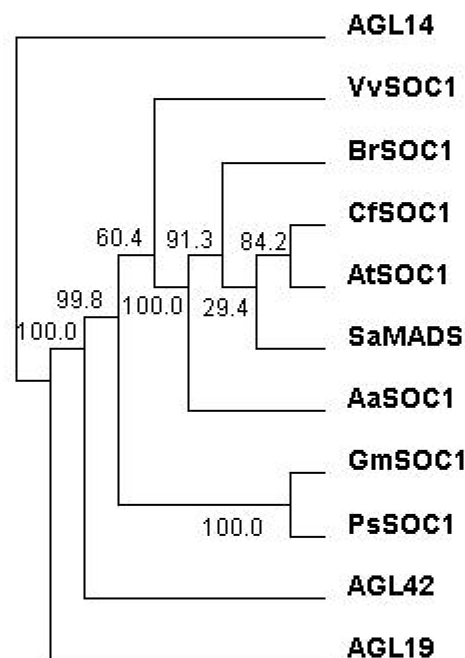


Figure 20. Phylogenetic analysis of AaSOC1.
Aa, *Arabis alpina*. At, *Arabidopsis thaliana*. Br, *Brassica rapa*. Cf, *Cardamine flexuosa*. Sa, *Sinapis alba*. Vv, *Vitis vinifera*. Gm, *Glycine max*. Ps, *Pisum sativum*. Bootstrap numbers shown at nodes are percentage of 1000 replicates.

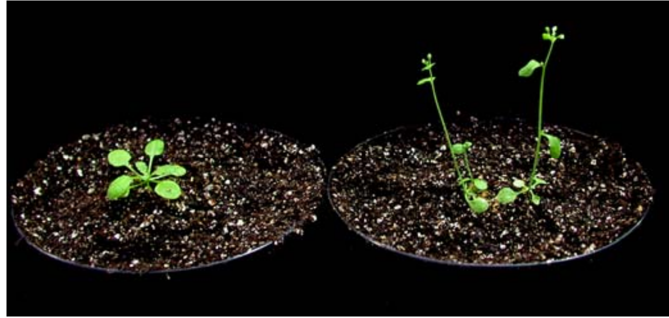


Figure 21. Ectopic expression of *AaSOC1* from the 35S promoter in Arabidopsis resulted in early flowering (right) compared to Col (left).
Plants were grown in LDs. The three *35S:AaSOC1* plants shown are representative T1 plants.

3.8. *AaSOC1* expression increased during vernalization treatment in both juvenile and adult plants

Expression of *AaSOC1* during vernalization treatment in juvenile and adult *Arabis alpina* plants was tested with the same set of plant materials as used to test *AaFLC* expression in Section 3.3. Before vernalization treatment, *AaSOC1* mRNA levels in apices of adult plants was slightly higher than in those of juvenile plants, which is similar to what was observed for *AaFLC* and *AaFT* expression (Figure 22A). Interestingly, during vernalization treatment, *AaSOC1* expression was upregulated gradually and to dramatically high levels at the end of vernalization in apices of both juvenile and adult plants (Figure 22A). This increase was not correlated with flowering as adult but not juvenile plants flowered under these conditions. The expression pattern of *AaSOC1* in leaves also increased in both juvenile and adult plants in response to vernalization, but not as dramatically as in apices at the end of the vernalization treatment (Figure 22A and 22B). At the end of the vernalization treatment, although expression of the potent floral promoter, *AaSOC1* increased to similarly high levels in juvenile and adult plants, floral transition only happened in adult but not juvenile plants. This suggests that floral signaling was blocked downstream of *AaSOC1* expression, possibly by the presence of repression mechanisms present specifically in young plants.

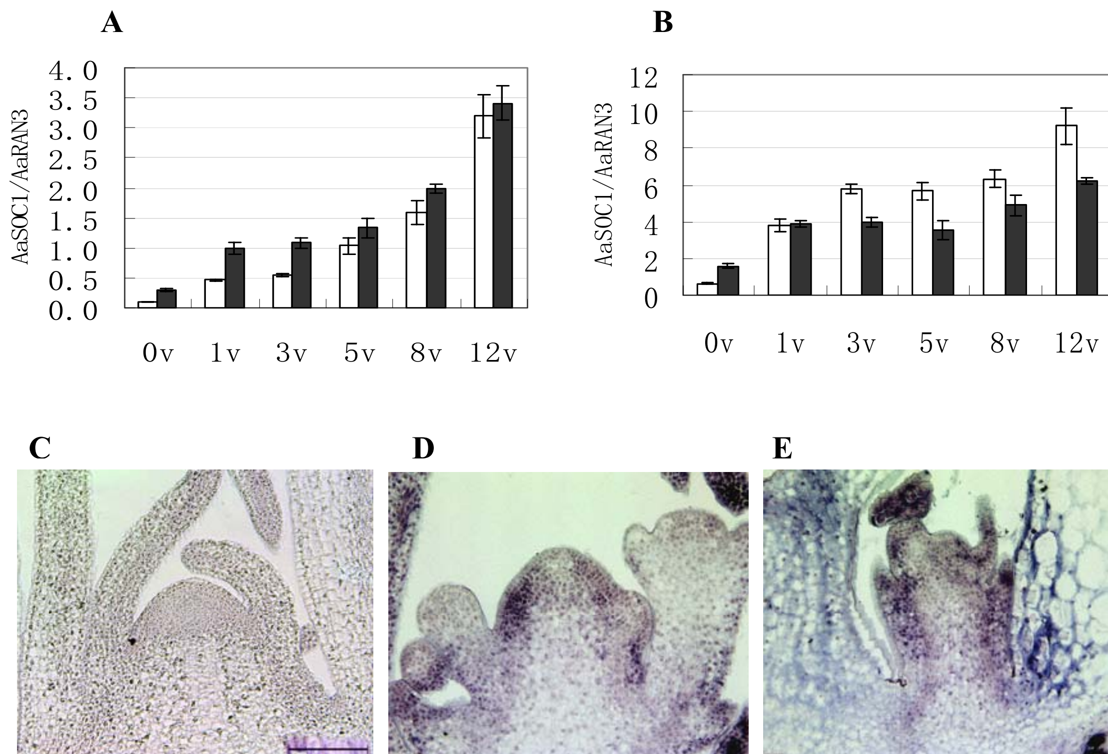


Figure 22. *AaSOC1* expression in juvenile and adult *Arabis alpina Pajares* plants in response to vernalization treatment.
(A and B) *AaSOC1* expression levels in apices **(A)** and expanded leaves **(B)** tested with quantitative real time PCR. Black bars, juvenile plants. White bars, adult plants. **(C-E)** *AaSOC1* mRNA levels tested by *in situ* hybridization in main shoot apices **(C and D)** and type II branches **(E)** of adult plants before **(C)** and at the end of **(D and E)** vernalization.

To test the detailed expression pattern of *AaSOC1*in apices, *in situ* hybridization was carried out. Before vernalization, *AaSOC1* was hardly detectable in the apex, but at the end of a 12 week vernalization treatment strong signals were detected in the apical meristems and young leaves of adult plants, and the signal was especially strong in the young leaves (Figure 22C and 22D). In developing flowers and floral meristems, *AaSOC1* signal was much weaker than in the flanking regions of the SAM. Furthermore, *AaSOC1* was also highly expressed in the vegetative type II axillary branches as in the main shoot apical meristem (Figure 22E).

3.9. *AaFT* and *AaSOC1* mRNA levels are increased in *AaFLC* dsRNAi lines

In Arabidopsis, AtFLC represses the expression of *AtSOC1* and *AtFT* by directly binding to the promoter or introns of these genes. Reduction of AtFLC expression causes increased *AtSOC1* and *AtFT* expression (Searle et al., 2006). To investigate whether in *Arabis alpina* this pattern of regulation is conserved between the corresponding orthologs, *AaFLC*, *AaSOC1* and *AaFT*, quantitative real-time PCR was carried out to test expression levels of *AaSOC1* and *AaFT* in *AaFLC* dsRNAi lines (Section 3.4). *AaSOC1* expression was increased 3 to over 10 fold in the leaves of all the other transgenic plants except line 4 in which *AaFLC* expression levels did not decreased (Figure 23A and Section 3.4). Furthermore, the levels of *AaSOC1* mRNA positively correlated with flowering time or the degree of the floral transition observed. The transgenic plant line L3 had highest *AaSOC1* mRNA levels (over 10 fold higher than in control plants) and correspondingly flowered the earliest and in most side shoot branches. Flowering happened about one month later and only in a few axillary branches of line L5, which had second highest *AaSOC1* expression level among the *AaFLC* dsRNAi plants (about 6.2 fold increase compared to wild-type). In line L1, where *AaSOC1* expression level was also over 6 fold higher than that in the control plants, floral transition happened to a transitional step where only RLs but not floral buds were produced (Figure 23A and Section 3.4). None of the lines in which *AaSOC1* mRNA levels increased less than 5 fold of that in the nontransgenic controls flowered or even produced any transition associated phenotypes. In L4, where *AaFLC* mRNA levels were not reduced, *AaSOC1* was expressed at similar levels as in wild type plants. *AaSOC1* mRNA levels also showed a close positive correlation with stem elongation, which may be an event closely related to the floral transition (Figure 23A and Figure 15I in Section 3.4). The close correlation between expression levels of *AaFLC*, *AaSOC1* and the flowering phenotypes suggests that, *AaFLC* regulates flowering via regulation of the expression of *AaSOC1*, which may therefore be as potent a floral promoter in *Arabis alpina* as in *Arabidopsis thaliana* (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000).

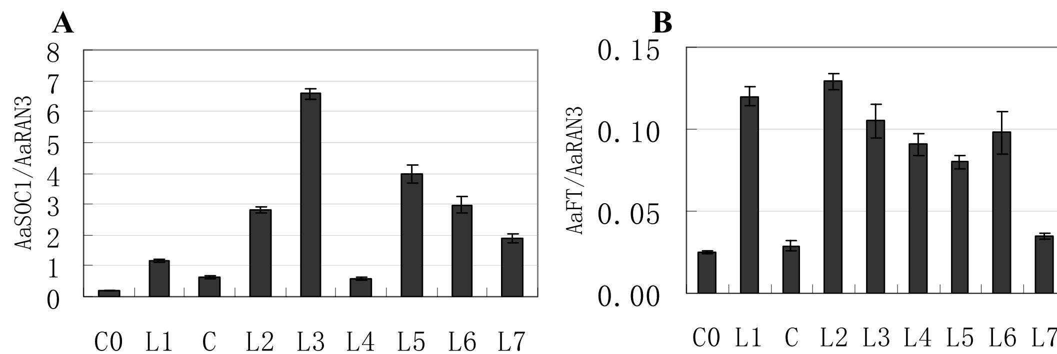


Figure 23. Expression of *AaSOC1* and *AaFT* in *AaFLC* dsRNAi lines.
(A) and *AaFT* **(B)** mRNA levels were tested with quantitative real-time PCR in individual *AaFLC* dsRNAi lines (L1-L7) and wild type controls (C0 for L1 and C for the other transgenic lines). RNA was extracted from expanding leaves of 6-week old plants.

Contrary to the situation of *AaSOC1*, expression levels of *AaFT* in *AaFLC* dsRNAi lines were not correlated with flowering time, although its expression levels did increase to various degrees in these lines compared to wild type controls (from slightly to nearly 5 fold higher)(Figure 23B). Especially, *AaFT* mRNA levels increased in L4 although *AaFLC* levels in this line did not increase at all. The inconsistency between decrease of *AaFLC* mRNA levels and upregulation of *AaFT* mRNA might be caused by some RNA degradation-independent effects or suggest that the regulation of *AaFT* expression by AaFLC might be indirect. The inconsistency between increase of *AaFT* expression and flowering phenotypes suggests the increase might still be under the threshold for floral promotion in *Arabis alpina*.

3.10. Cloning and sequence analysis of *AaTFL1*

AtTFL1 plays antagonistic roles with AtFT in regulation of flowering in Arabidopsis although they share high sequence similarity (Kardailsky et al., 1999; Kobayashi et al., 1999). The function of AtTFL1 includes at least two aspects, repression of flowering and maintenance of inflorescence indeterminancy. AtTFL1 exerts its function at least partially through repression of the upregulation of floral meristem identity genes, *LFY* and *AP1*, which function in quite late steps of the hierarchy of genes controlling flowering time. Our results described above suggest

that a possible repression mechanism exists downstream of *AaSOC1* transcription and contributes to the juvenility of young *Arabis alpina* plants. Based on the function of AtTFL1 in Arabidopsis, an ortholog of *AtTFL1* could play this repression role in *Arabis alpina*.

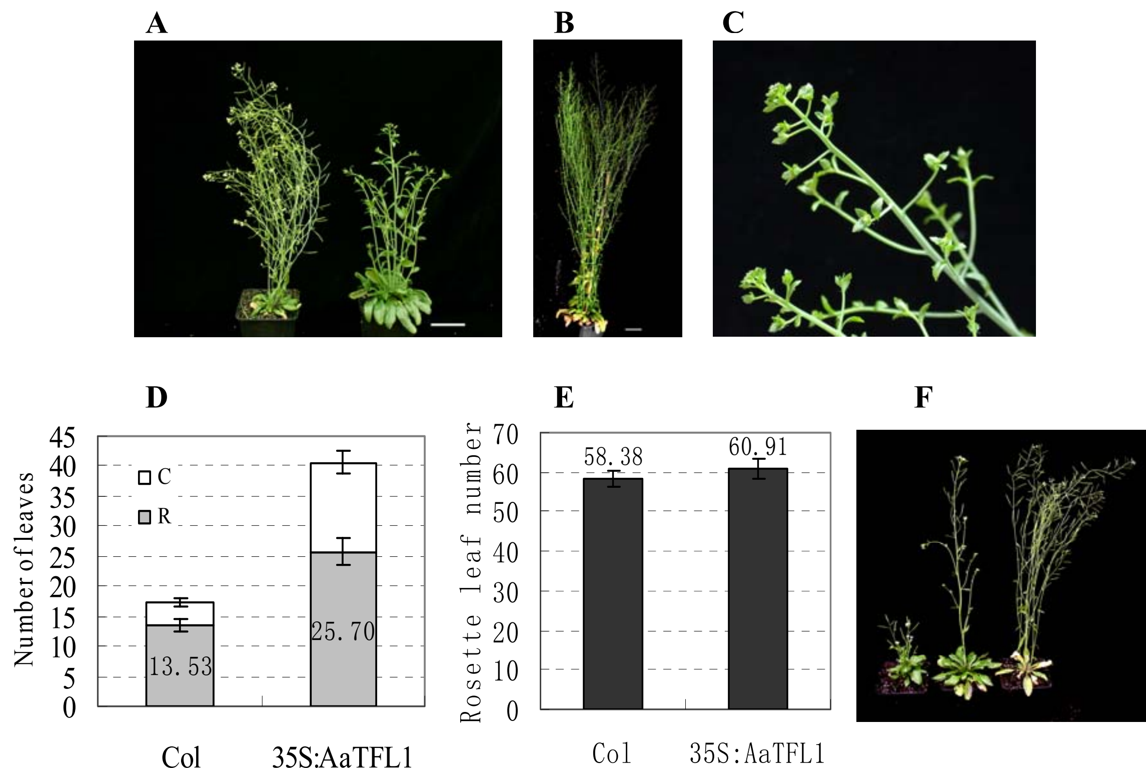


Figure 24. *AaTFL1* expression from either a 35S promoter or its native promoter delayed flowering in Arabidopsis.
(A) A *35S:AaTFL1* plant (right) and a Col plant (left), which were sown at the same time. **(B)** Highly branched phenotype in a *35S:AaTFL1* plants. **(C)** Transition of flowers to inflorescence branches in a *35S:AaTFL1* plant. **(D and E)** Under LDs **(D)** and SDs **(E)**, flowering time of *35S:AaTFL1* plants compared with that of wild type Col plants in terms of rosette (R) and cauline (C) leaves produced on the primary stems. **(F)** Comparison of flowering time in *tfl1-1*, *tfl1-1* carrying Pro_{AaTFL1}*:AaTFL1*, and Col. The photoperiod used in **(E)** was SDs, and LDs in all the rest.

To test this idea, an *AtTFL1* homolog was cloned with similar methods as used to clone *AaFLC* (See Materials and Methods for more details) and the gene was named *AaTFL1*. The predicted AaTFL1 protein consists of 177 amino acids, as does AtTFL1, and shows 94 percent identity with AtTFL1. Furthermore AaTFL1 was in the TFL1 clade in the phylogenetic tree (Figure 17). The amino acids at positions (His88 and Asp144) that were proven critical for the functionality of

AtTFL1 are conserved in AaTFL1 (Hanzawa et al., 2005; Ahn et al., 2006). The result of BAC sequencing suggested that the synteny between *Arabis alpina* and *Arabidopsis thaliana* is at least partially conserved: a homolog of gene At5g03850, which resides immediately upstream of *AtTFL1* gene in Arabidopsis genome, was also found immediately upstream of *AaTFL1*, but the sequencing of regions downstream *AaTFL1* has not been completed.

3.11. Expression of *AaTFL1* from the CaMV35S promoter or its own promoter caused late flowering in Arabidopsis

To provide an indication of the function of AaTFL1, the gene was expressed from the CaMV35S promoter in transgenic Arabidopsis plants. Under LDs, these plants showed phenotypes similar to those of *35S:AtTFL1*, principally delayed flowering at both the rosette and inflorescence stages (Figure 24A-24D)(Bradley et al., 1997; Ratcliffe et al., 1998). As a result, flowers were converted into small inflorescence branches and the plants finally developed a highly-branched structure (Figure 24 B and C). *35S:AaTFL1* plants produced almost twice as many rosette leaves as wild type plants under LDs (Figure 24D). Under SDs *35S:AaTFL1* plants flowered only slightly later than wild type plants (Figure 24E).
To further confirm the function of *AaTFL1*, a 5.2 kb *AaTFL1* genomic fragment including a 2.6 kb of promoter region, all the exons and introns, and a 1.5 kb fragment carrying the downstream sequences was introduced into the *tfl1-1* mutant (Bradley et al., 1997; Ratcliffe et al., 1998). The majority of the T1 plants showed various degrees of restoration of the indeterminate inflorescence phenotype and in addition about half of the T1 plants flowered dramatically later than wild-type plants (Figure 24F). Furthermore, compared to the wild type controls, the transgenic plants did not show any other phenotype except late flowering.
Together with the sequence comparison and phylogentic analysis, the phenotypes of expression of *AaTFL1* in Arabidopsis indicated that *AaTFL1* encodes a floral repressor and an ortholog of AtTFL1.

3.12. In response to vernalization, *AaTFL1* shows different expression patterns in juvenile and adult plants

To evaluate the role of *AaTFL1* in the meristem as part of the juvenility response, its expression was tested by *in situ* hybridization in juvenile and adult plants in response to vernalization treatment. Before the treatment, in both juvenile and adult plants *AaTFL1* was expressed highly in the sub-domain of the shoot apices, provasculature in apices and young leaves, adaxial basal part of the young leaf primordia and axillary meristems. The expression levels in the domain under the menistem dome, the axillary meristems and provasculature tissues connecting axillary meristems and the main body of the apices were much higher than in the flanking regions (Figure 25A and 25E). Images from successive sections indicated that the various tissues where AaTFL1 was expressed were interlinked with each other, forming a continuous zone in the shoot apex. Similar to the expression of *AtTFL1*, *AaTFL1* mRNA could not be detected (or only weakly) in several outer layers of cells in the shoot apex (Figure 25)(Bradley et al., 1997; Ratcliffe et al., 1998). During early stages of the vernalization treatment (up to 3 weeks), *AaTFL1* expression patterns did not change too much in apices of either juvenile or adult plants except that the expression in both cases extended a bit downward along the provascular tissues and the expression levels seemed to increase (Figure 25 A, B, E and F). Later during the treatment but before any indication of development of floral primordia, the expression region of *AaTFL1* in adult apices began to become restricted. At the end of 5 weeks in cold treatment, *AaTFL1* expression was already restricted to very limited regions in the apices of adult plants, including the domain under the meristem dome of the main shoot apex and axillary meristems, and these regions were clearly isolated from each other (Figure 25C). With still longer treatments, the region expressing *AaTFL1* in the sub-dome domain under the meristem was still more restricted. At the end of the 12 week cold treatment *AaTFL1* expression at the main shoot apex was restricted to very limited numbers of cells just below the meristem dome and floral meristems arose from the flanking regions where *AaTFL1* was undetectable. In contrast to what occurred in the apices of adult plants, *AaTFL1* expression patterns in juvenile did not change significantly throughout the cold treatment, even when an extremely long (30 weeks) cold

treatment was used (Figure 25E-25H). These results indicate that restriction of *AaTFL1* expression is an early step in flowering control of adult plants and that this is prevented in juvenile plants.

