ABSTRACT

Title of Dissertation:	TYPE II MADS-BOX GENES ASSOCIATED WITH POPLAR APICAL BUD DEVELOPMENT AND DORMANCY
	Kuang-Yu Chen, Doctor of Philosophy, 2008
Dissertation Directed By:	Associate Professor, Gary D. Coleman, Department of Plant Sciences and Landscape Architecture

MADS-box transcription factors regulate the development of vegetative and reproductive organs in plants. Little is known about the role of MADS-box genes in tree development. Using phylogenetic analysis, 57 putative type II MADS-box genes representing 14 functional classes were identified in the *Populus trichocarpa* genome. cDNA sequencing of the poplar type II MADS-box genes indicates that 28.1% of the transcripts differed in the intron-exon structures predicted in the genome database and 19.3% of the transcripts appear to be alternatively spliced. The majority of the poplar type II MADS-box genes were expressed in a wide variety of tissues including shoot apices, leaves, bark, xylem, root, and floral tissues and in shoot apices during bud development and dormancy. These results indicate that poplar MADS-box genes have diverse regulatory roles in a broad range of tissues and developmental processes.

Six poplar *FLC-like* genes, *PtFLC1–PtFLC6*, were identified in the poplar genome and expression of all six genes was detected in poplar shoot apices. The expression of one gene, *PtFLC2,* declined in apical buds during SD photoperiod and low temperature induced dormancy development suggesting a role in bud dormancy and may represent an analogous regulatory mechanism to the down-regulation of *FLC* during vernalization in *Arabidopsis*. In addition, several *PtFLC2* splice isoforms (*PtFLC2as1–9*) were identified that were associated with the later stages of bud dormancy. Overexpression of the *PtFLC2as1* isoform delayed photoperiod induced apical bud development and bud dormancy, growth cessation, and leaf senescence while

overexpression of the *PtFLC2as2* isoform appeared to accelerate bud development and dormancy and reduce the amount of chilling required to overcome dormancy.

These findings suggest that *PtFLC2*, unlike *Arabidopsis FLC*, could be an integration point for both photoperiod and cold signals that regulate bud development and dormancy. These results also suggest that in addition to transcriptional regulation, that cold-mediated production of *PtFLC2* splicing isoforms may have an important regulatory role in bud dormancy. The regulated production of splicing isoforms could regulate bud dormancy either by dominate negative interactions, by forming different protein complexes or regulating different pathways that regulate growth, dormancy, and dormancy release.

TYPE II MADS-BOX GENES ASSOCIATED WITH POPLAR APICAL BUD DEVELOPMENT AND DORMANCY

By

Kuang-Yu Chen

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2008

Advisory Committee:
Professor Gary D. Coleman, Chair
Professor Zhongchi Liu
Professor Stephen M. Mount
Professor Margaret Pooler
Professor Joseph H. Sullivan

Dedication

This dissertation is dedicated to my husband Tsyr-Huei Chiou who regularly tells me that nothing is beyond reach, my parents Chih-Hsuan Chen and Shu-Ching Chen who raised me to who I am, and my country Taiwan which has enriched and empowered my life.

Acknowledgements

Firstly, I would like to thank my advisor and mentor, Dr. Gary Coleman, who inspired me to study on tree dormancy and gave 100% trust and support for me to develop, design and carry out my project even in the difficult time of our lab.

I would like to acknowledge my committee for their continued support, advice, help and encouragement of my project and study over the past five years.

Many aspects of this project would not have been possible without the help and input of other people. Dr. Charles Delwiche drew my focus on MADS-box genes. Dr. Zhongchi Liu encouraged my interest on plant development and genetics and guided me in the study of *Arabidopsis*. Dr. Steve Mount brought to my attention on the study of alternatively spliced variants. Dr. Cecile Parmentier-Line guided me through the experimental world of molecular and genetic biology. Mr. Shaun Faulkner ensured that all the growth chambers and microcosms were functioning normally with plenty supplies for me to carry out my experiments. Dr. Kuang-Chi Chen offered her expertise and assisted on statistical analysis. Dr. Tsyr-Huei Chiou took almost all the photos, worked together with me in histology, and discussed with me daily whenever I wanted.

Finally I would like to thank my family, friends, and members in Dr. Coleman's lab who have given me endless support, love, and encouragement.

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2iP, N6-(2-isopenthy) adenine, cytokinins
ABA, abscisic acid
AG, AGamous
AP1, Apetala 1
AP3, Apetala 3
AS, alternative splicing
Basta, non-selective herbicide glufosinate
CArG-box, CC(A/T)6GG motif
CO, COnstans
Col, Columbia accession of Arabidopsis thaliana
FCA, Flowering time Control protein A
FLC, Flowering Locus C
FRI, Frigida
FUL, Fruitfull
FT, Flowering Locus T
GA, Gibberellic Acid
JGI, DOE Joint Genome Institute
LB, Luria-Bertani medium
LD, Long Day photoperiod
LER, Landsberg erecta accession of Arabidopsis thaliana
LS, Linsmair-Skoog's medium, a modified Murashige and Skoog medium
MADS, MCM1 (MiniChromosome Maintenance 1)-Agamous-Deficiens-SRF
MAF1–5, MADS Affecting Flowering 1–5
NAA, 1-naphthylacetic acid, auxin
PAR, Photosynthetically Active Radiation
PCR, Polymerase Chain Reaction
PI, Pistillata
PLSC, Plant Science Building
Pt, prefix in every gene from the JGI Populus trichocarpa genome database
PtFLC, Poplar FLC-like gene
RH, relative humidity
RT-PCR, Reverse Transcriptase PCR
SAM, Shoot Apical Meristem
SD, Short Day photoperiod
SD#, # week(s) of SD (8 hours light, 18°C)
SDC#, SD# with Chilling temperature (10/4°C day/night), there is 0, 256, 296, 752, 1024 chilling hours in SDC8, SDC10.3, SDC12.4, SDC14.7, and SDC17
SEP, SEPallata
SOC1, Suppressor of Overexpression of Constans 1
PtUBQL, Poplar Polyubiquitin-like gene
TDZ, 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea, cytokinins
VIN3, Vernalization INsensitive 3

Chapter 1: Literature review

Dormancy

Tree growth cycle and *Populus*

Trees are characterized by perennial growth, large size, complex crown architecture, extensive secondary xylem (wood) formation, and vegetative and floral bud dormancy (Taylor 2002; Wullschleger et al. 2002). The annual growth cycle of temperate trees is characterized by active shoot growth during spring and summer followed by a quiescent or dormant vegetative phase (Figure 1-1). The length of the growth period between spring bud break and fall bud set impacts tree productivity and wood quality. Dormancy is a survival strategy that enables