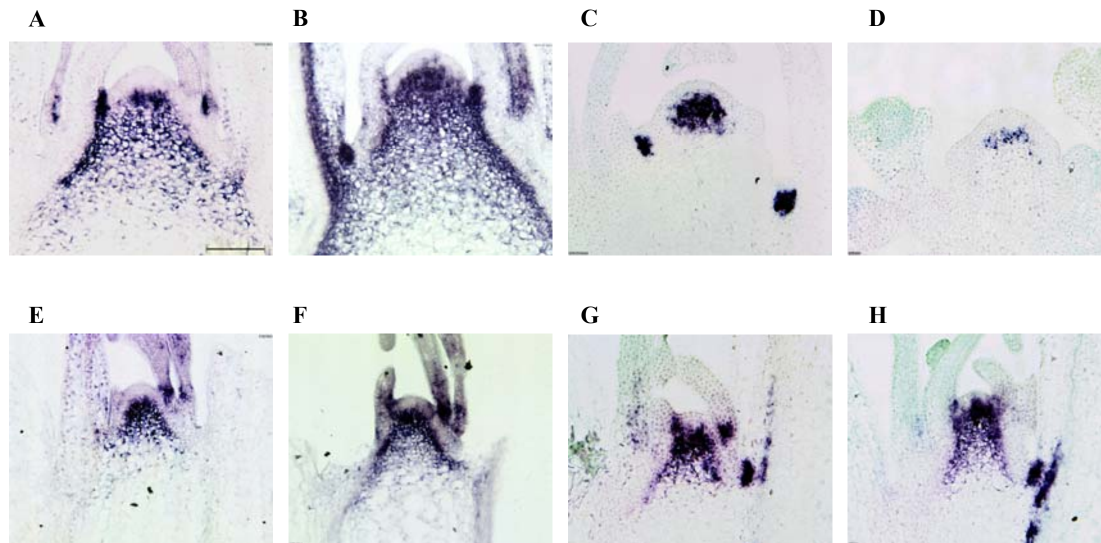


Figure 25. *AaTFL1* expression tested by *in situ* hybridization in apices of juvenile and adult *Arabis alpina Pajares* plants in response to vernalization treatment.
(A-D) Apices of plants which were grown in LDs for 8 weeks **(A)**, followed by 3 weeks **(B)**, 5 weeks **(C)** and 12 weeks **(D)** of cold treatment. **(E-H)**. Apices of plants which were grown in LDs for 2 weeks **(E)**, followed by 3 weeks **(F)**, 5 weeks **(G)** and 30 weeks **(H)** of cold treatment. Bar, 100 µm.

3.13. Discussion

3.13.1. Sequence and functional conservation of flowering time genes in *Arabis alpina* and *Arabidopsis thaliana*

The results of sequence comparisons and phylogenetic analysis showed that AaFLC shares 85 percent sequence identity with Arabidopsis FLC and that in the phylogenetic tree AaFLC resides in the FLC clade rather than the clade containing the MAF proteins (Figure 12, and also see the appendix). Therefore, AaFLC shares higher sequence similarity with FLC than with any other Arabidopsis protein. Similar to FLC, when constitutively expressed from the CaMV35S promoter,

AaFLC caused dramatic late flowering in Arabidopsis, which indicates that AaFLC also acts as a floral repressor (Figure 13B and 13C). Furthermore, in the Arabidopsis plants constitutively expressing *AaFLC*, both *FT* and *SOC1* expression were strongly repressed (Figure 13D and 13E), which is reminiscent of the effect of constitutive expression of *FLC*. This observation suggests that in transgenic Arabidopsis, *AaFLC* delays flowering by repressing the expression of the floral promoter genes *FT* and *SOC1*, which is also the mechanism by which *FLC* represses flowering (Searle et al., 2006). BAC sequencing results revealed that there is conservation of synteny between the *AaFLC* region of the *Arabis alpina* genome and the *FLC* region of the Arabidopsis genome (M.Albani, unpublished results). All these data indicate that both the sequence and the function of AaFLC and Arabidopsis FLC are conserved. The idea that *AaFLC* is an ortholog of Arabidopsis *FLC* and represses floral transition in *Arabis alpina* was further confirmed by the phenotypes of transgenic *Arabis alpina Pajares* plants in which *AaFLC* expression was reduced to various levels by a RNA interference method (dsRNAi). In *AaFLC* dsRNAi lines, strongest reduction of *AaFLC* transcript levels correlates with the earliest flowering (L3), whereas the lines in which *AaFLC* was not reduced (L4) or less efficiently reduced (e.g. L7) did not undergo flowering. Moreover, the expression levels of *AaFLC* in L5, in which partial floral transition occurred about one month later than L3, were slightly higher than in L3. Therefore, our data provide strong evidence that *AaFLC* acts as an ortholog of Arabidopsis *FLC* and represses flowering in *Arabis alpina Pajares* plants. The observations that even in L3, which showed strongest reduction in *AaFLC* expression, floral transition occurred very late, and that the lines with slightly less reduction in *AaFLC* expression only underwent partial flowering or did not flower at all (Table 1) suggest the existence of additional floral repressors. These additional repressors may also contribute to the obligate vernalization response of *Arabis alpina Pajares*, which is a more severe phenotype than that of *Arabidopsis thaliana* vernalization responsive accessions.

Phylogenetic analysis, together with very strong sequence similarity (90 percent similarity in amino acid sequences) between AaFT and Arabidopsis FT, suggest that AaFT might be an ortholog of FT in *Arabis alpina* (Figure 17, and also see the appendix). This speculation was further supported by the expression patterns of

AaFT in response to day length and also the phenotypes of transgenic Arabidopsis plants constitutively expressing *AaFT* under the control of a CaMV35S promoter. Under LDs *AaFT* expression exhibited diurnal fluctuation, whereas under SDs it is only expressed at background levels (Figure 18A). These expression patterns are very similar to those of Arabidopsis *FT* mRNA under the same photoperiods (Hayama and Coupland, 2004). Constitutive expression of *AaFT* in Arabidopsis caused not only very early flowering but also formation of a terminal flower independent of day length (Figure 18B, 18C and 18D), the same phenotypes exhibited by Arabidopsis plants overexpressing *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999). In transgenic Arabidopsis plants constitutively expressing *AaFT*, *SOC1* expression was dramatically elevated, which is similar to the observation that in Arabidopsis *FT* expression levels positively regulated *SOC1* expression (Yoo et al., 2005). These data indicate that AaFT is an ortholog of FT and its function as a potent floral activator is conserved in Arabidopsis.

AaSOC1 shares an extremely high level of identity (93 percent) to Arabidopsis SOC1 (see the appendix). Therefore, in the phylogenetic tree AaSOC1 resides in the same clade with SOC1 but not any other Arabidopsis MADS box proteins (Figure 20). The high level of sequence similarity suggested that AaSOC1 plays a similar role as SOC1, a strong floral promoter in Arabidopsis, in regulation of flowering. In support of this idea constitutive expression of *AaSOC1* in transgenic Arabidopsis plants caused extreme early flowering (Figure 21). Furthermore, during vernalization, in *Arabis alpina Pajares*, *AaSOC1* expression increased (Figure 22A and 22B), and this increase correlated with the floral promotion effect of vernalization in adult *Arabis alpina Pajares* plants. In *AaFLC* dsRNAi *Arabis alpina Pajares* transgenic lines, *AaSOC1* expression increased. Moreover, the extent of the increase of *AaSOC1* expression in these lines not only correlated well with the degree of *AaFLC* mRNA reduction, but also the flowering time and extent of flowering in individual lines (Figure 15, 16 and 23A). All these data indicate that like SOC1, AaSOC1 is a strong floral activator and mediate floral transition in *Arabis alpina*. In addition, *AaFLC* dsRNAi lines showed an interesting increase in stem elongation that correlated well with the reduction of *AaFLC* expression, the increase of *AaSOC1* expression and the flowering time of individual plants. The elongation of the stems, possibly involving GA activities, might be a direct effect of

the reduction of *AaFLC* expression or the increase of *AaSOC1* levels. Alternatively, increased stem elongation may be closely associated with the floral transition.
In *AaFLC* dsRNAi lines, the expression levels of *AaSOC1* negatively correlated with that of *AaFLC* and positively correlated very well with flowering times of individual plants. However, the correlation of expression levels of *AaFT* with either *AaFLC* levels or the flowering time is not high. The inconsistency between decreased *AaFLC* mRNA levels and upregulation of *AaFT* expression might be caused by some RNA degradation-independent effects or by the regulation of *AaFT* expression by other factors. The inconsistency between the increase of *AaFT* expression and flowering time suggests that the increased *AaFT* levels in *AaFLC* dsRNAi lines might still be under the threshold for floral promotion in *Arabis alpina*. Nevertheless, in most *AaFLC* DsRNAi lines, *AaFT* was up-regulated, suggesting the repressive effect of AaFLC on *AaFT* expression.
The amino acid sequence of AaTFL1 is 94 percent identical to that of Arabidopsis TFL1 (See the appendix). This striking similarity, plus the observation that all the amino acids that were shown to be crucial for TFL1 function (Hanzawa et al., 2005; Ahn et al., 2006), are conserved between AaTFL1 and TFL1 strongly suggests that they may have similar activities. Not surprisingly, AaTFL1 sequence is closer to TFL1 than any other Arabidopsis protein (Figure 17). Constitutive expression of *AaTFL1* gene in Arabidopsis under the control of a CaMV35S promoter strongly delayed all the developmental phases under LDs (Figure 24A-D), which is similar to the effect of overexpression of *TFL1* (Ratcliffe et al., 1998; Ratcliffe et al., 1999). Furthermore, a 5.2 kb *Arabis alpina* genomic DNA fragment carrying *AaTFL1* complemented both the early flowering and the terminal flower phenotypes of *tfl1* mutants (Figure 24F). These plants also flowered much later than wild-type Arabidopsis plants (Figure 2F), which may be caused by the differences in the activity of the AaTFL1 protein compared to TFL1 or in the regulatory sequences of *AaTFL1* and *TFL1*. These data, together with the conservation of synterny between chromatins containing *AaTFL1* and *TFL1*, suggest *AaTFL1* is very likely an ortholog of Arabidopsis *TFL1* and functions as a strong floral repressor gene. Interestingly, under LDs transgenic Arabidopsis plants overexpressing *AaTFL1* bolted much later than the wild type control plants (35S:AaTFL1 plants produced 12 more rosette leaves than Col plants, Figure 24D), however under SDs they

bolted only slightly later than the control plants (Figure 24E). This result suggests that overexpression of *AaTFL1* prolonged vegetative phase mainly through suppressing the floral induction signals produced by the photoperiod pathway. Under LDs, *35S:AaTFL1* plants produced many more cauline leaves than wild-type plants (the early inflorescence phase was prolonged), and also flowers in the transgenic plants were converted to small coflorescences. These phenotypes are similar to those observed in transgenic Arabidopsis plants overexpressing *TFL1* (Ratcliffe et al., 1998; Ratcliffe et al., 1999), suggesting that *AaTFL1* acts to delay floral transition in the whole developmental process. Under SDs, after bolting, *35S:AaTFL1* plants did not flower but just continuously produced aerial rosette leaves and cauline leaves (data not shown), suggesting that floral inductive signals generated from LD photoperiods are important to antagonize the repression of flower development caused by AaTFL1. Interestingly, in the late flowering *35S:AaTFL1* transgenic Arabidopsis plants, neither *FT* nor *SOC1* transcript levels were reduced (data not shown), suggesting that the repression of flowering by AaTFL1 occurred downstream of *FT* and *SOC1* transcription.
The data shown above indicates that, in addition to the conservation in the functions of the flowering time genes AaFLC, AaFT, AaSOC1 and AaTFL1 the regulatory relationships among them and their Arabidopsis counterparts are also basically conserved.

3.13.2. Juvenility in vernalization response is not caused by a failure to repress *AaFLC* expression

The highly functional conservation of flowering-time control genes in *Arabis alpina* and *Arabidopsis thaliana* suggests that a flowering-time-control network primarily similar to that illustrated in Arabidopsis exists in *Arabis alpina*. This led us to wonder whether juvenility in vernalization response of *Arabis alpina Pajares* is due to ineffectiveness in initiation of the vernalization process in juvenile plants in a similar way as the failure of vernalization response in Arabidopsis *vin3* mutants (Sung and Amasino, 2004). However, the similar patterns of *AaFLC* expression in both juvenile and adult plants during vernalization indicate that in both cases

AaFLC was efficiently repressed (Figure 14). Although this was a logical hypothesis it may not be surprising that juvenility does not involve *FLC* regulation, because juvenility is a wider concept in flowering control than vernalization and found more widely than *FLC* homologues. First, juvenility exists extensively in most perennials and biennials that are evolutionarily far from each other. Numerous physiological studies in various plant species implied that some common mechanisms underlying juvenility are shared by different species (Hackett, 1985; Zimmerman, 1985a; Meilan, 1997). However, *FLC*-mediated vernalization requirement is not conserved in many species. For example, in winter wheat the vernalization requirement is mediated by the activities of wheat *VRN1* and *VRN2* (Tranquilli and Dubcovsky, 2000; Yan et al., 2003; Yan et al., 2004). Furthermore, the recently-released poplar genome sequences indicated that no *FLC* homolog exists in the poplar genome (Bohlenius et al., 2006; Tuskan and al., 2006). Therefore, the mechanisms controlling juvenility are unlikely to have co-evolved with vernalization requirement, unless they have a very different molecular basis in different genera and families.

In vernalization requiring species, exposure to prolonged cold suppresses the expression of floral repressors, such as *FLC* in Arabidopsis and *VRN2* in wheat, and in turn activates the expression of floral promoters, consequently promoting flowering. However, although *AaFLC* is an important factor mediating vernalization requirement as demonstrated by RNAi and ectopic expression in Arabidopsis (Figure 12 and 15), suppression of its expression during vernalization of juvenile plants was not sufficient to induce flowering (Figure 2B and 2F). An interesting question arising now is how flowering of juvenile plants is repressed even after vernalization.

3.13.3. Repression of floral transition occurs at a relatively late step in juvenile plants

In Arabidopsis, *FT* is a target of *FLC*, which represses *FT* expression by directly binding to a region of its first intron (Searle et al., 2006). Suppression of *FLC* releases this repression, and the resulting high levels of FT promote floral transition

(Abe et al., 2005; Wigge et al., 2005). In *AaFLC* dsRNAi lines of *Arabis alpina* plants, increases in *AaFT* mRNA levels and decreases of *AaFLC* mRNA are not perfectly correlated, but the significant elevation of *AaFT* expression in all *AaFLC* dsRNAi lines still suggests that *AaFT* expression is regulated by *AaFLC* directly or indirectly (Figure 16 and 23B). *PtFT1* and *PdFT2*, two *FT* homologs in poplar, were proposed to play a role in juvenility based on the observation that they were expressed at higher levels in adult plants than in juvenile ones and that ectopic expression of either caused precocious flowering in poplar (Bohlenius et al., 2006; Hsu et al., 2006). Similarly, before vernalization, *AaFT* expression levels in leaves of adult *Arabis alpina* plants were 2-3 fold higher than those in juvenile plants (Figure 19A). However, involvement of *AaFT* in juvenility in vernalization response was not demonstrated by its expression pattern during vernalization. In leaves of both juvenile and adult *Arabis alpina Pajares* plants, *AaFT* was only expressed at background levels throughout vernalization treatment (Figure 19A). The rapid decrease of *AaFT* expression in leaves upon vernalization does not indicate that vernalization repressed *AaFT* expression, rather it was more likely caused by the short-day conditions used for vernalization (Sung et al., 2006a). Even in short days at normal growth temperatures, *AaFT* was only expressed at background levels (Figure 18A). Similarly, in apices of both adult and juvenile plants, *AaFT* transcript levels did not rise above background levels in the first 5 weeks of vernalization. However, floral transition must be initiated in adult plants at this stage because if adult plants were moved to normal growth temperatures immediately after a 5-week cold treatment, transitional leaves (TLs) and reproductive leaves (RLs), and in some plants even floral buds were visible several weeks later (Figure 19C and 19D). In contrast, when juvenile *Arabis alpina Pajares* plants were moved to normal growth temperature after a 5-week vernalization treatment, no phenotype related to the floral transition was ever observed even after being grown for months at normal growth temperatures (data not shown). The levels of *AaFT* mRNA in apices of neither adult nor juvenile plants which had been vernalized for 5 weeks were higher than background levels (Figure 19B), suggesting that at the stage when floral transition started in adult plants, *AaFT* mRNA expression had not increased. 8 weeks after vernalization treatment started *AaFT* mRNA levels in apices of adult plants were dramatically higher than those in

juvenile plants, but at this stage the apex of vernalized adult plants already shows morphological changes associated with the floral transition (data not shown). The increase in *AaFT* expression in apices of adult plants in these late stages of vernalization implies that *AaFT* may be upregulated during the early stages of development of the inflorescence.

In Arabidopsis, vernalization elevates *SOC1* expression by decreasing FLC levels (Searle et al., 2006). As argued in Chapter 3.14.1, the regulatory relationship between *AaFLC* and *AaSOC1* is conserved in *Arabis alpina Pajares*, similar to that between *FLC* and *SOC1* in Arabidopsis. Interestingly, although before vernalization *AaSOC1* transcript levels in adult plants were 2.5-3 fold higher than those in juvenile plants, the expression in both cases increased in response to vernalization and finally to similar levels (Figure 22A and 22B). These results indicate that upon vernalization treatment *AaSOC1* expression is up-regulated in juvenile plants as efficiently as in adult plants, very possibly through removing repression of AaFLC. In Arabidopsis, *SOC1* acts as a potent floral activator and elevated *SOC1* expression promotes floral transition possibly through activating the expression of the floral meristem identity gene *LFY* (Lee et al., 2000; Mouradov et al., 2002; Jack, 2004). Consistent with the increase in *AaSOC1* transcript levels and its role as a floral activator, floral transition indeed occurred in adult *Arabis alpina Pajares* plants at the end of vernalization treatment (Figure 4I). However, flowering has never been observed in juvenile plants during or after vernalization, suggesting that in juvenile plants a downstream repression event prevents AaSOC1 from activating the expression of floral meristem identity genes.

3.13.4. *AaTFL1* expression pattern differs between *Arabis alpina* juvenile and adult plants

In Arabidopsis, the floral repressor TFL1 can prolong all the developmental phases including vegetative, early inflorescence and late inflorescence phases (Ratcliffe et al., 1998; Ratcliffe et al., 1999). In part these effects are caused by TFL1 repressing both the upregulation of the transcripts and the activities of floral meristem identity genes *LFY* and *AP1* (Weigel et al., 1992; Bowman et al., 1993; Gustafson-Brown et

al., 1994; Bradley et al., 1997), but the detailed mechanism by which this occurs remains elusive. Nevertheless, *AaTFL1*, an ortholog of Arabidopsis *TFL1*, was a candidate for repressing the floral transition in juvenile *Arabis alpina* plants even when *AaSOC1* was expressed at high levels, as described above. In contrast to the weak expression of *TFL1* in the apex of vegetative Arabidopsis plants (Bradley et al., 1997; Ratcliffe et al., 1998), *AaTFL1* is strongly expressed in the inner cells of the whole apex of vegetative *Arabis alpina* plants (Figure 25A and 25E). Moreover, expression of *AaTFL1* from either a constitutive promoter or its own promoter dramatically delayed flowering in Arabidopsis (Figure 24). These data suggest that the high levels of *AaTFL1* mRNA present in the apex of *Arabis alpina* plants may contribute to regulating the transition of the apex from vegetative to reproductive phase, and that this could have a role in juvenility.

In the early stages of vernalization treatment, up to 3 weeks after the start of the treatment, in apices of both juvenile and adult *Arabis alpina Pajares* plants, *AaTFL1* expression levels significantly increased and the region of expression also appeared to extend (Figure 25B and 25F). Interestingly, this increase of *AaTFL1* expression and extension of its expression region during the early stages of vernalization coincides with the observation that cold treatments shorter than 3 weeks could not cause any morphological change linked to the floral transition in either adult or juvenile plants (data not shown). This correlation implies that in *Arabis alpina Pajares*, AaTFL1 might be involved in preventing premature vernalization in response to brief cold spells, as proposed for MAF2 plays in Arabidopsis (Ratcliffe et al., 2003). Five weeks after the cold treatment started was the earliest time during vernalization when restriction in *AaTFL1* expression in adult plants was observed, and this restriction coincided with the observation that a 5 week vernalization treatment could promote floral transition in adult plants (Figure 19C, 19D, 25C and 25G). Furthermore, in adult *Arabis alpina Pajares* plants, the restriction of *AaTFL1* mRNA to the center of the meristem dome was followed by the development of floral meristems on the flancks of the meristem (Figure 25D). In contrast to what was observed in adult plants, the restriction of *AaTFL1* expression regions have never been observed in juvenile plants even when the cold treatment was extended for a very long time (Figure 25F-25H). Coincidently, even an extremely long period of cold treatment (e.g. 1 year) did not

induce juvenile *Arabis alpina* plants to flower (Figure 2B). The correlation between the differential changes in *AaTFL1* expression patterns in juvenile and adult plants during vernalization and the diverse effects of vernalization on promoting flowering in juvenile and adult plants suggests a role for regulation of AaTFL1 expression in juvenility. During vernalization, in adult *Arabis alpina Pajares* plants, *AaTFL1* expression in regions other than the inner cells of the sub-dome domain of the SAM and axillary meristems is suppressed, allowing the initiation of floral meristem on the flanks of the SAM, where *AaTFL1* expression is low or absent. In contrast, in juvenile plants, since *AaTFL1* mRNA levels remain high in the inner cells of the whole SAM throughout vernalization, activity of floral promoters like AaSOC1 is suppressed and floral meristems can not be initiated on the flanks of the SAM. The mechanism of restricting *AaTFL1* expression in adult *Arabis alpina Pajares* plants during vernalization may be mediated through up-regulation of unknown genes by vernalization, and the products of these genes in turn repress *AaTFL1* expression. Alternatively, repression of unknown genes by vernalization could restrict *AaTFL1* expression if their products are necessary for maintaining *AaTFL1* expression at high levels in some regions of the shoot apex. Since this up-regulation of a suppressor or suppression of an activator of *AaTFL1* expression during vernalization only occurs when *Arabis alpina* plants reach a certain developmental stage, floral transition can not proceed in juvenile plants which have not reached the appropriate developmental stage.

In *AaFLC* dsRNAi lines, although in most of them (except L4) both *AaSOC1* and *AaFT* were strongly up-regulated, complete floral transition only occurred in one line L3 in which *AaSOC1* mRNA level increased over 10 fold. Furthermore even L3 flowered very late, after more than 3-month growth in LDs. These data indicate that in these *AaFLC* dsRNAi lines a strong floral repression function antagonizes the activity of floral promoters such as *AaSOC1* and *AaFT*. One possible candidate floral repressor is *AaTFL1*. Possibly, during development of *Arabis alpina Pajares* plants that are grown at normal temperatures, a gradual decrease in *AaTFL1* expression (Figure 25A and 25E) increases the competence of the SAM to respond to floral induction. However, floral transition does not occur in wild type *Arabis alpina Pajares* plants as other floral repressors such as AaFLC are also present. In some strong *AaFLC* dsRNAi lines (e.g. L3), since *AaFLC* expression levels are

dramatically decreased, expression of floral activator genes such as *AaSOC1* and *AaFT* is released. Consequently, high levels of floral activator promoted flowering in these lines when the plants became older and *AaTFL1* mRNA is decreased to a lower level. Furthermore, once floral transition has proceeded to a later step, the resulting activation of some factors (e.g. the counterparts of *LFY* and *AP1*) might in turn further repress the expression of *AaTFL1* in some regions of the SAM to reinforce the floral transition. As speculated in some *AaFLC* dsRNAi lines, a similar mechanism may underlie the suppression of *AaTFL1* in *Arabis alpina Bonn* accessions during the floral transition. In all cases, after floral transition, the expression patterns of *AaTFL1* in *Arabis alpina* (Figure 25C, 25D, 31C and 31D) were reminiscent of those of *TFL1* in the inflorescence of Arabidopsis. This observation implies that at this stage the interactions between *AaTFL1* and other genes, possibly including counterparts of *LFY* and *AP1*, are the same as those between *TFL1*, *LFY* and *AP1* in Arabidopsis (Bradley et al., 1997). In this case in the dome of the SAM of *Arabis alpina AaTFL1* expression would be expected to repress the up-regulation and activities of *AaLFY* and *AaAP1* genes to maintain the vegetative identity of this region, whereas in the flanking regions, AaLFY and AaAP1 would repress *AaTFL1* expression to establish floral meristem identities.

Therefore, the functional divergence of *AaTFL1* and *TFL1* in floral repression may be caused by the difference in the regulation of their expression patterns in vegetative shoot apices. Furthermore, the strong floral repression effect of AaTFL1, caused by its expression in more extensive regions of the shoot apex at higher levels, may make it an important factor controlling juvenility. In fact, several reports have described that variations in function between functional homologs arose from the divergence in their expression patterns. Among *TFL1* functional homologs, the amino acid sequences (especially those amino acid residues which are key to their activity as a floral repressors) are highly conserved (Hanzawa et al., 2005; Ahn JH, 2006). However, there are notable differences in the function of these homologs. Some of these differences have been suggested to result from divergence in their expression patterns. In Arabidopsis, *TFL1* is expressed in both vegetative and inflorescence phases, although in vegetative shoot apices its expression levels are very low and it acts to both prolong vegetative phase and maintain indeterminacy of the inflorescence apical meristem (Bradley et al., 1997).

In contrast, *CENTRORADIALIS* (*CEN*), the ortholog of *TFL1* in *Antirrhinum*, is only expressed in inflorescence meristems, and loss-of-function mutants only altered the determinacy of the meristem but not flowering time (Bradley et al., 1996). However, constitutive expression of CEN from the CaMV35S promoter in tobacco plants caused severe late flowering (Amaya et al., 1999). Moreover, two *TFL1* homologs were cloned from pea: *DETERMINATE* (*DET*) which exclusively controls inflorescence determination, and *LATE FLOWERING* (*LF*), which acts only to delay flowering time (Foucher et al., 2003). Although detailed analyses of their spatial expression patterns have not been described, RT-PCR result showed that *DET* is only expressed in inflorescence apices, while *LF* is expressed in both vegetative and inflorescence apices (Foucher et al., 2003). Another good example illustrating the importance of differential regulation of expression pattern was described by Hay and Tsiantis (2006). They showed that the divergent roles of *Knotted-like homeobox* (*KNOX*) genes from *Cardamine hirsuta* and their *Arabidopsis* homologs in controlling leaf shapes are due to differences in their expression patterns and that the specific expression patterns of *KNOX* genes is mediated by regulatory modules in its promoter (Hay and Tsiantis, 2006).

In summary, through analysis of the function of *AaTFL1*, we demonstrated that AaTFL1 is a potent floral repressor. The observations that during vernalization treatment, the maintenance of extensive expression of *AaTFL1* in vernalized juvenile plants correlated with insensitivity to floral transition and that restricted expression patterns correlated with floral transition in adult plants suggest that regulation of *AaTFL1* expression plays a role in controlling juvenility in *Arabis alpina* plants. Formally we can not completely exclude the possibility that the restriction of *AaTFL1* expression pattern in adult plants during vernalization was an effect rather than a cause of the floral transition. However, this possibility may be less likely because in strong *AaFLC* dsRNAi lines, where floral promoters including *AaFT* and *AaSOC1* are highly expressed, flowering still occurred very late, and an *AaTFL1* genomic fragment, including the promoter, the coding region and the terminator, not only complemented the flowering defects of Arabidopsis *tfl1* mutant, but also caused the mutant plants to flower dramatically later than wild type Arabidopsis control plants.

4. Roles of flowering time genes in polycarpic traits and seasonal flowering in *Arabis alpina Pajares*

4.1. Introduction

In monocarpic annual species such as *Arabidopsis thaliana*, floral transition occurs in all apical meristems and the whole plant undergoes senescence after seed set. However, a reproductively mature polycarpic perennial plant does not initiate flowers in all apical meristems. To study the molecular mechanisms underlying this perennial-specific characteristic, it is first necessary to describe which meristems flower and which do not under defined conditions. This analysis of the connection between the differential identities and the diversity of developmental and environmental factors that the meristems are exposed to may give an indication of the underlying mechanisms controlling the fate of individual meristems.

One example in which the perennial flowering behavior of individual apical meristems has been well characterized is the woody perennial poplar (Yuceer et al., 2003). During floral transition in spring , a mature branch in its second growing season can be subdivided acropetally into three zones: the vegetative zone I (VZI) bearing leaves preformed during the last growing season before dormancy, the floral zone (FZ) bearing leaves initiated at the end of the last growing season or maybe produced during late autumn and winter, and the forming vegetative zone II (VZII), initiation of which has started from the beginning of the second growing season through the activity of the vegetative terminal apical meristem of the branch. The axillary buds that were initiated in the first growing season at axils of the preformed leaves in the VZI and those that have been initiated or will be initiated at axils of leaves in the VZII will remain vegetative throughout the second growing season, whereas the buds produced in the FZ will undergo floral transition at the end of the spring of the second growing season. The floral buds (those in FZ) will flush in the early spring of the third growing season. The terminal meristem of the branch will stay vegetative throughout the whole developmental process (Yuceer et al., 2003).

Recently, the mechanism underlying the differential fates of axillary meristems

produced along a mature branch of poplar has been proposed to be due to the difference in *PdFT2* expression observed in these axillary buds during floral transition (Hsu et al., 2006). *PdFT2* mRNA levels were dramatically upregulated in buds from the FZ of mature branches from both male and female trees during floral transition, whereas the expression in buds from the VZI was upregulated less dramatically in both types of trees, and also later in the case of male trees. However, the expression of *PnFT2* increased strongly in leaves from both the FZ and the VZI. In both Arabidopsis and rice, FT protein and its rice ortholog Hd3a have already been demonstrated to be a component of the mobile signal florigen that has been shown to have a universal nature (Corbesier et al., 2007; Tamaki et al., 2007). Furthermore, the relationship between poplar and Arabidopsis is evolutionarily much closer than that between Arabidopsis and rice (Jansson and Douglas, 2007). Therefore, PdFT2, as an ortholog of Arabidopsis FT, is likely also to act as a mobile signal that induces flowering through translocation from a leaf to the apical meristem of the bud at the leaf axil. In this case, it would be difficult to explain that during floral transition *PdFT2* expression increased in a similar pattern in leaves in both the VZI and the FZ whereas only the buds formed at the axils of leaves in the FZ but not those in the VZI flowered. One possibility is that these axillary buds differed in their response to the PdFT2 signal.

In *Arabis alpina*, we have already described the differential floral competence in different apical meristems (Chapter 2.3). To elucidate the mechanisms underlying variation in the competence of meristems to flower, it would be helpful to test the expression of important flowering-time genes in different apical meristems during floral induction. Especially, comparing the expression patterns of flowering-time genes in meristems of different types of side branches with those in Arabidopsis would help to understand the relationship between flowering-time control and life history. Comparing the possible mechanism controlling the fates of different branches in *Arabis alpina* with those in other polycarpic perennials such as poplar could lead to a better understanding of the conservation and diversity of perenniality in different species.

Polycarpic perennial plants show seasonality in their growth and development in response to the seasonal changes of environmental factors, especially day length and temperature (Jansson and Douglas, 2007). *Arabis alpina Pajares* flowers

annually in early spring. However, our preliminary work indicates that after each flowering, if the plants are not exposed again to a prolonged cold treatment they will retain vegetative growth. Therefore, a vernalization requirement is very important to maintain seasonal flowering in *Arabis alpina Pajares*.
In this chapter, the relationship between expression of flowering time genes and differential competence of meristems to flower will be introduced. Moreover, the mechanisms underlying seasonal flowering in *Arabis alpina* will be analyzed.

4.2. *AaTFL1* expression patterns differ in shoots showing different flowering behaviors

As described in Section 3.8, at the end of vernalization *AaSOC1* mRNA was highly expressed in both the SAM and type II branches, but only the SAM underwent the floral transition. This result, together with the observation that *AaTFL1*, which codes for a potent floral repressor, was highly expressed in axillary meristems, led us to consider the role of *AaTFL1* in polycarpy of *Arabis alpina*. To answer this question, firstly *AaTFL1* expression patterns in different types of side branches were tested with *in situ* hybridization. During growth at normal temperatures, axillary branches were produced continuously from axils of leaves along the main shoot. *AaTFL1* was highly expressed in the axillary meristems from the beginning when they detached from the SAM (Figure 25A). During the early stages of development of the axillary meristems, *AaTFL1* maintained high expression in the whole meristem and later in the axillary shoot primordia and young shoots (Figure 26A and 26B). When axillary shoots grew bigger, *AaTFL1* expression was restricted to regions which were similar to those in the main shoot before vernalization (Figure 26C and Figure 25A). At the end of a 12-week vernalization treatment, *AaTFL1* was expressed in different patterns in different types of axillary branches. The expression patterns in the apices of type I and type IV branches were similar to that in the main shoot apex (Figure 26H, 26G and Figure 25D), being restricted to the sub-dome domain and floral meristems were produced from the flanking regions. Interestingly, *AaTFL1* was expressed all over vegetative type II branches (Figure 26E). Whereas in the transitional type III branches *AaTFL1*

expression pattern was between those observed in type II and type IV branches (Figure 26F). After vernalization, in type II branches, with the branches growing bigger, AaTFL1 expression in stems gradually disappeared but was retained in the meristem similar to the main shoot before vernalization, and the branches maintained vegetative growth (Figure 26H). In type III branches, the residual expression of *AaTFL1* in stems gradually faded and finally *AaTFL1* was only expressed in the sub-dome domain of the apices (Figure 26I).

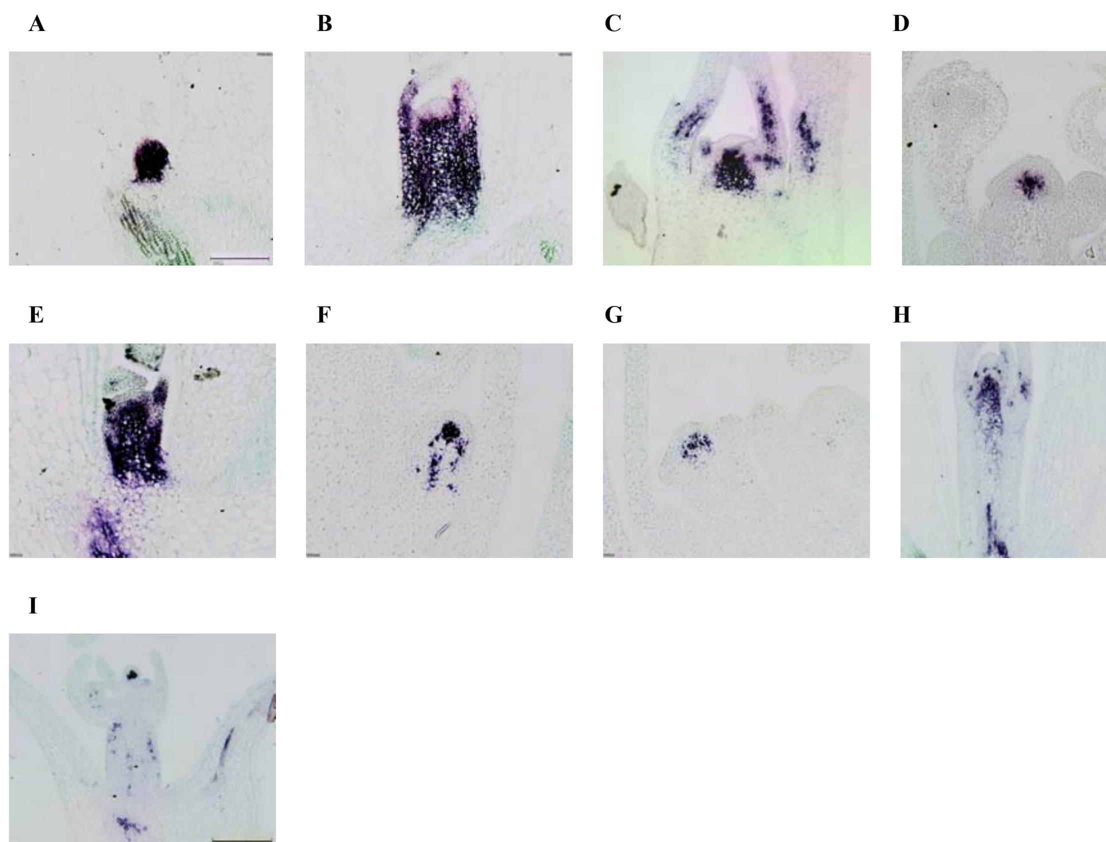


Figure 26. *AaTFL1* expression in different types of axillary shoots of *Arabis alpina Pajares* plants.
(A-C) *AaTFL1* expression in type I axillary branches at different developmental stages before vernalization. **(D-G)** *AaTFL1* expression in different types of axillary branches at the end of the vernalization treatment: **(D)**, type I; **(E)**, type II; **(F)**, type III; **(G)**, type IV. **(H and I)** *AaTFL1* expression in axillary branches 1 week after vernalization. **(H)**, type II. **(I)**, type III. Magnifications in **(A-G)** are the same. Bar, 100µm. Magnifications in **(H and I)** are the same. Bar, 200µm.

4.3. *AaSOC1* expression decreased after vernalization

As described in Section 2.3, after vernalization treatment flowering will occur at the apical meristems of the main shoot, the type I and the type IV axillary shoots and also possibly the type III axillary shoots while vegetative shoots form from type II axillary shoots, the secondary meristems at the axils of leaves on type II branches, as well as the vegetative part of type I, and, in some cases, type III branches. Flowering does not occur in these vegetative shoots until the plants are vernalized again after the shoots grow out. In these newly grown-out vegetative branches, before being vernalized again, it was not known whether expression of a strong floral promoter such as *AaSOC1* that was upregulated in the previous vernalization treatment is maintained or not. To resolve this issue, *AaSOC1* expression in apices of type II axillary branches was checked after vernalization.

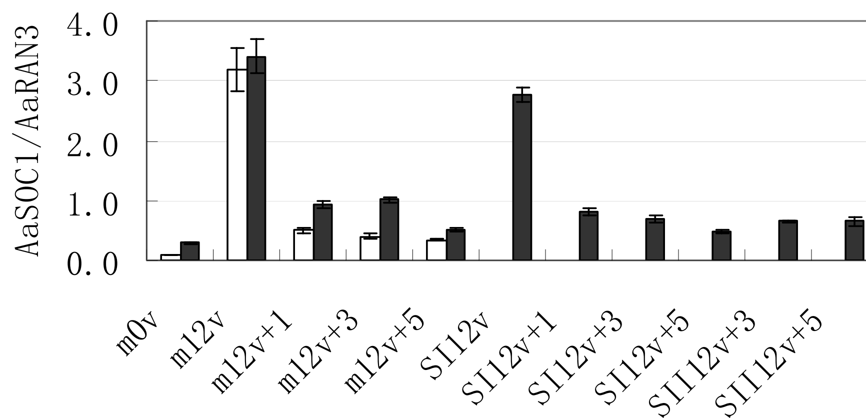


Figure 27. *AaSOC1* mRNA levels in main shoot and axillary branch apices of *Arabis alpina Pajares* plants in response to vernalization.
Plants were grown under LDs until 2 weeks (0v, open bars) or 8 weeks old (0v, close bars) followed by a 12 week vernalization treatment (12v). After the vernalization treatment plants were put back to LDs and apices of main shoot (m) and axillary shoots (SI, type I; SII, type II) were harvested 1 week (12v+1), 3 weeks (12v+3) and 5 weeks (12v+5) after the vernalization treatment.

As shown in Figure 27, at the end of vernalization treatment, *AaSOC1* was expressed at similarly high levels in the apices of type I branches as in main shoot apices of juvenile and adult plants. After vernalization, *AaSOC1* mRNA expression levels in apices of all these shoots decreased quickly and finally to much lower

levels. The expression levels of *AaSOC1* mRNA in the vegetative type II branches after the vernalization treatment was similarly low as in type I branches. This result suggested that *AaSOC1* expression in type II branches decreased rapidly once the cold treatment was removed, although it had already been upregulated to very high levels during the treatment (Figure 27 and Figure 22E).

4.4. The repression of *AaFLC* expression by vernalization was unstable after vernalization

In the majority of *Arabidopsis thaliana* accessions that require vernalization, once *AtFLC* expression was repressed by vernalization, the repression is maintained when plants are returned to normal temperatures (Sung and Amasino, 2005). The expression patterns of *AaSOC1* in *Arabis alpina* suggested that after vernalization the observed decrease of *AaSOC1* mRNA levels might have resulted from increased AaFLC expression, so that the effect of vernalization on *AaFLC* expression was unstable. To answer this question, total RNA were extracted from the same materials used to test *AaSOC1* expression in Section 4.3 and *AaFLC* expression levels were tested with quantitative real-time PCR. Indeed, *AaFLC* mRNA expression in apices of primary shoots of juvenile and adult plants increased gradually after vernalization treatment (Figure 28A). Five weeks after the treatment, *AaFLC* expression in the apices of juvenile plants were already restored to the original levels as in apices of adult plants before vernalization, but in primary apices of adult plants, which were flowering, the restoration was not so complete. The incomplete restoration of *AaFLC* mRNA expression in the primary apices of adult plants might be caused by the difference in tissues constituting apices before and 5 weeks after vernalization. *AaFLC* expression levels in apices of type I axillary branches were similar to that in the primary apices both at the end and after vernalization. Three weeks after vernalization *AaFLC* expression in apices of type II branches had already reached levels similar to the original ones in the primary apices before vernalization (Figure 28A). After vernalization, *AaFLC* expression was also tested in the primary leaves which were produced before the vernalization treatment and had already expanded at the time when the treatment started. Similar

to the situations in the shoot apices, *AaFLC* expression in leaves was also restored after vernalization treatment (Figure 28B).

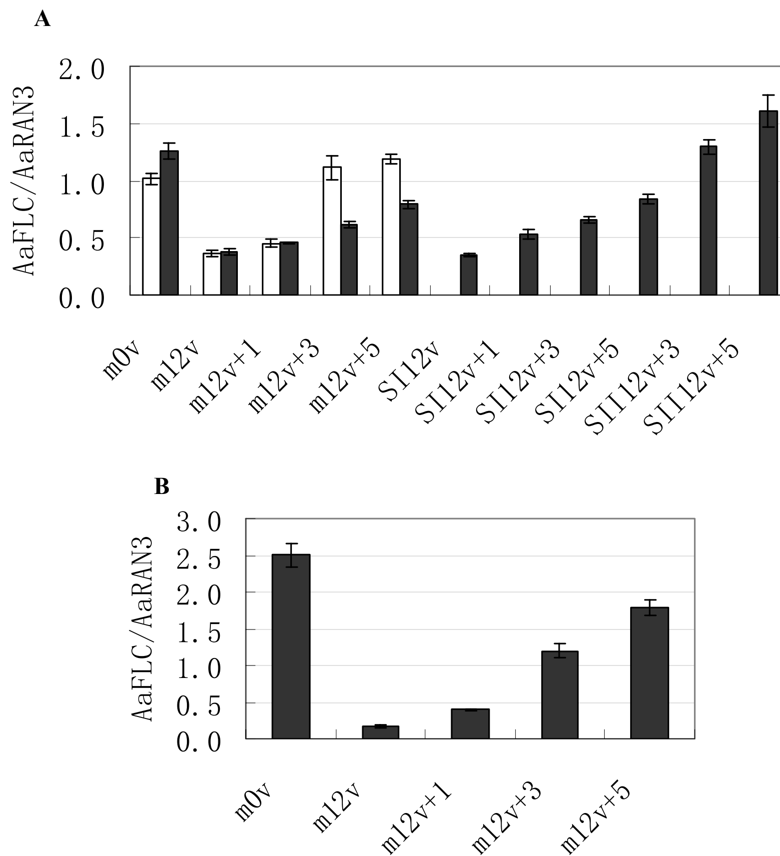


Figure 28. *AaFLC* expression in apices and leaves of *Arabis alpina Pajares* plants in response to vernalization.
(A) *AaFLC* expression in apices of the main shoots (m) of juvenile (open bars) and adult (close bars) plants and apices of axillary branches (SI, II and III represent type I, II and III branches respectively) of adult plants. **(B)** *AaFLC* expression in leaves which were produced on the primary shoots of adult plants and had already expanded before the vernalization treatment.

4.5. Discussion

4.5.1. *AaTFL1* may be involved in differential competence of apical meristems to flower in *Arabis alpina*

During vernalization of adult *Arabis alpina* plants increases in the mRNA encoding the potent floral promoter *AaSOC1* correlated with floral transition at the shoot

apical meristem. In the same plant, floral transition occurs in the main shoot apical meristem as well as type I, type IV and also partially in type III branches, whereas type II branches stay vegetative. Detailed analysis of *AaSOC1* expression (Figure 22D and 22E) revealed that *AaSOC1* transcript levels were similarly elevated in the apical meristem of the main shoot and all types of side branches, although being higher in the region where floral meristems were being initiated (Figure 22D and 22E, and data not shown). The inconsistency between lack of floral competence and the presence of high levels of a floral promoter in type II branches suggests the existence of an additional floral repressor that antagonizes the activity of the floral promoter *AaSOC1*. Interestingly, broad expression patterns and high expression levels of *AaTFL1* correlated with the vegetative fate of type II branches. The observation that in apices of the main shoot, the type I, and the type IV branches *AaTFL1* was exclusively expressed in the limited sub-dome domain of the apical meristems, further support the speculation that *AaTFL1* levels and expression patterns are involved in determination of the fate of different meristems within an individual *Arabis alpina* plant. In *Arabis alpina Pajares* plants grown at normal temperatures, axillary meristems arose on the flanks of the vegetative SAM. Moreover, in very early stages of the establishment of the axillary meristems *AaTFL1* expression was detectable (Figure 25A). At the early stages, the *AaTFL1*-expressing regions in the main shoot apex formed a continuous region including the inner cells of the SAM, all the axillary meristems on its flanks and the pro-vasculature tissues (Figure 25A). This extensive expression pattern of *AaTFL1* may reflect a mechanism that coordinately represses floral evocation in all the tissues of the apex. *AaTFL1* expression in the axillary meristems is especially high, which suggests that different mechanisms may exist in axillary meristems to regulate *AaTFL1* expression, thereby maintaining axillary meristems in vegetative phase. During the growth of axillary branches at normal growth temperatures, *AaTFL1* expression retreated from the whole young buds to the inner cells of the whole apex of the axillary shoot (Figure 26A-26C). The reduction in the *AaTFL1* expressing regions may result in an increase of competence to respond to floral induction in these side branches. Axillary branches are continuously produced so that when vernalization treatment started, axillary branches of various sizes had already formed along the stem of an adult *Arabis alpina* plant. In larger branches

(type I), the pattern of expression of *AaTFL1* is reminiscent of that in the main shoot apex, in terms of both the patterns and the levels. Therefore, similar to what was observed in the main shoot apex, during vernalization *AaTFL1* expression in apices of axillary branches became restricted to a very localized sub-dome domain, allowing formation of floral meristems on the flanks of the axillary shoot apical meristems (Figure 26D). In contrast, in small axillary branches, buds and axillary meristems *AaTFL1* was highly expressed everywhere (Figure 26A and 26B), and therefore during vernalization treatment floral transition was blocked in these branches or meristems (the type II branches, Figure 26E). Ater vernalization, along with the growth of these branches, *AaTFL1* expression became restricted gradually to a similar pattern as observed in type I branches and the main shoot apex before vernalization (Figure 26H) and flowering occurred on these branches if they were exposed to vernalization. Type IV branches were initiated after the SAM of the main shoot was vernalized. Therefore, they acquired similar identity as the SAM of the main shoot, and AaTFL1 expression was restricted to a localized sub-dome domain as in the main shoot meristem, possibly by similar mechanisms (Figure 26G). As in the SAM of the main shoot, localized expression of *AaTFL1* in type IV branches did not repress formation of floral meristems on the flanks of the apical meristem, but only maintained indeterminate development of these branches. The axillary meristems of type III branches were being formed when vernalization treatment started and at the time they had not an independent identity. Therefore, the mechanism that maintains *AaTFL1* expression was not completely established in these branches when vernalization treatment started.

Although *AaTFL1* expression patterns correlate with the fate of individual axillary branches, the mechanisms that maintain *AaTFL1* expression in young axillary branches remain unknown. These mechanisms might be similar to or different from those that maintain *AaTFL1* expression in the SAM of juvenile plants. Nevertheless, the observation that the expression of *AaTFL1* at high levels and in extensive regions correlates with both juvenility and incompetence of some axillary meristems to respond to floral induction supports the speculation that juvenility and differential competence to flower in adult meristems of polycarpic perennials may be controlled by similar mechanisms (Brunner and Nilsson, 2004; Tan and Swain, 2006).

However, we can not rule out the possibility that the different expression patterns of *AaTFL1* in different axillary branches are not the cause of their different behaviors but a consequence of their distinct identities acquired by a different mechanism. However, high expression level of *AaTFL1* in type II branches appeared to prevent AaSOC1 in these branches from promoting flowering during vernalization, and this supports the idea that the expression patterns and levels of *AaTFL1* are the cause rather than an effect of different identities of axillary branches. Furthermore, a recent report on the effect of transgenic apple trees expressing antisense *MdTFL1*, a *TFL1* homolog in apple, also supports the idea *TFL1* homologs control juvenility and maintain vegetative identity of some apical meristems in polycarpic perennials (Kotoda et al., 2006). Reduction of *MdTFL1* expression not only caused very early flowering (8 month vs. 6 years in wild type controls) but also resulted in conversion of vegetative axillary meristems (the water sprout in apples) to flowering meristems.

One possible mechanism by which *AaTFL1* represses the floral transition may be through repressing the activation of floral meristem identity genes such as *AaLFY* by AaSOC1. The repression of the floral transition in juvenile *Arabis alpina Pajares* plants and type II branches of adult plants during vernalization could occur by this mechanism. Another possibility is that *AaTFL1* represses activation of floral meristem identity genes by competing with the floral promoter AaFT for interacting factors. In Arabidopsis, *TFL1* and *FT* are homologs, but they have antagonistic functions (Kardailsky et al., 1999; Kobayashi et al., 1999). FT promotes AP1 expression through interacting with FD. Whether TFL1 interacts with FD is controversial and conflicting results were reported (Abe et al., 2005; Wigge et al., 2005), but both TFL1 and FT interact with SPGB, a tomato FD homolog (Pnueli et al., 2001). Moreover, TFL1 represses *AP1* upregulation whereas FT activates *AP1* expression. TFL1 and FT have similar structures (Ahn et al., 2006). All these data suggest that TFL1 and FT may interact with and compete for a common factor such as FD. Considering the conservation of both sequence and function between AaTFL1 and AaFT and their Arabidopsis counterparts, a similar mechanism may exist to mediate the antagonistic functions of AaTFL1 and AaFT. If such a mechanism does indeed exist, it might be important in repression of the floral transition in juvenile *Arabis alpina Bonn* plants in which AaFT is expressed at high

levels (Figure 30C). Recently, FT and Hd3a were reported to be components of the transmissible floral signal florigen in Arabidopsis and rice respectively (Corbesier et al., 2007; Tamaki et al., 2007). Therefore, AaFT or an unidentified homolog may also be a component of a mobile signal in *Arabis alpina*. In addition to antagonizing AaFT activity in promoting expression of target genes, possibly high levels of AaTFL1 protein present in the pro-vasculature might block AaFT movement to the SAM through competitive binding to a protein that is essential for AaFT transmission.

Furthermore, the role of *TFL1* homologs in controlling competence of different meristems to respond to floral induction may be conserved among polycarpic perennials. In poplar, PdFT2 was highly expressed in leaves in both VZI and FZ along a mature branch, but in the second growing season only shoot buds produced at the axils of leaves in the FZ undergo the floral transition while those at the axils of leaves in the VZI maintain vegetative. Local structures were proposed to contribute to the transportation of floral signals such as PdFT2 mRNA from leaves to individual buds at their axils, since it was also described that the PdFT2 mRNA levels in buds formed in FZ were much higher than those in VZI during floral transition (Hsu et al., 2006). However, another possibility is that the fate of shoot buds (or axillary meristems) is already decided originally at the end of the first growing season or during the winter. Possibly, the developmental stage of individual axillary meristems at the onset of winter is a critical factor that determines whether a meristem is competent to undergo floral transition next spring when PdFT2 protein levels are high in leaves of the whole plant. Differences in the developmental stage of individual axillary meristems at the time when winter starts may cause the difference in abundance and pattern of the expression of a *TFL1* homolog in the axillary meristem. In the buds formed in VZI, a putative poplar *TFL1* homolog may be expressed at high levels and in broad regions, and therefore antagonize the floral inductive effect of some floral promoters such as PdFT2 in the spring of the second growing season.

4.5.2. Seasonal flowering is maintained in *Arabis alpina Pajares* by resetting expression of flowering-time genes after flowering

In addition to timing the first flowering, polycarpic perennials must also develop mechanisms to decide when to flower each year. In both annual and perennial plants during the floral transition, floral promoter genes are activated while floral repressor genes are suppressed. In perennials, there must be some mechanism to reset the vegetative state after each flowering so that the plants can return to vegetative growth and flower in the same season the following year. In temperate regions, photoperiod and temperature are the most important environmental factors that exhibit stable annual fluctuation. Therefore, to maintain seasonal flowering behavior, perennial plants must synchronise their development with these factors. The photoperiodic inductive signal output is transient in well studied annual plants, and will disappear when the inductive photoperiod is removed. A similar mechanism may exist in perennial species such as *Arabis alpina* considering that *AaFT* is only expressed at high levels under LD conditions (Figure 18A). However, in Arabidopsis, repression of *FLC*, the major gene mediating vernalization requirement in Arabidopsis, is mitotically stable and this repression can only be reset via sexual reproduction. Perennial plants such as *Arabis alpina Pajares*, which has a vernalization requirement, must develop a mechanism to reset this requirement each year. In *Arabis alpina Pajares*, *AaFLC* expression decreased during vernalization while *AaSOC1* transcript levels increased (Figure 27 and 28). However, once the plants were moved back to normal growth temperatures, *AaFLC* expression was restored rapidly and correspondingly *AaSOC1* levels decreased (Figure 27 and 28). In Arabidopsis, this unstable repression of *FLC* was only observed in vernalization defective mutants such as *vrn2* (Gendall et al., 2001). Consistent with the unstable repression of *AaFLC* after vernalization, flowering *Arabis alpina Pajares* plants did not flower again until they were again exposed to prolonged cold. Therefore, our data suggest that the mitotically unstable repression of *AaFLC* and activation of *AaSOC1* expression make an important contribution to maintaining the spring flowering behavior of *Arabis alpina Pajares* plants. In agreement with their role in mediating seasonal flowering, in strong *AaFLC* RNAi lines (L3), where *AaFLC* is suppressed and *AaSOC1* is expressed at high levels,

floral transition continuously occurs in apical meristems without vernalization.
Since the vernalization requirement mediated by *FLC* homologs is not conserved throughout higher plants, the seasonal flowering behavior mediated by unstable repression of *FLC* homologs (such as *AaFLC* in *Arabis alpina Pajares*) can not be common for all polycarpic perennial plant species. However, similar mechanisms in which other floral repressors are unstably repressed by vernalization may underlie seasonal flowering of polycarpic perennial plants.

5. The correlation between expression of flowering-time genes and natural genetic variation in vernalization requirement

5.1. Introduction

Extensive naturally occuring genetic variation affecting development occurs within many plant species, and possibly reflects adaptive fitness to different natural environments during evolution. Furthermore, the genetic variation within species may also be the origin of plant species differentiation (Linhart and Grant, 1996). Timing of the floral transition is an important adaptive trait because aligning reproduction with favorable environmental conditions is crucial for ensuring reproductive success. Photoperiod and temperature are among the most important environmental factors and vary within a year and also between different geographic locations. Genetic and molecular studies with the model species Arabidopsis have revealed a complex network that integrates the environmental cues to control the developmental transition to flowering (Mouradov et al., 2002; Boss et al., 2004).
As introduced above, Arabidopsis is a facultative LDP that includes winter annual and summer annual accessions. Winter annuals have a requirement for a long cold winter to accelerate flowering, whereas summer annuals do not. Through analysis of 8 crosses derived between different Arabidopsis accessions, at least 14 quantitative trait loci (QTL) that contribute to naturally occurring flowering variation have been identified (Koornneef et al., 2004; Alonso-Blanco et al., 2005). So far, six of these QTLs have been cloned: *FRI* and *FLC* involved in vernalization requirement (Sung and Amasino, 2005); *CRY2*, *FLOWERING LOCUS M* (*FLM*, also as *MAF1* or *FLW1*), and *PHYTOCHROME D* (*PHYD*) involved in photoperiod response (Imaizumi and Kay, 2006); the *ENHANCER OF ELF4* (*EEL*, also as *HUA2*) affecting both responses (Alonso-Blanco et al., 2005).
Analysis of accessions with different vernalization requirement has suggested that summer annual Arabidopsis accessions evolved from winter annuals through multiple independent loss-of-function mutations in *FRI* and/or *FLC* (Le Corre et al., 2002; Gazzani et al., 2003; Michaels et al., 2003a; Liu et al., 2004; Stinchcombe et

al., 2004; Shindo et al., 2005). These mutations in either *FRI* or *FLC* or both result in decrease in *FLC* expression, converting a late flowering winter annual accession to a fast cycling summer annual type. Therefore, *FRI* and *FLC* appear to be major targets for natural selection. Mutations in *FRI* are often deletions and reside both in the promoter and the coding sequence, whereas mutations in *FLC* are often caused by natural insertions of transposable elements in the first intron.

FLM encodes a floral repressor that was suggested to be involved in the photoperiod pathway. Recently, a natural deletion removing the complete transcribed region was shown to be responsible for the early flowering of the Niederzenz (Nd) accession (Werner et al., 2005). A 14-bp deletion in the coding sequence of *PHYD* was found in WS accession and this deletion resulted in early flowering (Aukerman et al., 1997). In contrast to *PHYD*, *CRY2* functions as a floral activator. A strong gain-of-function allele of *CRY2* was identified to be caused by a single amino acid substitution (El-Assal et al., 2001). *HUA2* acts as a floral repressor and encodes a protein involved in RNA processing of several MADS box genes including *FLC* and *FLM*. Therefore, mutations in *HUA2* influence flowering response to both vernalization and photoperiod (Doyle et al., 2005). A natural loss-of-function mutation that causes early flowering was identified in Landsberg *erecta* (Ler) (Doyle et al., 2005).

In other species, naturally occurring genetic variation has also been described in flowering-time genes. For example, in rice, a set of QTLs, including *Hd1* and *Hd3a* that affect photoperiod response, were mapped (Yano et al., 1997; Yano et al., 2000). In addition, naturally occurring variation in *VRN1* and *VRN2* was shown to mediate the major difference for vernalization response in winter and spring wheat varieties (see Chapter 1). In this chapter, the expression patterns of flowering time genes will be compared in *Arabis alpina Pajares* and *Arabis alpina Bonn* accessions, which have distinct vernalization requirement to flower.

5.2. In *Arabis alpina Bonn* and *Arabis alpina Pajares*, *AaFLC* mRNA levels were similar

To check whether like *AtFLC* in Arabidopsis differentiated *AaFLC* expression

levels in *Arabis alpina* also contribute to differences in flowering time and vernalization requirement for floral transition in *Arabis alpina Bonn* and *Arabis alpina Pajares* accessions, *AaFLC* mRNA levels were tested in apices and leaves of plants from each accession. Surprisingly, *AaFLC* mRNA levels in plants of both accessions were similar (Figure 29). To find out whether the early flowering in the Bonn accession was caused by mutations in *AaFLC*, *AaFLC* coding sequences (CDS) from the Pajares and the Bonn accessions were compared and it was found that the CDS of *AaFLC* from both accessions were identical. These data suggested that the difference in vernalization requirement for flowering in these two accessions was not caused by differences in *AaFLC* transcript levels.

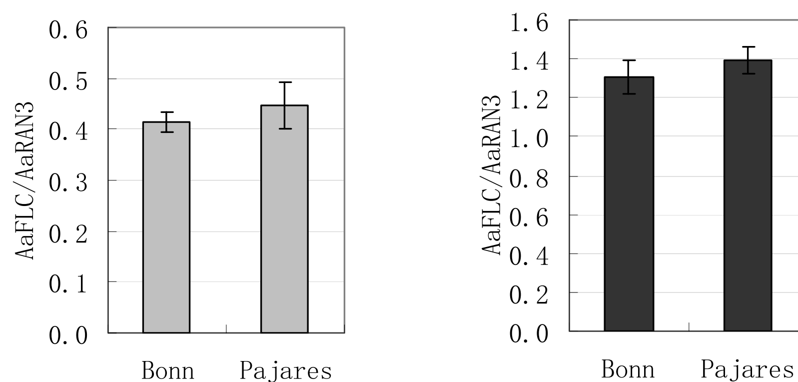


Figure 29. *AaFLC* expression in apices and leaves of *Arabis alpina Pajares* and *Arabis alpina Bonn* accessions.
(A) *AaFLC* expression in apices of primary shoots. **(B)** *AaFLC* expression in expanded leaves on primary shoots. Plants were grown under LDs and materials were harvested 4 weeks after germination.

5.3. Both *AaFT* and *AaSOC1* were expressed at higher levels in the *Arabis alpina Bonn* plants than in the *Arabis alpina Pajares* plants

Since in Arabidopsis, *AtSOC1*and *AtFT* are two direct targets of AtFLC, and low *AtFLC* levels correlates with high *AtSOC1* and *AtFT* levels, expression levels of *AaSOC1* and *AaFT* were compared in Bonn and Pajares. *AaSOC1* and *AaFT* mRNAs were expressed at dramatically higher levels in Bonn than in Pajares

(Figure 30). The expression of these genes therefore correlated with the early flowering phenotype of Bonn and the non-flowering phenotype of Pajares.

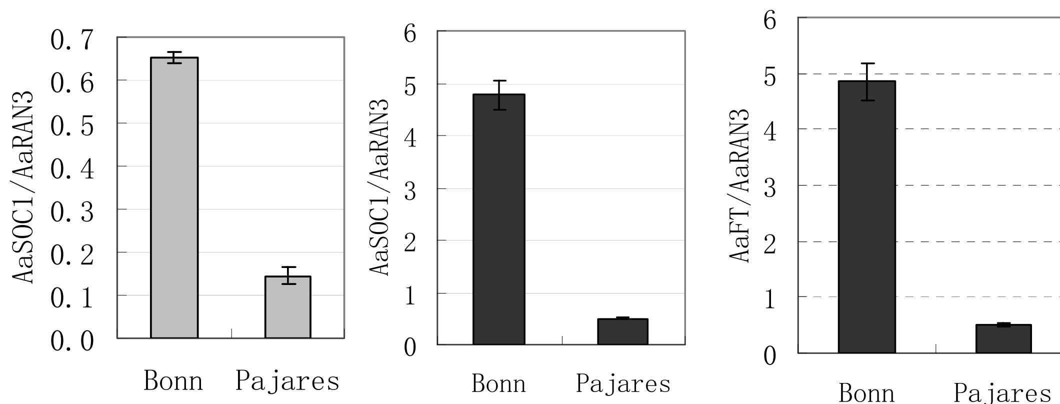


Figure 30. *AaSOC1* and *AaFT* expression in *Arabis alpina Pajares* and *Arabis alpina Bonn* plants.
(A and B) *AaSOC1* expression in apices of primary shoots **(A)** and expanded primary leaves **(B)**. **(C)** *AaFT* expression in expanded primary leaves. Plants were grown under LDs and materials were harvested 4 weeks after germination.

5.4. *AaTFL1* expression patterns in *Arabis alpina Bonn* and *Arabis alpina Pajares* accessions during development

AaTFL1 expression was also compared in the apices of the Bonn and the Pajares accessions during development (Figure 31). Within the first two weeks after germination, the expression patterns of *AaTFL1* were similar in both accessions, including the sub-domain of the shoot apex, the provascular tissues in the apex and young leaves, the adaxial basal part of young leaf primordia and axillary meristems (Figure 31A, 31B and 31E). However, in *Arabis alpina Bonn* plants, the expression in the sub-domain of the shoot apex did not spread to the basal part so much as in *Arabis alpina Pajares* plants. In later stages, the *AaTFL1* expression region became restricted in *Arabis alpina Bonn* plants (Figure 31C and 31D). Four weeks after germination, *AaTFL1* expression in the Bonn accession had already become restricted to isolated regions including a very limited part of the sub-dome domain of the main shoot apex and axillary meristems (Figure 31D). Furthermore, the expression in the sub-dome domain had become much weaker compared with

earlier developmental stages tested. At this time point, the main shoot apex had already elongated, implying the early steps of floral transition were proceeding. Five weeks after germination, in *Arabis alpina Bonn* plants, *AaTFL1* was weakly expressed in a few cells of the sub-dome domain of the main shoot apex, while on the flanking region where *AaTFL1* expression was not detectable floral meristems had already been formed. In contrast to the restriction observed in the Bonn accession *AaTFL1* expression patterns in the Pajares accession maintained almost unchanged throughout the 5 week time course tested, but the expression levels decreased slightly in 5-week old plants (Figure 31E and 31F).

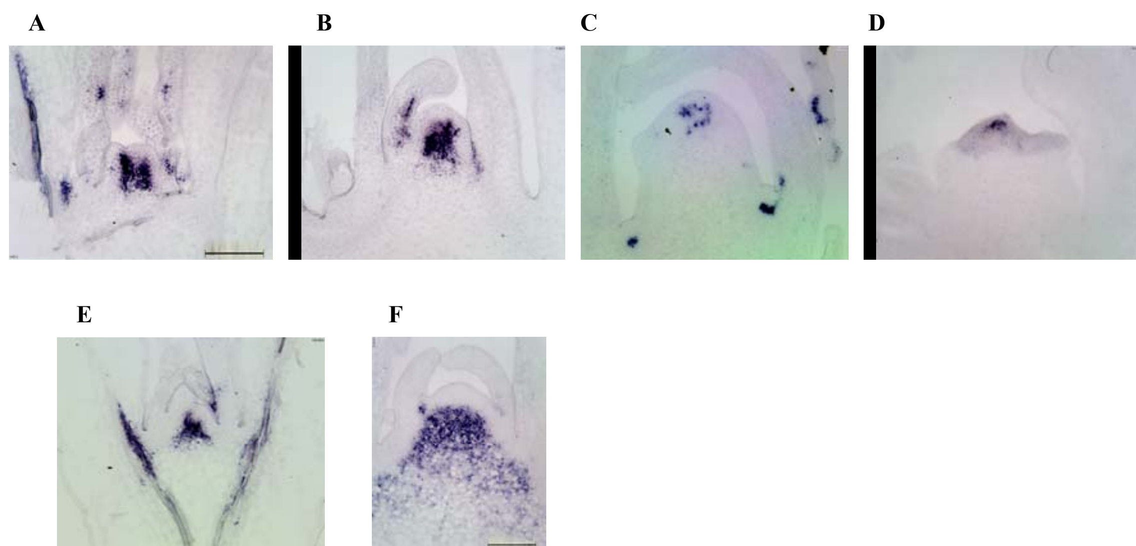


Figure 31. *AaTFL1* expression detected by *in situ* hybridization in apices of *Arabis alpina Bonn* and *Arabis alpina Pajares* accessions during development.
(A-D) Apices of *Arabis alpina Bonn* plants harvested 1 week **(A)**, 2 weeks **(B)**, 4 weeks **(C)** and 5 weeks **(D)** after germination, respectively. **(E and H)** Apices of *Arabis alpina Pajares* plants harvested 1 week **(E)** and 5 weeks **(F)** after germination, respectively. All the plants were grown under LDs. Bars, 100 µm.

5.5. Discussion

In Arabidopsis, *FRI* and *FLC* are two important genes that account for much of the natural genetic variation that converts a winter annual Arabidopsis accession to a summer annual. This led us to wonder whether the difference in vernalization requirement for flowering in the *Arabis alpina Pajares* and *Arabis alpina Bonn*

accessions resulted from loss-of-function mutation in *Arabis alpina* counterparts of *FRI*, *FLC* or other genes that regulate *AaFLC* expression. However, it was found that *AaFLC* mRNA was expressed at similar levels in both accessions (Figure 29). Furthermore, the coding sequences of *AaFLC* from these two accessions are identical. Thus, there must be some other mechanism that causes the loss of vernalization requirement in the Bonn accession. The data acquired from the analysis of *AaFLC* dsRNAi lines indicated that *AaFLC* represses both *AaSOC1* and *AaFT* expression in wild type plants. As shown above, our data suggested that *AaSOC1* and *AaFT* are orthologs of *SOC1* and *FT*, respectively, and function as crucial floral promoters (see Chapter 3.14.1 for detailed argument). Therefore, we wondered whether the early flowering phenotypes in *Arabis alpina Bonn* plants are dependent on upregulation of *AaSOC1* and/or *AaFT* or not. Indeed in this accession both *AaSOC1* and *AaFT* are expressed at dramatically higher levels than in *Arabis alpina Pajares* accession. In terms of the activities of *AaFT* and *AaSOC1* as floral promoters, the early flowering of the Bonn accession was attributed, at least partially, to the high levels of expression of these genes. Considering that in *Arabis alpina Pajares*, AaFLC suppresses the expression of *AaSOC1* and possibly also *AaFT*, the high levels of *AaSOC1* and *AaFT* expression in the Bonn accession could be caused by a repression of *AaFLC* function that occurs downstream of *AaFLC* transcription or by an activity that can bypass the repression of *AaFLC*.
We also compared *AaTFL1* expression in *Arabis alpina Bonn* and *Arabis alpina Pajares* during development from a juvenile to an adult plant. Consistent with the early-flowering phenotype observed in the Bonn accession, *AaTFL1* expression in this accession was high and extensive in the apices of juvenile plants (Figure 31A and 31B), which is similar to what was observed in juvenile *Arabis alpina Pajares* plants (Figure 31 E), but was restricted to very localized sub-dome domain of the SAM and axillary meristems in older plants that were undergoing the floral transition (Figure 31C and 31D). The restriction of *AaTFL1* expression in the *Arabis alpina Bonn* accession may be achieved by AaSOC1 and AaFT, which are strongly active in this accession and could promote flowering by overcoming the repression of *AaTFL1*. In turn, the floral transition restricts *AaTFL1* expression to the sub-dome domain of the SAM and the axillary meristems. Furthermore, a mechanism may exist in *Arabis alpina Bonn* to repress *AaTFL1* expression after the

plants have developed to a certain stage. This mechanism would not occur in Arabidopsis, since *TFL1* is already repressed at low levels in vegetative plants. Such a speculation would be consistent with the late flowering caused by *AaTFL1* in Arabidopsis.

Crosses of *Arabis alpina Pajares* with *Arabis alpina Bonn* have been done in our group (M. albani, unpublished) and several QTLs involved in the difference in flowering time in these two accessions have been mapped on the genetic linkage map established by N. Warthmann and D. Weigel..

6. General conclusions and perspectives

The extensive studies performed in recent years using the monocarpic annual model species *Arabidopsis thaliana* have revealed a complex network controlling flowering time, a vital developmental step in higher plants. However, little is known so far about the mechanisms underlying flowering time control and a closely related trait, perenniality in polycarpic perennial plants. In this thesis, I described our work in developing *Arabis alpina* as a polycarpic perennial model species to study flowering time control and perenniality. The molecular mechanisms underlying two prerequisites of polycarpic perenniality, juvenility and divergent competence of apical meristems to flower, have been investigated. Furthermore, the studies on the molecular mechanisms underlying the naturally occurring variation in flowering-time control in two *Arabis alpina* accessions have also been introduced.

Establishment of *Arabis alpina* as a model species for studying flowering-time control and perenniality

Many advantages have been revealed in *Arabis alpina*, which make it a promising perennial model for exploring various biological issues including flowering time and perenniality. These advantages include its close relationship with *Arabidopsis thaliana*, diploidy, self compatibility, small genome size, small plant size, existence of a large number of accessions distributed in various climatic environments and well-studied phylogeography. Furthermore, we have developed a floral-dip transformation method for *Arabis alpina*, analogous to that used for Arabidopsis.

An approximately 3-4-week-long juvenility period has been revealed in both accessions we studied. The flowering physiology of two accessions of this species in response to various floral inductive signals has been studied. By characterizing flowering behaviours of individual meristems within the same plant, four types (types I-IV) of side branches with different degrees of vegetative or reproductive identities were illustrated in *Arabis alpina Pajares* in response to vernalization. The identities of individual meristems appeared to be decided by the developmental stages they had reached when vernalization treatment started. The identities of individual axillary meristems in the early flowering accession *Arabis alpina Bonn*

was described. In addition, localized senescence was found to be closely related to flowering/seed-setting, implying that signals derived from flowering or seed-setting play a role in localized senescence.

At least part of the flowering-time control network is conserved between *Arabis alpina* and *Arabidopsis thaliana*

To study molecular mechanisms underlying flowering and perenniality, several genes, including *AaFLC*, *AaFT*, *AaSOC*1 and *AaTFL*, were cloned from *Arabis alpina*. Sequence analysis and the effect on flowering time and expression of other regulated genes when ectopically expressed indicated that they are orthologs of Arbidopsis *FLC*, *FT*, *SOC1* and *TFL1* respectively. When *AaFLC* expression was strongly reduced in transgenic plants, the flowering transition occurred, but only after several months, suggesting *AaFLC* is important, but not the only contributor to confer late flowering to this accession. The up-regulation of *AaFT*, especially of *AaSOC1* expression indicates that in *Arabis alpina Pajares*, AaFLC suppresses flowering through reducing the expression of the floral activator gene *AaSOC1* and maybe also *AaFT*, such a mechanism would be similar to that described for *FLC* in Arabidopsis (Searle et al., 2006).

Involvement of the regulation of *AaTFL1* expression in juvenility and the divergent competence of different meristems to flower

Similar expression patterns of flowering-time genes *AaFLC*, *AaFT* and *AaSOC1* in the apices of juvenile and adult plants during the early stages of floral transition suggest that a repression mechanism exists downstream of these genes that plays a role in the repression of floral induction in juvenile plants. In the early stages of the floral transition, the observation that restriction of *AaTFL1* expression occurred only in the SAM of adult plants but not in that of juvenile plants suggests that differential regulation of *AaTFL1* in the SAMs of juvenile and adult plants may underlie juvenility. This idea is further supported by the demonstration that *AaTFL1* expression decreases during plant development, and that a genomic fragment carrying *AaTFL1* not only complements the defects of Arabidopsis *tfl1* mutants, but also causes dramatic late flowering compared with wild-type controls.
During the development of a vegetative side branch of *Arabis alpina*, the

expression levels of *AaTFL1* mRNA decreased and the region of expression was restricted from the whole shoot bud to limited regions in the apex. Furthermore, during floral induction by vernalization, extensive expression of *AaTFL1* at high levels in side branches closely correlated with vegetative identity, whereas a restricted expression at lower levels was associated with reproductive identity of the side branch. These data suggest that *AaTFL1* may be involved in controlling the competence of individual axillary meristems to respond to floral induction.

Involvement of mitotically unstable repression of *AaFLC* by vernalization in seasonal flowering of *Arabis alpina*

Repetitive seasonal flowering is a very important aspect of seasonality that is unique to perennial plant species. In perennial plants vernalization response is maintained so that flowering occurs in the spring after winter over many years. Such responses are adopted by many polycarpic perennial plants to align their reproduction with a favorable season. Therefore, for these plants it is necessary to reset vernalization requirement in the vegetative tissue after each floral induction. The unstable repression of *AaFLC* observed in *Arabis alpina* plants may allow such resetting of vernalization requirement. After vernalization, *AaFLC* expression in most (if not all) parts of an *Arabis alpina* plant increase. In flowering apical meristems, restoration of *AaFLC* expression can not reverse flowering since some late and irreversible step has already taken place. However, in the vegetative shoots, restored *AaFLC* expression would block the floral transition. Correspondingly, after vernalization, *AaSOC1* expression was reduced to low levels. Thus, the combination of repressors mediating vernalization requirement and those controlling the differential competence of meristems to respond to floral induction seems to maintain seasonal flowering in polycarpic perennials with a vernalization requirement.

Reduced levels of *AaFLC* transcription do not account for early flowering of *Arabis alpina Bonn* plants

In contrast to the situation in Arabidopsis, where naturally occurring mutations in *FRI* and *FLC* cause reduction in *FLC* expression and account for a large part of the naturally occurring variation in flowering time, in *Arabis alpina Bonn* accession,

AaFLC is expressed at similarly high levels as in *Arabis alpina Pajares*. Mapping carried out in our group using populations generated by crossing the two accessions has attributed the late flowering of the Pajares accession to several QTLs.
The *AaTFL1* dsRNAi lines that are being made will provide a better understanding of the function of *AaTFL1* in flowering-time control and perenniality. Similarly, ongoing EMS mutagenesis treatments of *Arabis alpina* are expected to generate mutants with flowering-time phenotypes or affecting perenniality-related traits. Analysis of *Arabis alpina* homologs of more Arabidopsis flowering-time genes will help understand the flowering time control in this perennial species. The late-flowering observed in *tfl1* mutants complemented with a 5.2 kb *AaTFL1* genomic fragment suggests that regulatory elements may contribute to the difference in expression patterns of *AaTFL1* and *TFL1*. Such changes in regulatory sequences may enable rapid evolution of the flowering time network and thereby change life histories from perennial to annual in a relatively short time. Similar processes have been described in pigmentation of the wings of *Drosophila* relatives (Prud'homme et al., 2006). If this is confirmed for *Arabis alpina AaTFL1* gene, then yeast one hybrid may be adopted to screen for proteins that regulate *AaTFL1*, and whose activity could vary between species.

7. Materials and Methods

7.1. Materials

7.1.1. Chemicals

Chemicals used were purchased from Sigma, Merck, Roth, Amersham, Gibico and Boehringer unless otherwise stated. General solutions and buffers used were prepared as described by Sambrook and Russell (Sambrook and Russell, 2001) unless otherwise stated.

7.1.2. Enzymes

All enzymes were used following the instructions of the manufacturers unless otherwise stated.

Tag DNA polymerase	Homemade
Platinum Taq Polymerase High Fidelity	Invitrogen, Karlsruhe, Germany
Superscript II Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany
Restriction enzymes	New England Biolabs, Frankfurt
Gateway BP Clonase Enzyme	Invitrogen, Karlsruhe, Germany
Gateway LR Clonase Enzyme	Invitrogen, Karlsruhe, Germany

7.1.3. Kits

Qiagen plasmid preparation kits	Qiagen, Hilden, Germany
Plant DNA extraction kits	Qiagen, Hilden, Germany
PCR purification kit	Roche , Mannheim, Germany
RNeasy plant mini kit	Qiagen, Hilden, Germany
TA cloning kit	Invitrogen, Karlsruhe, Germany

7.1.4. Vectors

TA and Gateway cloning vectors

TOPO-pCRII	Invitrogen, Karlsruhe, Germany
pDONR221	Invitrogen, Karlsruhe, Germany
pDONR207	Invitrogen, Karlsruhe, Germany

Vectors for plant expression

pLeela	Gateway compatible with 35S-promoter
pMDC99	Gateway compatible for complementation test
pJawohl8	Gateway compatible for dsRNAi

7.1.5. Bacterial Strains

***E coli* strains**

DH5α	Invitrogen, Karlsruhe, Germany
DB 3.1	Invitrogen, Karlsruhe, Germany

***Agrobacterium tumefaciems* strains**

GV3101/pMP90RK	rifampicin, gentamicin and kanamycin resistant
GV3101/pMP90	rifampicin and gentamicin resistant

7.1.6. Oligonucleotides

Degenerated primers for cloning *Arabis alpina* genes

RH9	CAT(C/T)GAGAAAGCTCGTCAGCT
RH10	GAAGCTT(C/T)AACA(T/A)(T/G)AGTTC(T/G)G(C/T)C
RH98	AACG(T/G)AAACT(C/T)(T/A)TGGGAGAAGG
W100	TCCTT(C/T)T(C/G)(C/T)TT(A/G)AG(C/T)TGCTCAATTTG
RH87	GATGTTCC(A/T)(G/A)(G/A)TCCTAGTGA
RH88	TTGGC(C/T)TTGGCA(A/G/T)(C/T)TCATAG

Gateway entry cloning

GWF	GGGGACAAGTTTGTACAAAAAAGCAGGCT
GWR	GGGGACCACTTTGTACAAGAAAGCTGGGT
XAaFLC5-F	(GWF)GAGACAGAAGCCATGGGTAG
XAaFLC3-R	(GWR)GGCTTAATTGAGTAGTGGGAG
XAtFLC5-F	(GWF)CGGAGAGAAGCCATGGGAAG
XAtFLC3-R	(GWR)TGGCTAATTAAGTAGTGGGAGA
W221	(GWF)CCTAAAAAATGGAGAATATTGGAAG
W222	(GWR)GATGAACAATAATGTAACTAGCGTT
W217	(GWF)ATATGTCTATAAGTCCGAGAGA
W218	(GWR)GGGACAAGCCATCTCTAACT
W246	(GWF)TCGAAGACTATTCGTTCGCAGA
W247	(GWR)CACTCGACTTTAGTGCCCATA
W270	(GWF)ACAAAGGAATAAGAAatgGTGAG
W271	(GWR)TTTGTTGGAAGAATAGTG
RH63	(GWF)AAGCTCGTGGCACCAAATGTC
RH64	(GWR)AGTCTCTCAGCCATAGAGAGT
W155	(T3F)AGGCCAAGCATAGGGATACA
W156	(T7R)TGGTTACATTCTTCTCCCTCA
W274	(T3F)CGAGCAAGAAAGACTCAAGTGT
W275	(T7R)GTTGGAAGAATAGTGTTTCTTCAC

Real-time quantitative RT-PCR

AaFLC

W132	CTTGTCGTCTCCTCCTCTGG
W133	ACTACGGCGAGAGCAGTTTC

AaFT

W141	GATCTAAGGCCTTCTCAAGTCCAA
W142	CTGTCGGAACAATATCAGCACGATA

AaSOC1

W268	GCTTTCAGTGCTTTGTGATGC
W269	GGATGCTTCGAGTTGTTCGAT

AaRAN3

W113	CACAGGAAAAACCACATTCGT
W114	CCATCCCTAAGACCACCAAAT

Southern blot and screening of BAC library

At(a)extron2-F	CCTGGTCAAGATCCTTGATCGA
At(a)extron6-R	CTGGCTAGCCAAAACCTGGTT
At(a)extron2-F	CCTGGTCAAGATCCTTGATCGA
ArabisAUTR3	AGTCTCTCAGCCATAGAGAG
RH78	GGAACTAGAGTGATAGAGCCA
RH79	GTCTTTGCTTCTGCCTGAACAG
RH94	AGGCATACTAAGGATCGAGTCAG
RH95	CACTTTCTTGAAGAACAAGGTAAC

7.1.7. Plant Materials

Seeds of *Arabis alpina Pajares* were kindly provided by Dr. Carlos Alonso-Blanco from Instituto Nacional de Investigaciony Tecnologia Spain.
Arabis alpina Bonn seeds were from Bonn botany garden. Seeds of *tfl1-1* mutant were a kind gift from Dr. Desmond Bradley from the John Innes Centre UK. Arabidopsis thaliana Columbia wild type seeds were from the stocks in our lab.

7.2. Methods

7.2.1. General molecular biological techniques

Standard molecular biological techniques such as PCR amplification, agarose gel separation, restriction enzyme digestion, and DNA purification were performed as described by Sambrook and Russell (Sambrook and Russell, 2001)

7.2.2. RNA isolation

Total RNA was isolated from plant tissues with the RNeasy Plant Mini Kit from Qiagen following the manufacture's instruction. An on-column DNase treatment was performed during the RNA extraction. The quality of RNA was tested with formaldehyde agarose gel electrophoresis. The quantity of RNA was measured with a UV photometer.

7.2.3. Reverse transcription

Reverse transcription was carried out in 20µL final volume using the Superscript II reverse transcriptase from Invitrogen according to the manufacturer's instruction.

7.2.4. Cloning of *AaFLC*, *AaSOC1* and *AaTFL1*

Degenerated primers, RH9 and RH10, were designed based on the alignment of the coding sequences (CDS) of Arabidopsis *FLC* and its homologs from *Brassica napus* (*BnFLC1-5*). Similarly, RH98 and W100 were designed based on the alignment of *SOC1* and its homologs from *Cardamine flexuosa* (*CfSOC1*), *Sinapis alba* (*SaMADSA*), *Brassica rapa* (*BrSOC1*), *Draba nemorosa* (*DnSOC1*) and *Brassica juncea* (*BjSOC1*), and RH87 and RH88 on the alignment of *TFL1* and its homologs from *Arabidopsis lyrata* (*AlTFL1*), *Brassica rapa* (*BrTFL1-1 and BrTFL1-2*), *Brassica napus* (*BnTFL1-1 and BnTFL1-2*), *Eriobotrya japonica* (*EjTFL1-1*), *Pyrus communis* (*PcTFL1-2*), *Malus x domestica* (*MdTFL1*), *Lotus japonicus* (*LjCEN/TFL1-like*), *Antirrhinum majus* (*CEN*) and *Nicotiana tabacum* (*CET2*). cDNA synthesized with RNA extracted from vegetative apices of *Arabis alpina Pajares* was used as templates in PCR to clone *AaFLC*, *AaSOC1* and *AaTFL1* with respective primer pairs. The resulting fragments were linked to cloning vector TOPO-pCRII and sequenced at ADIS (MPIZ).

7.2.5. BAC library screening and sequencing of BAC DNA

A ^{32}P labeled 547 bp cDNA fragment of *FLC* (amplified with primers At(a)extron2-F and ArabisAUTR3) lacking the MADS-box-domain coding region was used to screen the an *Arabis alpina Pajares* BAC library, which was constructed in our lab. Under intermediate stringency, 2 colonies were obtained. The result of restriction enzyme digestion of the BAC DNA isolated from these colonies suggested that they are independent ones. BAC DNA was sequenced at ADIS (MPIZ) using primers designed according to cDNA sequence cloned with degenerated primers.

To identify colonies carrying homologs of *SOC1* or *TFL1*, similar screenings were carried out using a *SOC1* cDNA fragment without the MADS-box-domain coding sequence (amplified with primers RH94 and RH95) or the full length CDS of *TFL1* (amplified with primers RH78 and RH79) as probes respectively. A single colony was identified with the *SOC1* probe, and four with the *TFL1* probe. Primers were designed according to the partial cDNA sequences of *AaSOC1* and *AaTFL1* cloned using degenerate primers. BACs carrying *AaSOC* or *AaTFL1* were sequenced at ADIS (MPIZ).

7.2.6. Phylogenetic analysis

To identify the related sequences in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST), protein–protein BLAST was conducted using the deduced amino acid sequence from *AaFLC*, *AaFT*, *AaTFL1* and *AaSOC1* respectively. Representative full length sequences were selected to construct phylogenetic trees. ClustalX was used to create multiple alignments (Thompson et al., 1997). The resulting alignment was used to generate the phylogenetic tree by the neighbor-joining method with Splitstree (Huson, 1998). Bootstrap analysis was performed to estimate nodal support based on 1000 replicates.

7.2.7. Southern blot

BamHI, BglII, EcoRI, HindIII and SpeI were used respectively to digest 10 μg of genomic DNA extracted from *Arabis alpina Pajares*. Southern blot was carried out primarily following the standard protocol (Sambrook and Russell, 2001). A 1296 bp fragment of the genomic sequence of *AaFLC* including the region from the beginning of the second exon to the end of the sixth exon was amplified with primers At(a)extron2-F and At(a)extron6-R. This fragment was labeled with ^{32}P and used as the probe.

7.2.8. Construction of expression vectors

To make constructs for constitutive expression of *AaFLC*, *AtFLC, AaFT*, *AaSOC1* and *AaTFL1*, the coding sequences of each gene (using primers XAaFLC5-F and XAaFLC3-R for *AaFLC*, XAtFLC5-F and XAtFLC3-R for *AtFLC*, W217 and W218 for *AaFT*, W270 and 271 for *AaSOC1*, and W221 and W222 for *AaTFL1*) were amplified and introduced into the Gateway cloning vector pDONR221 via BP reaction according to the manufacture's instruction of the BP clonase. The resulting entry clone vectors carrying *AaFLC*, *AtFLC, AaFT*, *AaSOC1* and *AaTFL1* were named as pENT221-AaFLC, pENT221-AtFLC, pENT221-W217-218, pENT221-W270-271 and pENT221-W221-222 respectively. LR reactions were performed to transfer the CDSs of these genes from the entry clone vectors to the destination vector pLeela. The resulting expression constructs carrying *AaFLC*, *AtFLC, AaFT*, *AaSOC1* and *AaTFL1* were named pLeela-AaFLC, pLeela-AtFLC, pLeela-W217-218, pLeela-W270-271 and pLeela-W221-222, respectively.

To make constructs for generating *AaFLC* dsRNAi lines, primers RH63 and RH64 were used to amplify a 399 bp fragment covering a region from exon 4 to 3'UTR of *AaFLC* cDNA sequence. Through BP and LR reactions similar to what was described above, two copies of this fragment were ligated to the intron of the destination vector pJawohl8 for dsRNAi in reverse orientations. The resulting expression construct was named PJawohl8-RH63-64.

A 5.2 kb genomic fragment consisting of a 2.6 kb promoter, a 1.1 kb reading frame and a 1.5 kb terminator of the *AaTFL1* gene from *Arabis alpina Pajares* was

amplified with primers W246 and W247. BP and LR reactions were conducted following the manufacture's instructions of BP and LR clonases respectively. This fragment was finally introduced into the destination vector pMDC99 to make an expression construct, pMDC99-W246-247.

7.2.9. Agrobacterium-mediated transformation of Arabidopsis and *Arabis alpina* plants

Transformation of *Agrobacterium tumefaciens*

An aliquot (50µl) of an electro-competent Agrobacterium cells (strains GV3101 pMP90 or pMP90RK) was thawn on ice. 1µl of plasmid DNA was added; cell and DNA were mixed by flicking the tube and then transferred to an electroporation cuvette. An electric pulse was applied (2.5kv/cm, 25pF, 400Ω for 8-12ms), and then cells were resuspended with 1ml LB medium and transferred to a 15ml Falcon tube. After incubation at 28°C with constant shaking for 2 hours, 100µl were plated on LB plates containing appropriate antibiotics.

Transformation of Arabidopsis and *Arabis alpina* plants

Arabidopsis transformation was conducted primarily as described as by Clough and Bent (Clough and Bent, 1998).

For transformation of *Arabis alpina*, an adapted method was used. Plasmid carrying Agrobacterium strains were grown overnight in one liter LB medium and appropriate antibiotics. Cells were harvested by a 30 minute centrifugation at 4,000prm and pellets were resuspended in one liter transformation buffer (50g sucrose, 500µl silvet-L77, pH5.7) to OD 0.8. *Arabis alpina* plants with as many unopened flowers as possible were dipped in the transformation mixture for two minutes and then put into a plastic tray with a cover. After 16-24 hour incubation, the cover was removed and plants were grown in LDs in the greenhouse until the seeds were ready.

7.2.10. *In situ* hybridization

Longitudinal sections of plant tissue were probed with digoxigenin-labelled *AaTFL1* or *AaSOC1* antisense RNA. cDNA fragments amplified with primers W155 and W156 (*AaTFL1*), or primers W274 and W275(*AaSOC1*) were used as templates to label the probes in vitro as described by Searle et al. (Searle et al., 2006). RNA *in situ* hybridyzation was carried out as described by Jackson (Jackson, 1992).

7.2.11. Plant growth conditions and measurement of flowering time

For growing both *Arabis alpina* and Arabidopsis plants, seeds were sown in soil, cold-treated for 3 days at 4 °C, transferred to Percival growth chambers or the greenhouse and grown in LD (16 hours light/8 hours dark) or SD (8 hours light/16 hours dark) at 22°C/18°C day/night temperature. For vernalization treatment, plants were grown at 4°C in SD, since this condition was thought to be similar to the natural winter (Sung et al., 2006a). For measuring flowering time, leaf numbers from at least 20 Arabidopsis plants or 15 *Arabis alpina* plants were counted in each experiment.

7.2.12. GA treatment

100µM GA3 or GA4 solution was sprayed to the aerial part including shoot apical meristems of *Arabis alpina* plants once or twice a week. In all experiments, GA treatment started when plants were one week old. In the experiment where vernalization treatment was also used, GA treatment lasted until the end of vernalization. In the other cases, plants were treated with GAs for at least two months.

7.2.13. Quantitative RT-PCR

For real-time quantitative PCR, 3.5µl of a nine-fold dilution of the cDNA produced

by reverse transcription was used. The amplification mix contained the following: 2µl PCR buffer containing 0.5µl/ml SYBR-green, 2µl 10µmM of each primer, 2µl 2mM dNTPs, 0.25µl Taq polymerase and 10.25µl water. Quantification was performed on an iCycler iQ real-time PCR detection system (BIO-RAD).

8. References

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., and Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science **309,** 1052-1056.

Achard, P., Herr, A., Baulcombe, D.C., and Harberd, N.P. (2004). Modulation of floral development by a gibberellin-regulated microRNA. Development **131,** 3357-3365.

Achard, P., Baghour, M., Chapple, A., Hedden, P., Van Der Straeten, D., Genschik, P., Moritz, T., and Harberd, N.P. (2007). The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes. PNAS **104,** 6484-6489.

Ahn, J., Miller, D., and Winter VJ, B.M., Lee JH, Yoo SY, Henz SR, Brady RL, Weigel D. (2006). A divergent external loop confers antagonistic activity on floral regulators FT and TFL1. Embo J **25,** 605–614.

Alonso-Blanco, C., Mendez-Vigo, B., and Koornneef, M. (2005). From phenotypic to molecular polymorphisms involved in naturally occurring variation of plant development. The International Journal of Developmental Biology **49** 717 - 732.

Amasino, R. (2004). Vernalization, Competence, and the Epigenetic Memory of Winter. Plant Cell **16,** 2553-2559.

Amaya, I., Ratcliffe, O.J., and Bradley, D.J. (1999). Expression of CENTRORADIALIS (CEN) and CEN-like Genes in Tobacco Reveals a Conserved Mechanism Controlling Phase Change in Diverse Species. Plant Cell **11,** 1405-1418.

An, H.L., Roussot, C., Suarez-Lopez, P., Corbesler, L., Vincent, C., Pineiro, M., Hepworth, S., Mouradov, A., Justin, S., Turnbull, C., and Coupland, G. (2004). CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development **131,** 3615-3626.

Aukerman, M.J., Hirschfeld, M., Wester, L., Weaver, M., Clack, T., Amasino, R.M., and Sharrock, R.A. (1997). A Deletion in the PHYD Gene of the Arabidopsis Wassilewskija Ecotype Defines a Role for Phytochrome D in Red/Far-Red Light Sensing. Plant Cell **9,** 1317-1326.

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L., and Martinez-Zapater, J.M. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat Genet **36,** 162-166.

Bailey, C.D., Koch, M.A., Mayer, M., Mummenhoff, K., O'Kane, S.L., Jr., Warwick, S.I., Windham, M.D., and Al-Shehbaz, I.A. (2006). Toward a Global Phylogeny of the Brassicaceae. Mol Biol Evol **23,** 2142-2160.

Balasubramanian, S., Sureshkumar, S., Lempe, J., and Weigel, D. (2006). Potent

Induction of Arabidopsis thaliana Flowering by Elevated Growth Temperature. PLoS Genetics **2,** e106.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A., and Dean, C. (2004). Vernalization requires epigenetic silencing of FLC by histone methylation. Nature **427,** 164-167.

Battey, N.H., and Tooke, F. (2002). Molecular control and variation in the floral transition. Current Opinion in Plant Biology **5,** 62-68.

Baurle, I., and Dean, C. (2006). The Timing of Developmental Transitions in Plants. Cell **125,** 655-664.

Blazquez, M.A., and Weigel, D. (2000). Integration of floral inductive signals in Arabidopsis. Nature **404,** 889-892.

Blazquez, M.A., Ahn, J.H., and Weigel, D. (2003). A thermosensory pathway controlling flowering time in *Arabidopsis thaliana*. Nat Genet **33,** 168-171.

Blazquez, M.A., Soowal, L.N., Lee, I., and Weigel, D. (1997). LEAFY expression and flower initiation in Arabidopsis. Development **124,** 3835-3844.

Blazquez, M.A., Green, R., Nilsson, O., Sussman, M.R., and Weigel, D. (1998). Gibberellins promote flowering of Arabidopsis by activating the LEAFY promoter. Plant Cell **10,** 791-800.

Bohlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A.M., Jansson, S., Strauss, S.H., and Nilsson, O. (2006). CO/FT Regulatory Module Controls Timing of Flowering and Seasonal Growth Cessation in Trees. Science **312,** 1040-1043.

Borner, R., Kampmann, G., Chandler, J., Gleissner, R., Wisman, E., Apel, K., and Melzer, S. (2000). A MADS domain gene involved in the transition to flowering in *Arabidopsis*. Plant Journal **24,** 591-599.

Boss, P.K., Bastow, R.M., Mylne, J.S., and Dean, C. (2004). Multiple pathways in the decision to flower: Enabling, promoting, and resetting. Plant Cell **16,** S18-S31.

Bowman, J.L., Alvarez, J., Weigel, D., Meyerowitz, E.M., and Smyth, D.R. (1993). Control of flower development in Arabidopsis thaliana by APETALA1 and interacting genes. Development **119,** 721-743.

Bradley, D., Vincent, C., Carpenter, R., and Coen, E. (1996). Pathways for inflorescence and floral induction in Antirrhinum. Development **122,** 1535-1544.

Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R., and Coen, E. (1997). Inflorescence commitment and architecture in *Arabidopsis*. Science **275,** 80-83.

Brunner, A.M., and Nilsson, O. (2004). Revisiting tree maturation and floral initiation in the poplar functional genomics era. New Phytologist **164,** 43-51.

Burn, J.E., Smyth, D.R., Peacock, W.J., and Dennis, E.S. (1993). Genes conferring late flowering in Arabidopsis thaliana. Genetica **90,** 147-155.

Cerdan, P.D., and Chory, J. (2003). Regulation of flowering time by light quality. Nature **423,** 881-885.

Choi, K., Kim, S., Kim, S.Y., Kim, M., Hyun, Y., Lee, H., Choe, S., Kim, S.-G., Michaels, S., and Lee, I. (2005). SUPPRESSOR OF FRIGIDA3 Encodes a Nuclear ACTIN-RELATED PROTEIN6 Required for Floral Repression in Arabidopsis. Plant Cell **17,** 2647-2660.

Chou, M.-L., and Yang, C.-H. (1998). FLD interacts with genes that affect different developmental phase transitions to regulate Arabidopsis shoot development. Plant Journal **15,** 231-242.

Clarke, J.H., and Dean, C. (1994). Mapping FRI, a locus controlling flowering time and vernalization response in Arabidopsis thaliana. Molecular and General Genetics **242,** 81-89.

Clauss, M.J., and Koch, M.A. (2006). Poorly known relatives of Arabidopsis thaliana. Trends in Plant Science **11,** 449-459.

Cleland, C.F., and Zeevaart, J.A.D. (1970). Gibberellins in Relation to Flowering and Stem Elongation in the Long Day Plant Silene armeria. Plant Physiol. **46,** 392-400.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method forAgrobacterium-mediated transformation ofArabidopsis thaliana. The Plant Journal **16,** 735-743.

Coles, J.P., Phillips, A.L., Croker, S.J., Garcia-Lepe, R., Lewis, M.J., and Hedden, P. (1999). Modification of gibberellin production and plant development in Arabidopsis by sense and antisense expression of gibberellin 20-oxidase genes. Plant Journal **17,** 547-556.

Corbesier, L., Vincent, C., and Jang S, F.F., Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G. (2007). FT Protein Movement Contributes to Long-Distance Signaling in Floral Induction of Arabidopsis. Science **316,** 1030-1033.

Danyluk, J., Kane, N.A., Breton, G., Limin, A.E., Fowler, D.B., and Sarhan, F. (2003). TaVRT-1, a Putative Transcription Factor Associated with Vegetative to Reproductive Transition in Cereals. Plant Physiol. **132,** 1849-1860.

Deal, R.B., Kandasamy, M.K., McKinney, E.C., and Meagher, R.B. (2005). The Nuclear Actin-Related Protein ARP6 Is a Pleiotropic Developmental Regulator Required for the Maintenance of FLOWERING LOCUS C Expression and Repression of Flowering in Arabidopsis. Plant Cell **17,** 2633-2646.

Deal, R.B., Topp, C.N., McKinney, E.C., and Meagher, R.B. (2007). Repression of Flowering in Arabidopsis Requires Activation of FLOWERING LOCUS C Expression by the Histone Variant H2A.Z. Plant Cell **19,** 74-83.

Doyle, M.R., Bizzell, C.M., Keller, M.R., Michaels, S.D., Song, J., Noh, Y.-S., and Amasino, R.M. (2005). HUA2 is required for the expression of floral repressors in Arabidopsis thaliana. The Plant Journal **41,** 376-385.

El-Assal, S.E.D., Alonso-Blanco, C., Peeters, A.J.M., Raz, V., and Koornneef, M. (2001). A QTL for flowering time in *Arabidopsis* reveals a novel allele of *CRY2*. Nat Genet **29,** 435-440.

Endo, T., Shimada, T., Fujii, H., Kobayashi, Y., Araki, T., and Omura, M. (2005). Ectopic Expression of an FT Homolog from Citrus Confers an Early Flowering Phenotype on Trifoliate Orange (Poncirus trifoliata L. Raf.). Transgenic Research **14,** 703-712.

Eriksson, S., Bohlenius, H., Moritz, T., and Nilsson, O. (2006). GA4 Is the Active Gibberellin in the Regulation of LEAFY Transcription and Arabidopsis Floral Initiation. Plant Cell **18,** 2172-2181.

Foucher, F., Morin, J., Courtiade, J., Cadioux, S., Ellis, N., Banfield, M.J., and Rameau, C. (2003). DETERMINATE and LATE FLOWERING Are Two TERMINAL FLOWER1/CENTRORADIALIS Homologs That Control Two Distinct Phases of Flowering Initiation and Development in Pea. Plant Cell **15,** 2742-2754.

Fu, D., Szűcs, P., Yan, L., Helguera, M., Skinner, J.S., von Zitzewitz, J., Hayes, P.M., and Dubcovsky, J. (2005). Large deletions within the first intron in VRN-1 are associated with spring growth habit in barley and wheat. Molecular Genetics and Genomics **273,** 54-65.

Fuchs, J., Demidov, D., Houben, A., and Schubert, I. (2006). Chromosomal histone modification patterns - from conservation to diversity. Trends in Plant Science **11,** 199-208.

Garrison, R. (1955). Studies in the Development of Axillary Buds. American Journal of Botany **42,** 257-266.

Gazzani, S., Gendall, A.R., Lister, C., and Dean, C. (2003). Analysis of the Molecular Basis of Flowering Time Variation in Arabidopsis Accessions. Plant Physiol. **132,** 1107-1114.

Gendall, A.R., Levy, Y.Y., Wilson, A., and Dean, C. (2001). The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell **107,** 525-535.

Giavalisco, P., Kapitza, K., Kolasa, A., Buhtz, A., and Kehr, J. (2006). Towards the proteome of *Brassica napus* phloem. Proteomics **6,** 896-909.

Gocal, G.F., Sheldon, C.C., Gubler, F., Moritz, T., Bagnall, D.J., MacMillan, C.P., Li, S.F., Parish, R.W., Dennis, E.S., Weigel, D., and King, R.W. (2001). GAMYB-like genes, flowering, and gibberellin signaling in Arabidopsis. Plant Physiol **127,** 1682-1693.

Gomez-Mena, C., Pineiro, M., Franco-Zorrilla, J.M., Salinas, J., Coupland, G., and Martinez-Zapater, J.M. (2001). EARLY BOLTING IN SHORT DAYS: An Arabidopsis mutation that causes early flowering and partially suppresses the floral phenotype of leafy. Plant Cell **13,** 1011-1024.

Greb, T., Clarenz, O., Schafer, E., Muller, D., Herrero, R., Schmitz, G., and Theres, K. (2003). Molecular analysis of the LATERAL SUPPRESSOR gene in Arabidopsis reveals a conserved control mechanism for axillary meristem formation. Genes Dev. **17,** 1175-1187.

Greb, T., Mylne, J.S., Crevillen, P., Geraldo, N., An, H., Gendall, A.R., and Dean, C. (2007). The PHD Finger Protein VRN5 Functions in the Epigenetic Silencing of Arabidopsis FLC. Current Biology **17,** 73-78.

Gustafson-Brown, C., Savidge, B., and Yanofsky, M.F. (1994). Regulation of the Arabidopsis floral homeotic gene APETALA1. Cell **76,** 131-143.

Hackett, W. (1985). Juvenility, maturation, and rejuvenation in woody plants. Horticultural Reviews **7,** 109-155.

Hanzawa, Y., Money, T., and Bradley, D. (2005). A single amino acid converts a repressor to an activator of flowering. PNAS **102,** 7748-7753.

Hay, A., and Tsiantis, M. (2006). The genetic basis for differences in leaf form between Arabidopsis thaliana and its wild relative Cardamine hirsuta. Nat Genet **38** 942 - 947.

Hayama, R., and Coupland, G. (2004). The molecular basis of diversity in the photoperiodic flowering responses of Arabidopsis and rice. Plant Physiol **135,** 677-684.

He, Y., Michaels, S.D., and Amasino, R.M. (2003). Regulation of Flowering Time by Histone Acetylation in Arabidopsis. Science **302,** 1751-1754.

He, Y., Doyle, M.R., and Amasino, R.M. (2004a). PAF1-complex-mediated histone methylation of FLOWERING LOCUS C chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. Genes Dev. **18,** 2774-2784.

He, Y., Tang, R.-H., Hao, Y., Stevens, R.D., Cook, C.W., Ahn, S.M., Jing, L., Yang, Z., Chen, L., Guo, F., Fiorani, F., Jackson, R.B., Crawford, N.M., and Pei, Z.-M. (2004b). Nitric Oxide Represses the Arabidopsis Floral Transition. Science **305,** 1968-1971.

Hsu, C.-Y., Liu, Y., Luthe, D.S., and Yuceer, C. (2006). Poplar FT2 Shortens the Juvenile Phase and Promotes Seasonal Flowering. Plant Cell **18,** 1846-1861.

Hu, F.Y., Tao, D.Y., Sacks, E., Fu, B.Y., Xu, P., Li, J., Yang, Y., McNally, K., Khush, G.S., Paterson, A.H., and Li, Z.K. (2003). Convergent evolution of perenniality in rice and sorghum. PNAS **100,** 4050-4054.

Huang, S.S., Raman, A.S., Ream, J.E., Fujiwara, H., Cerny, R.E., and Brown, S.M. (1998). Overexpression of 20-oxidase confers a gibberellin-overproduction phenotype in Arabidopsis. Plant Physiol **118,** 773-781.

Huson, D.H. (1998). SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics **14,** 68-73.

Imaizumi, T., and Kay, S.A. (2006). Photoperiodic control of flowering: not only by coincidence. Trends in Plant Science **11,** 567-567.

Jack, T. (2004). Molecular and genetic mechanisms of floral control. The Plant Cell **S16,** 1 - 17.

Jackson, D.P. (1992). *In situ* hybridization in plants. . Molecular Plant pathology: A Practical Approach (Bowles D.J., Gurr S.J. and McPhereson M.). Oxford

University Press, Oxford, 163-174.

Jansson, S., and Douglas, C.J. (2007). Populus: A Model System for Plant Biology. Annual Review of Plant Biology **58,** 435-458.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. (2000). Molecular analysis of FRIGIDA, a major determinant of natural variation in Arabidopsis flowering time. Science **290,** 344-347.

Johnston, J.S., Pepper, A.E., Hall, A.E., Chen, Z.J., Hodnett, G., Drabek, J., Lopez, R., and Price, H.J. (2005). Evolution of Genome Size in Brassicaceae. Ann Bot **95,** 229-235.

Jones, C.S. (1999). An essay on juvenility, phase change, and heteroblasty in seed plants. International Journal of Plant Sciences **160,** S105-S111.

Kania, T., Russenberger, D., Peng, S., Apel, K., and Melzer, S. (1997). FPF1 promotes flowering in Arabidopsis. Plant Cell **9,** 1327-1338.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J., and Weigel, D. (1999). Activation tagging of the floral inducer *FT*. Science **286,** 1962-1965.

Kim, J.S., Chung, T.Y., King, G.J., Jin, M., Yang, T.-J., Jin, Y.-M., Kim, H.-I., and Park, B.-S. (2006a). A Sequence-Tagged Linkage Map of Brassica rapa. Genetics **174,** 29-39.

Kim, S., Choi, K., Park, C., Hwang, H.-J., and Lee, I. (2006b). SUPPRESSOR OF FRIGIDA4, Encoding a C2H2-Type Zinc Finger Protein, Represses Flowering by Transcriptional Activation of Arabidopsis FLOWERING LOCUS C. Plant Cell **18,** 2985-2998.

Kim, S.Y., and Michaels, S.D. (2006). SUPPRESSOR OF FRI 4 encodes a nuclear-localized protein that is required for delayed flowering in winter-annual Arabidopsis. Development **133,** 4699-4707.

Kim, S.Y., He, Y., Jacob, Y., Noh, Y.-S., Michaels, S., and Amasino, R. (2005). Establishment of the Vernalization-Responsive, Winter-Annual Habit in Arabidopsis Requires a Putative Histone H3 Methyl Transferase. Plant Cell **17,** 3301-3310.

King, K.E., Moritz, T., and Harberd, N.P. (2001). Gibberellins are not required for normal stem growth in Arabidopsis thaliana in the absence of GAI and RGA. Genetics **159,** 767-776.

King, R.W., Moritz, T., Evans, L.T., Junttila, O., and Herlt, A.J. (2001b). Long-day induction of flowering in Lolium temulentum involves sequential increases in specific gibberellins at the shoot apex. Plant Physiol **127,** 624 - 632.

King, R.W., Evans, L.T., Mander, L.N., Moritz, T., Pharis, R.P., and Twitchin, B. (2003). Synthesis of gibberellin GA6 and its role in flowering of Lolium temulentum. Phytochemistry **62,** 77-82.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. Science **286,**

1960-1962.

Kobor, M.S., Venkatasubrahmanyam, S., Meneghini, M.D., Gin, J.W., Jennings, J.L., Link, A.J., Madhani, H.D., and Rine, J. (2004). A Protein Complex Containing the Conserved Swi2/Snf2-Related ATPase Swr1p Deposits Histone Variant H2A.Z into Euchromatin. PLoS Biology **2,** e131.

Koch, M., Bishop, J., and Mitchell-Olds, T. (1999). Molecular systematics and evolution of Arabidopsis and Arabis. Plant Biology **1,** 529-537.

Koch, M., Haubold, B., and Mitchell-Olds, T. (2001). Molecular systematics of the Brassicaceae: evidence from coding plastidic matK and nuclear Chs sequences. American Journal of Botany **88,** 534-544.

Koch, M.A., Haubold, B., and Mitchell-Olds, T. (2000). Comparative Evolutionary Analysis of Chalcone Synthase and Alcohol Dehydrogenase Loci in Arabidopsis, Arabis, and Related Genera (Brassicaceae). Mol Biol Evol **17,** 1483-1498.

Koch, M.A., Kiefer, C., Ehrich, D., Vogel, J., Brochmann, C., and Mummenhoff, K. (2006). Three times out of Asia Minor: the phylogeography of *Arabis alpina* L. (*Brassicaceae*). Molecular Ecology **15,** 825-839.

Koornneef, M., Hanhart, C.J., and Van Der Veen, J.H. (1991). A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Molecular and General Genetics **229,** 57-66.

Koornneef, M., Alonso-Blanco, C., and Vreugdenhil, D. (2004). Naturally occurring genetic variation in *Arabidopsis thaliana*. Annual Review of Plant Biology **55,** 141-172.

Kotoda, N., and Wada, M. (2005). MdTFL1, a TFL1-like gene of apple, retards the transition from the vegetative to reproductive phase in transgenic Arabidopsis. Plant Science **168,** 95-104.

Kotoda, N., Iwanami, H., Takahashi, S., and Abe, K. (2006). Antisense expression of MdTFL1, a TFL1-like gene, reduces the juvenile phase in apple. Journal of the American Society for Horticultural Science **131,** 74-81.

Krogan, N.J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Ryan, O.W., Golshani, A., Johnston, M., Greenblatt, J.F., and Shilatifard, A. (2003). The Paf1 Complex Is Required for Histone H3 Methylation by COMPASS and Dot1p: Linking Transcriptional Elongation to Histone Methylation. Molecular Cell **11,** 721-729.

Lammer, D., Cai, X., Arterburn, M., Chatelain, J., Murray, T., and Jones, S. (2004). A single chromosome addition from Thinopyrum elongatum confers a polycarpic, perennial habit to annual wheat. J. Exp. Bot. **55,** 1715-1720.

Lang, A. (1957). The effect of gibberellin upon flower formation. Proc Natl Acad Sci U S A **43,** 709-717.

Lang, A. (1965). Physiology of flower initiation. Encyclopedia of Plant Physiology ed. W. Ruhland**,** 1380-1536.

Langridge, J. (1957). Effect of day-length and gibberellic acid on the flowering of

Arabidopsis. Nature **180,** 36-37.

Le Corre, V., Roux, F., and Reboud, X. (2002). DNA polymorphism at the FRIGIDA gene in Arabidopsis thaliana: Extensive nonsynonymous variation is consistent with local selection for flowering time. Molecular Biology and Evolution **19,** 1261-1271.

Lee, H., Suh, S.-S., Park, E., Cho, E., Ahn, J.H., Kim, S.-G., Lee, J.S., Kwon, Y.M., and Lee, I. (2000). The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in *Arabidopsis*. Gene Dev **14,** 2366-2376.

Lee, I., Aukerman, M.J., Gore, S.L., Lohman, K.N., Michaels, S.D., Weaver, L.M., John, M.C., Feldmann, K.A., and Amasino, R.M. (1994). Isolation of LUMINIDEPENDENS: A gene involved in the control of flowering time in Arabidopsis. Plant Cell **6,** 75-83.

Lee, I.A.B., and Amasino, R. (1993). Analysis of naturally occurring late flowering in Arabidopsis thaliana. Molecular and General Genetics **237,** 171-176.

Lee, J.H., Yoo, S.J., Park, S.H., Hwang, I., Lee, J.S., and Ahn, J.H. (2007). Role of SVP in the control of flowering time by ambient temperature in Arabidopsis. Genes Dev. **21,** 397-402.

Lee, S., Cheng, H., King, K.E., Wang, W., He, Y., Hussain, A., Lo, J., Harberd, N.P., and Peng, J. (2002). Gibberellin regulates Arabidopsis seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition. Genes Dev. **16,** 646-658.

Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendall, A.R., and Dean, C. (2002). Multiple roles of Arabidopsis VRN1 in vernalization and flowering time control. Science **297,** 243-246.

Lifschitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z., Alvarez, J.P., and Eshed, Y. (2006). The tomato FT ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. PNAS **103,** 6398-6403.

Liljegren, S.J., Gustafson-Brown, C., Pinyopich, A., Ditta, G.S., and Yanofsky, M.F. (1999). Interactions among APETALA1, LEAFY, and TERMINAL FLOWER1 specify meristem fate. Plant Cell **11,** 1007-1018.

Lim, M.-H., Kim, J., Kim, Y.-S., Chung, K.-S., Seo, Y.-H., Lee, I., Kim, J., Hong, C.B., Kim, H.-J., and Park, C.-M. (2004). A New Arabidopsis Gene, FLK, Encodes an RNA Binding Protein with K Homology Motifs and Regulates Flowering Time via FLOWERING LOCUS C. Plant Cell **16,** 731-740.

Linhart, Y.B., and Grant, M.C. (1996). Evolutionary significance of local genetic diffenrentiation in plants. Annual Review of Ecology and Systematics **27,** 237-277.

Liu, J., He, Y., Amasino, R., and Chen, X. (2004). siRNAs targeting an intronic transposon in the regulation of natural flowering behavior in Arabidopsis. Genes Dev. **18,** 2873-2878.

Longman, K.A. (1976). Some experimental approaches to the problem of phase change in

forest trees. Acta Hort. (ISHS) **56,** 81-90.

Lysak, M., and Lexer, C. (2006). Towards the era of comparative evolutionary genomics in Brassicaceae. Plant Systematics and Evolution **259,** 175-198.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997). FCA, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. Cell **89,** 737-745.

Martin-Trillo, M., Lazaro, A., Poethig, R.S., Gomez-Mena, C., Pineiro, M.A., Martinez-Zapater, J.M., and Jarillo, J.A. (2006). EARLY IN SHORT DAYS 1 (ESD1) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates FLC accumulation in Arabidopsis. Development **133,** 1241-1252.

Martinez, C., Pons, E., Prats, G., and Leon, J. (2004). Salicylic acid regulates flowering time and links defence responses and reproductive development. The Plant Journal **37,** 209-217.

McSteen, P., and Leyser, O. (2005). Shoot branching. Annual Review of Plant Biology **56,** 353-374.

Meilan, R. (1997). Floral induction in woody angiosperms. New Forests **14,** 179-202.

Metzger, J. (1995). Hormones and Reproductive development. In: Davies PJ, ed. Plant hormones: physiology, biochemistry and molecular biology. Dordrecht, The Netherlands: Kluwer Academic Publishers, **,** 617–648.

Michaels, S.D., and Amasino, R.M. (1999). *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11,** 949-956.

Michaels, S.D., and Amasino, R.M. (2001). Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. Plant Cell **13,** 935-941.

Michaels, S.D., Bezerra, I.C., and Amasino, R.M. (2004). FRIGIDA-related genes are required for the winter-annual habit in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **101,** 3281-3285.

Michaels, S.D., He, Y., Scortecci, K.C., and Amasino, R.M. (2003a). Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. PNAS **100,** 10102-10107.

Michaels, S.D., Ditta, G., Gustafson-Brown, C., Pelaz, S., Yanofsky, M., and Amasino, R.M. (2003b). AGL24 acts as a promoter of flowering in Arabidopsis and is positively regulated by vernalization. The Plant Journal **33,** 867-874.

Moon, J., Suh, S.S., Lee, H., Choi, K.R., Hong, C.B., Paek, N.C., Kim, S.G., and Lee, I. (2003). The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. Plant Journal **35,** 613-623.

Mouradov, A., Cremer, F., and Coupland, G. (2002). Control of flowering time: interacting pathways as a basis for diversity. Plant Cell **14 Suppl,** S111-130.

Murai, K., Miyamae, M., Kato, H., Takumi, S., and Ogihara, Y. (2003). WAP1, a Wheat APETALA1 Homolog, Plays a Central Role in the Phase Transition from Vegetative to Reproductive Growth. Plant Cell Physiol. **44,** 1255-1265.

Mylne, J.S., Barrett, L., Tessadori, F., Mesnage, S., Johnson, L., Bernatavichute, Y.V., Jacobsen, S.E., Fransz, P., and Dean, C. (2006). LHP1, the Arabidopsis homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of FLC. PNAS **103,** 5012-5017.

Nakajima, M., Shimada, A., Takashi, Y., Kim, Y.-C., Park, S.-H., Ueguchi-Tanaka, M., Suzuki, H., Katoh, E., Iuchi, S., Kobayashi, M., Maeda, T., Matsuoka, M., and Yamaguchi, I. (2006). Identification and characterization of Arabidopsis gibberellin receptors. The Plant Journal **46,** 880-889.

Napp-Zinn, K. (1987). Vernalization. Environmental and genetic regulation. In Manipulation of Flowering (ed. J.G.Atherton). Butterworths, London.**,** 123-132.

Ng, H.H., Robert, F., Young, R.A., and Struhl, K. (2003). Targeted Recruitment of Set1 Histone Methylase by Elongating Pol II Provides a Localized Mark and Memory of Recent Transcriptional Activity. Molecular Cell **11,** 709-719.

Nilsson, O., Lee, I., Blazquez, M.A., and Weigel, D. (1998). Flowering-time genes modulate the response of LEAFY activity. Genetics **150,** 403-410.

Oh, S., Zhang, H., Ludwig, P., and van Nocker, S. (2004). A Mechanism Related to the Yeast Transcriptional Regulator Paf1c Is Required for Expression of the Arabidopsis FLC/MAF MADS Box Gene Family. Plant Cell **16,** 2940-2953.

Okazaki, K., Sakamoto, K., Kikuchi, R., Saito, A., Togashi, E., Kuginuki, Y., Matsumoto, S., and Hirai, M. (2007). Mapping and characterization of FLC homologs and QTL analysis of flowering time in Brassica oleracea. Theoretical and Applied Genetics **114,** 595-608.

Olszewski, N., Sun, T., and Gubler, F. (2002). Gibberellin signaling: biosynthesis, catabolism and response pathways. Plant Cell **14,** S61-80.

Osborn, T.C., Kole, C., Parkin, I.A.P., Sharpe, A.G., Kuiper, M., Lydiate, D.J., and Trick, M. (1997). Comparison of Flowering Time Genes in Brassica rapa, B. napus and Arabidopsis thaliana. Genetics **146,** 1123-1129.

Parcy, F., Nilsson, O., Busch, M.A., Lee, I., and Weigel, D. (1998). A genetic framework for floral patterning. Nature **395,** 561-566.

Pena, L., Martin-Trillo, M., Juarez, J., Pina, J.A., Navarro, L., and Martinez-Zapater, J.M. (2001). Constitutive expression of Arabidopsis LEAFY or APETALA1 genes in citrus reduces their generation time. Nature Biotechnology **19,** 263-267.

Peng, J.R., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P., and Harberd, N.P. (1997). The Arabidopsis GAI gene defines a signaling pathway that negatively regulates gibberellin responses. Gene Dev **11,** 3194-3205.

Pillitteri, L.J., Lovatt, C.J., and Walling, L.L. (2004). Isolation and Characterization of a TERMINAL FLOWER Homolog and Its Correlation with Juvenility in Citrus. Plant Physiol. **135,** 1540-1551.

Pineiro, M., Gomez-Mena, C., Schaffer, R., Martinez-Zapater, J.M., and Coupland, G. (2003). EARLY BOLTING IN SHORT DAYS is related to chromatin remodelling factors and regulates flowering in Arabidopsis by repressing FT. Plant Cell **15,** 1552-1562.

Plogander, A.Z. (2005). Analysis of the Regulatory Sequences of FT, a Flowering-Time Gene of Arabidopsis Thaliana. Diplomarbeit.

Pnueli, L., Gutfinger, T., Hareven, D., Ben-Naim, O., Ron, N., Adir, N., and Lifschitz, E. (2001). Tomato SP-Interacting Proteins Define a Conserved Signaling System That Regulates Shoot Architecture and Flowering. Plant Cell **13,** 2687-2702.

Poethig, R.S. (1990). Phase-Change and the Regulation of Shoot Morphogenesis in Plants. Science **250,** 923-930.

Poethig, R.S. (2003). Phase Change and the Regulation of Developmental Timing in Plants. Science **301,** 334-336.

Prud'homme, B., Gompel, N., Rokas, A., Kassner, V.A., Williams, T.M., Yeh, S.-D., True, J.R., and Carroll, S.B. (2006). Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. Nature **440,** 1050-1053.

Ratcliffe, O.J., Bradley, D.J., and Coen, E.S. (1999). Separation of shoot and floral identity in Arabidopsis. Development **126,** 1109-1120.

Ratcliffe, O.J., Nadzan, G.C., Reuber, T.L., and Riechmann, J.L. (2001). Regulation of flowering in Arabidopsis by an FLC homologue. Plant Physiol **126,** 122-132.

Ratcliffe, O.J., Kumimoto, R.W., Wong, B.J., and Riechmann, J.L. (2003). Analysis of the Arabidopsis MADS AFFECTING FLOWERING Gene Family: MAF2 Prevents Vernalization by Short Periods of Cold. Plant Cell **15,** 1159-1169.

Ratcliffe, O.J., Amaya, I., Vincent, C.A., Rothstein, S., Carpenter, R., Coen, E.S., and Bradley, D.J. (1998). A common mechanism controls the life cycle and architecture of plants. Development **125,** 1609-1615.

Razem, F.A., El-Kereamy, A., Abrams, S.R., and Hill, R.D. (2006). The RNA-binding protein FCA is an abscisic acid receptor. Nature **439,** 290-294.

Reeves, P.A., He, Y., Schmitz, R.J., Amasino, R.M., Panella, L.W., and Richards, C. (2006). Evolutionary conservation of the FLC mediated vernalization response: evidence from the sugar beet (Beta vulgaris). Genetics**, 176,** 295-307.

Reyes, J.L., and Chua, N.-H. (2007). ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. The Plant Journal **49,** 592-606.

Richards, D.E., King, K.E., Ait-ali, T., and Harberd, N.P. (2001). How gibberellin regulates plant growth and development: a molecular genetic analysis of gibberellin signaling. Annual Review of Plant Physiology and Plant Molecular Biology **52,** 67-88.

Ringrose, L., and Paro, R. (2004). Epigenetic regulation of cellular memory by the polycomb and trithorax group proteins. Annual Review of Genetics **38,** 413-443.

Roux, F., Touzet, P., Cuguen, J., and Le Corre, V. (2006). How to be early flowering: an

evolutionary perspective. Trends in Plant Science **11,** 375-381.

Sakakibara, H. (2006). CYTOKININS: Activity, Biosynthesis, and Translocation. Annual Review of Plant Biology **57,** 431-449.

Salisbury, F. (1985). Photoperiodism. Hortic Rev (Am Soc Hortic Sci) **4,** 66-105.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science **288,** 1613-1616.

Sambrook, J., and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press.

Schmitz, G., and Theres, K. (2005). Shoot and inflorescence branching. Current Opinion in Plant Biology **8,** 506-511.

Schmitz, R.J., Hong, L., Michaels, S., and Amasino, R.M. (2005). FRIGIDA-ESSENTIAL 1 interacts genetically with FRIGIDA and FRIGIDA-LIKE 1 to promote the winter-annual habit of Arabidopsis thaliana. Development **132,** 5471-5478.

Schomburg, F.M., Patton, D.A., Meinke, D.W., and Amasino, R.M. (2001). FPA, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. Plant Cell **13,** 1427-1436.

Schonrock, N., Bouveret, R., Leroy, O., Borghi, L., Kohler, C., Gruissem, W., and Hennig, L. (2006). Polycomb-group proteins repressthe floral activator AGL19 in the FLC-independent vernalization pathway. Genes Dev. **20,** 1667-1678.

Schranz, M.E., Quijada, P., Sung, S.-B., Lukens, L., Amasino, R., and Osborn, T.C. (2002). Characterization and Effects of the Replicated Flowering Time Gene FLC in Brassica rapa. Genetics **162,** 1457-1468.

Schumacher, K., Schmitt, T., Rossberg, M., Schmitz, G., and Theres, K. (1999). The Lateral suppressor (Ls) gene of tomato encodes a new member of the VHIID protein family. PNAS **96,** 290-295.

Scortecci, K., Michaels, S.D., and Amasino, R.M. (2003). Genetic interactions between FLM and other flowering-time genes in Arabidopsis thaliana. Plant Molecular Biology **52,** 915-922.

Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Krober, S., Amasino, R.A., and Coupland, G. (2006). The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. Genes Dev. **20,** 898-912.

Shannon, S., and Meeks-Wagner, D.R. (1991). A Mutation in the Arabidopsis Tfl1 Gene Affects Inflorescence Meristem Development. Plant Cell **3,** 877-892.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999). The FLF MADS box gene: A repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell **11,** 445-458.

Shindo, C., Aranzana, M.J., Lister, C., Baxter, C., Nicholls, C., Nordborg, M., and Dean, C. (2005). Role of FRIGIDA and FLOWERING LOCUS C in Determining

Variation in Flowering Time of Arabidopsis. Plant Physiol. **138,** 1163-1173.

Silverstone, A.L., Jung, H.S., Dill, A., Kawaide, H., Kamiya, Y., and Sun, T.P. (2001). Repressing a repressor: Gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. Plant Cell **13,** 1555-1565.

Simon, R., Igeno, M.I., and Coupland, G. (1996). Activation of floral meristem identity genes in *Arabidopsis*. Nature **384,** 59-62.

Simpson, G.G., and Dean, C. (2002). *Arabidopsis*, the Rosetta stone of flowering time? Science **296,** 285-289.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I., and Dean, C. (2003). FY Is an RNA 3' End-Processing Factor that Interacts with FCA to Control the Arabidopsis Floral Transition. Cell **113,** 777-787.

Snow, M., and Snow, R. (1942). The determination of axillary buds. New Phytologist **41,** 13-22.

Stinchcombe, J.R., Weinig, C., Ungerer, M., Olsen, K.M., Mays, C., Halldorsdottir, S.S., Purugganan, M.D., and Schmitt, J. (2004). A latitudinal cline in flowering time in Arabidopsis thaliana modulated by the flowering time gene FRIGIDA. Proceedings of the National Academy of Sciences of the United States of America **101,** 4712-4717.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and Coupland, G. (2001). *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. Nature **410,** 1116-1120.

Sung, S., and Amasino, R.M. (2005). Remembering winter: toward a molecular understanding of vernalization. Annual Review of Plant Biology **56,** 491-508.

Sung, S., Schmitz, R.J., and Amasino, R.M. (2006a). A PHD finger protein involved in both the vernalization and photoperiod pathways in Arabidopsis. Genes Dev. **20,** 3244-3248.

Sung, S., He, Y., Eshoo, T.W., Tamada, Y., Johnson, L., Nakahigashi, K., Goto, K., Jacobsen, S.E., and Amasino, R.M. (2006b). Epigenetic maintenance of the vernalized state in Arabidopsis thaliana requires LIKE HETEROCHROMATIN PROTEIN 1. Nat Genet **38,** 706-710.

Sung, S.B., and Amasino, R.M. (2004). Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature **427,** 159-164.

Tadege, M., Sheldon, C.C., Helliwell, C.A., Stoutjesdijk, P., Dennis, E.S., and Peacock, W.J. (2001). Control of flowering time by FLC orthologues in Brassica napus. The Plant Journal **28,** 545-553.

Takada, S., and Goto, K. (2003). TERMINAL FLOWER2, an Arabidopsis homolog of HETEROCHROMATIN PROTEIN1, counteracts the activation of *FLOWERING LOCUS T* by CONSTANS in the vascular tissues of leaves to regulate flowering time. Plant Cell **15,** 2856-2865.

Tamaki, S., Matsuo, S., and Wong HL, Y.S., Shimamoto K. . (2007). Hd3a Protein Is a Mobile Flowering Signal in Rice. Science **316,** 1033-1036.

Tan, F.-C., and Swain, S.M. (2006). Genetics of flower initiation and development in annual and perennial plants. Physiologia Plantarum **128,** 8-17.

Thomas, H., Thomas, H.M., and Ougham, H. (2000). Annuality, perenniality and cell death. J. Exp. Bot. **51,** 1781-1788.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. **25,** 4876-4882.

Townsend, T; Albani, M; Wilkinson, M; Coupland, G; Battey, N. (2006). The diversity and significance of flowering in perennials. Flowering and its manipulation (Annual plant reviews vol. 20), 181-197.

Tranquilli, G., and Dubcovsky, J. (2000). Epistatic interaction between vernalization genes Vrn-A(m)1 and Vrn-A(m)2 in diploid wheat. J Hered **91,** 304-306.

Trevaskis, B., Bagnall, D.J., Ellis, M.H., Peacock, W.J., and Dennis, E.S. (2003). MADS box genes control vernalization-induced flowering in cereals. PNAS **100,** 13099-13104.

Tuskan, G.A., and al., e. (2006). The Genome of Black Cottonwood, Populus trichocarpa (Torr. & Gray). Science **313,** 1596-1604.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T.-y., Hsing, Y.-i.C., Kitano, H., Yamaguchi, I., and Matsuoka, M. (2005). GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. Nature **437,** 693-698.

Wagner, D., Sablowski, R.W.M., and Meyerowitz, E.M. (1999). Transcriptional activation of APETALA1 by LEAFY. Science **285,** 582-584.

Warwick, S., Francis, A., and Al-Shehbaz, I. (2006). Brassicaceae: Species checklist and database on CD-Rom. Plant Systematics and Evolution **259,** 249-258.

Weigel, D., and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. Nature **377,** 495-500.

Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F., and Meyerowitz, E.M. (1992). Leafy Controls Floral Meristem Identity in Arabidopsis. Cell **69,** 843-859.

Wellensiek, S.J. (1962). Dividing Cells as the Locus for Vernalization. Nature **195,** 307-308.

Wellensiek, S.J. (1964). Dividing Cells as the Prerequisite for Vernalization. Plant Physiol. **39,** 832-835.

Wen, C.-K., and Chang, C. (2002). Arabidopsis RGL1 encodes a negative regulator of gibberellin responses. Plant Cell **14,** 87-100.

Werner, J.D., Borevitz, J.O., Warthmann, N., Trainer, G.T., Ecker, J.R., Chory, J., and Weigel, D. (2005). Quantitative trait locus mapping and DNA array hybridization identify an FLM deletion as a cause for natural flowering-time variation. PNAS **102,** 2460-2465.

Whittaker, R.J. (1993). Plant population patterns in a glacier foreland succession: pioneer

herbs and later-colonizing shrubs. Ecography **16,** 117-136.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U., and Weigel, D. (2005). Integration of spatial and temporal information during floral induction in Arabidopsis. Science **309,** 1056-1059.

William, D.A., Su, Y., Smith, M.R., Lu, M., Baldwin, D.A., and Wagner, D. (2004). Genomic identification of direct target genes of LEAFY. PNAS **101,** 1775-1780.

Willige, B.C., Ghosh, S., Nill, C., Zourelidou, M., Dohmann, E.M.N., Maier, A., and Schwechheimer, C. (2007). The DELLA Domain of GA INSENSITIVE Mediates the Interaction with the GA INSENSITIVE DWARF1A Gibberellin Receptor of Arabidopsis. Plant Cell**,** tpc.107.051441.

Wilson, R.N., Heckman, J.W., and Somerville, C.R. (1992). Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. Plant Physiol **100,** 403-408.

Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T., and Dubcovsky, J. (2003). Positional cloning of wheat vernalization gene *VRN1*. Proc Natl Acad Sci USA **100,** 6263–6268.

Yan, L., Loukoianov, A., Blechl, A., Tranquilli, G., Ramakrishna, W., SanMiguel, P., Bennetzen, J.L., Echenique, V., and Dubcovsky, J. (2004). The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. Science **303,** 1640-1644.

Yano, M., Harushima, Y., Nagamura, Y., Kurata, N., Minobe, Y., and Sasaki, T. (1997). Identification of quantitative trait loci controlling heading date in rice using a high-density linkage map. Theor Appl Genet **95,** 1025-1032.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y., and Sasaki, T. (2000). *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. Plant Cell **12,** 2473-2483.

Yoo, S.K., Chung, K.S., Kim, J., Lee, J.H., Hong, S.M., Yoo, S.J., Yoo, S.Y., Lee, J.S., and Ahn, J.H. (2005). CONSTANS Activates SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 through FLOWERING LOCUS T to Promote Flowering in Arabidopsis. Plant Physiol. **139,** 770-778.

Yu, H., Xu, Y., Ling Tan, E., and Kumar, P.P. (2002). AGAMOUS-LIKE 24, a dosage-dependent mediator of the flowering signals. Proc. Natl. Acad. Sci. USA **99,** 16336-16341.

Yuceer, C., Land, S.B., Jr., Kubiske, M.E., and Harkess, R.L. (2003). Shoot morphogenesis associated with flowering in Populus deltoides (Salicaceae). Am. J. Bot. **90,** 196-206.

Zimmerman, R.H. (1985a). Juvenility and flowering in woody plants: a review. Hortscience **7,** 447-455.

Zimmerman, R.H., Hackett, W.P., and Pharis, R.P. . (1985b). Hormonal aspects of phase change and precocious flowering. Encycl. Plant Physiol. **11,** 79–115.

9. Appendix

9.1. Alignment of AaFLC and AtFLC

```
SeqA Name    Len(aa)  SeqB Name    Len(aa)  Score
==================================================
 1   AaFLC     207      3    AtFLC    196       85
==================================================

AaFLC           MGRKKLEIKRIENKSSRQVTFSKRRNGLIEKARQLSVLCDASVALLVVSSSGKLYSFSSG 60
AtFLC           MGRKKLEIKRIENKSSRQVTFSKRRNGLIEKARQLSVLCDASVALLVVSASGKLYSFSSG 60
                *************************************************:**********

AaFLC           DNLVKILDRYGKRHADDLKALDLQSLLHGQDLQSKALNYGSHHELLEIVESKLVAPNVNN 120
AtFLC           DNLVKILDRYGKQHADDLKALDHQ---------SKALNYGSHYELLELVDSKLVGSNVKN 111
                ************:********* *         *********:****:*:****..**:*

AaFLC           VSFDTLVQLEKHLETALAVVRAKKTELMLKLLESLKEKEKLLKEENQVLASQMEKKTLVG 180
AtFLC           VSIDALVQLEEHLETALSVTRAKKTELMLKLVENLKEKEKMLKEENQVLASQMENNHHVG 171
                **:*:*****:******:*.***********:*.******:*************::  **

AaFLC           AEADDNMEISP-GEISDINLPVTLPLLN 207
AtFLC           AEAE--MEMSPAGQISD-NLPVTLPLLN 196
                ***:  **:** *:*** **********
```

9.2. CDS of *AaFLC*

**ATGGGTAGAAAAAAACTCGAAATCAAACGAATTGAGAACAAAA
GTAGCCGACAAGTCACCTTCTCTAAACGTCGCAACGGTCTCAT
CGAGAAAGCTCGTCAGCTTTCTGTTCTATGTGATGCCTCCGTA
GCTCTCCTTGTCGTCTCCTCCTCTGGCAAACTCTACAGCTTCTC
CTCCGGTGATAACCTGGTCAAGATCCTTGATCGATATGGAAAA
CGACATGCAGATGATCTCAAAGCCCTGGATCTTCAGTCCCTTC
TCCATGGACAGGATCTTCAGTCAAAAGCTCTGAACTATGGTTC
ACACCATGAGCTACTAGAAATTGTTGAAAGCAAGCTCGTGGCA
CCAAATGTCAATAACGTAAGTTTCGACACTCTCGTTCAGCTGG
AAAAACACCTTGAAACTGCTCTCGCCGTAGTTAGAGCTAAGAA
GACAGAACTAATGTTGAAGCTTCTTGAGAGCCTTAAAGAAAAG
GAGAAATTGCTGAAAGAAGAAAACCAGGTTTTGGCTAGCCAGA
TGGAGAAGAAGACTCTTGTGGGAGCAGAAGCTGATGATAATAT
GGAGATATCACCTGGAGAAATCTCCGACATCAATCTTCCGGTA
ACTCTCCCACTACTCAATTAA**

9.3. Alignment of AaFT, AaTSF1, AaTSF2, AtFT, AtTSF, AaTFL1 and AtTFL1

```
SeqA Name   Len(aa) SeqB Name    Len(aa) Score
===================================================
1    AaFT     175      2    AaTSF1   176      84
1    AaFT     175      3    AaTSF2   175      84
1    AaFT     175      4    AtFT     175      90
1    AaFT     175      5    AtTSF    175      85
1    AaFT     175      6    AaTFL1   177      55
1    AaFT     175      7    AtTFL1   177      54
2    AaTSF1   176      3    AaTSF2   175      86
2    AaTSF1   176      4    AtFT     175      80
2    AaTSF1   176      5    AtTSF    175      83
2    AaTSF1   176      6    AaTFL1   177      53
2    AaTSF1   176      7    AtTFL1   177      53
3    AaTSF2   175      4    AtFT     175      81
3    AaTSF2   175      5    AtTSF    175      81
3    AaTSF2   175      6    AaTFL1   177      52
3    AaTSF2   175      7    AtTFL1   177      51
4    AtFT     175      5    AtTSF    175      81
4    AtFT     175      6    AaTFL1   177      56
4    AtFT     175      7    AtTFL1   177      56
5    AtTSF    175      6    AaTFL1   177      53
5    AtTSF    175      7    AtTFL1   177      53
6    AaTFL1   177      7    AtTFL1   177      94
===================================================
```

```
AaFT            ---MSISPRDPLVVGGVVGDVLDAFTRSISLRVTYGQREVTNGLDLRPSQVQNKPRVEIG 57
AtFT            ---MSINIRDPLIVSRVVGDVLDPFNRSITLKVTYGQREVTNGLDLRPSQVQNKPRVEIG 57
AaTSF1          ---MSLSRTDPLVVGRVVGDVLDPFTRLISLRVVYGQRQVTNGLDLRSSQVLNRPTVEIG 57
AaTSF2          ---MSLSGKDPHVVGRVVGDVVDPFTRLISLKVTYGQRQVTNGLDLRSSQVVNKPMVEIG 57
AtTSF           ---MSLSRRDPLVVGSVVGDVLDPFTRLVSLKVTYGHREVTNGLDLRPSQVLNKPIVEIG 57
AaTFL1          MENIGSRLIEPLIVGRVVGEVLDYFTPTIKMNVSYNKKQVSNGHELFPSTVSSKPRVEIH 60
AtTFL1          MENMGTRVIEPLIIGRVVGDVLDFFTPTTKMNVSYNKKQVSNGHELFPSSVSSKPRVEIH 60
                    :.   :* ::. ***:*:* *.   .:.* *.:::*:** :* .* * .:* ***

AaFT            GEDLRNFYTLVMVDPDVPSPSNPHLREYLHWLVTDIPATTGTTFGNEIVCYENPSPTSGI 117
AtFT            GEDLRNFYTLVMVDPDVPSPSNPHLREYLHWLVTDIPATTGTTFGNEIVCYENPSPTAGI 117
AaTSF1          GDDLRNFYTLVMVDPDVPSPSNPYLREYLHWLVTDIPATTGTAFGNEMICYENPCPTSGI 117
AaTSF2          GFDLRNFYTLVMVDLDVPSPSNPYLREYLHWLVTDIPATTGTAFGNEMVCYENPCPTSGI 117
AtTSF           GDDFRNFYTLVMVDPDVPSPSNPHQREYLHWLVTDIPATTGNAFGNEVVCYESPRPPSGI 117
AaTFL1          GGDLRSFFTLVMIDPDVPGPSDPFLKEHLHWIVTNIPGTTDATFGKEVVSYELPRPSIGI 120
```

```
AtTFL1          GGDLRSFFTLVMIDPDVPGPSDPFLKEHLHWIVTNIPGTTDATFGKEVVSYELPRPSIGI 120
                * *:*.*:****:* ***.**:*. :*:***:**:**.**. :**:*::.** * *. **

AaFT            HRIVLILFRQLGRQTVYA-PGWRQQFNTREFAEIYNLGHPVAAVFFNCQRESGCGGRRS- 175
AtFT            HRVVFILFRQLGRQTVYA-PGWRQNFNTREFAEIYNLGLPVAAVFYNCQRESGCGGRRL- 175
AaTSF1          HRVVLVLFRQLGRQTVYP-PEWRQRFNTRGFAEMYNLGHPVAAVFFNCQKENGCGGRRTN 176
AaTSF2          HRLVLIMFRQLGRQTVYA-PEWRQRFNTREFAENYNLGLPLAAVFFNCQRQNGCGGRRS- 175
AtTSF           HRIVLVLFRQLGRQTVYA-PGWRQQFNTREFAEIYNLGLPVAASYFNCQRENGCGGRRT- 175
AaTFL1          HRFVFVLFKQKQRRVIFPNIPSRDHFNTREFAVEYDLGLPVAAVFFNAQRETAARKR--- 177
AtTFL1          HRFVFVLFRQKQRRVIFPNIPSRDHFNTRKFAVEYDLGLPVAAVFFNAQRETAARKR--- 177
                **.*:::*:*  *:.::.    *:.**** **  *:** *:** ::*.*::...  *
```

9.4. CDS of *AaTFL1*

ATGGAGAATATTGGAAGTAGACTGATAGAGCCATTGATAGT
TGGAAGAGTGGTAGGAGAAGTTCTTGATTACTTCACTCCAA
CAATTAAAATGAATGTGAGTTACAACAAGAAGCAAGTCTCC
AATGGCCATGAGCTTTTCCCTTCCACTGTCTCCTCCAAGCCT
AGGGTTGAGATCCATGGTGGTGATCTCAGATCCTTCTTCAC
CTTGGTGATGATAGACCCTGATGTTCCAGGTCCTAGTGACC
CCTTCCTAAAAGAACACCTGCATTGGATCGTTACAAACATCC
CCGGTACAACAGATGCTACATTTGGAAAAGAGGTGGTGAGC
TATGAATTGCCAAGGCCAAGCATAGGGATACATAGGTTTGT
GTTTGTTCTCTTCAAGCAGAAGCAAAGACGCGTCATCTTCCC
CAATATACCTTCGAGAGATCACTTCAACACTCGTGAATTCGC
AGTCGAGTATGATCTTGGTCTTCCTGTCGCTGCGGTGTTCTT
TAACGCTCAAAGGGAAACCGCGGCTCGCAAACGCTAG

9.5. Alignment of AaSOC1 and AtSOC1

```
SeqA Name     Len(aa)  SeqB Name     Len(aa)  Score
===================================================
 1   AaSOC1    216       2    AtSOC1    214       93
===================================================
```

```
AaSOC1          MVRGKTQMKRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVSLIIFSPKGKLYEFASS 60
AtSOC1          MVRGKTQMKRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVSLIIFSPKGKLYEFASS 60
                ************************************************************

AaSOC1          NMQETIDRYVRHTKDRIINKPVSEENMQHLKHEAANMMKKIEQLEASKRKLLGEGIGSCS 120
AtSOC1          NMQDTIDRYLRHTKDRVSTKPVSEENMQHLKYEAANMMKKIEQLEASKRKLLGEGIGTCS 120
```

```
                  ***:*****:******:  .************:*************************:**
AaSOC1            IEELQQIEQQLEKSVKCIRARKTQVFKEQIEQLKQKEKALAAENEKLSEKWGSSNEVVVW 180
AtSOC1            IEELQQIEQQLEKSVKCIRARKTQVFKEQIEQLKQKEKALAAENEKLSEKWG-SHESEVW 179
                  **************************************************** *:*  **

AaSOC1            SNKNEESGRGTGDEESSPSSEVETQLFIGLPCSSRK 216
AtSOC1            SNKNQES-TGRGDEESSPSSEVETQLFIGLPCSSRK 214
                  ****:**  * *************************
```

9.6. CDS of *AaSOC1*

**ATGGTGAGGGGGAAAACTCAAATGAAGCGAATAGAGAATGC
AACAAGTAGACAAGTGACATTCTCTAAGAGAAGAAATGGTT
TATTGAAGAAAGCTTTTGAGCTTTCAGTGCTTTGTGATGCTG
AAGTTTCTCTAATCATCTTCTCTCCTAAAGGAAAACTTTATG
AATTCGCCAGTTCCAATATGCAAGAAACCATAGATCGTTATG
TGAGGCATACTAAAGATCGAATCATCAACAAACCGGTTTCT
GAAGAAAATATGCAGCATTTGAAACATGAAGCAGCAAATAT
GATGAAGAAAATCGAACAACTCGAAGCATCCAAACGTAAAC
TTTTGGGAGAAGGTATAGGTTCATGCTCAATAGAGGAGCTT
CAACAAATTGAGCAACAACTTGAGAAAAGTGTCAAATGTATT
CGAGCAAGAAAGACTCAAGTGTTTAAGGAACAAATTGAGCA
GCTCAAGCAAAAGGAGAAAGCTCTTGCTGCAGAAAATGAGA
AGCTCTCTGAAAAGTGGGGATCATCTAATGAAGTGGTGGTT
TGGTCGAATAAGAATGAAGAAAGTGGAAGAGGTACTGGTGA
TGAAGAGAGTAGCCCAAGTTCTGAAGTAGAGACACAATTGT
TCATTGGGTTACCTTGTTCTTCAAGAAAGTGA**

Erklaerung

“Ich versichere, das ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeiteinschließlich Tabellen, Karten und Abbildungen, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung Kenntlich gemacht habe; daß diese Disseertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie-abgeseben Teiöpublikationen-noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bdkannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. George Coupland betreut worden”

Lebenslauf

Persönliche Informationen

Name	Wang
Vorname	Renhou
Geburtsort	Jilin China
Geburtstag	11. April 1976
Nationalität	chinesisch
Familienstand	verheirat

Ausbildung

Seit 2003	Doktorand am Max-Planck-Institute für Züchtungsforschung in Köln unter der Leitung von Prof. Dr. George Coupland, Mitglied der "Max Planck Research School (IMPRS)", Schwerpunkt: Genetik, Molekularbiologie
2000- 2003	"Postgraduate research" am Institut für Genetik und Zytologie der "Northeast Normal University" in Changchun unter der Leitung von Prof. Dr. Xingzhi Wang. Schwerpunkt: Zellbiologie
1996-2000	"Undergraduate study" an der "School of Life Sciences Northeast Normal University" in Changchun. Schwerpunkt: Biotechnologie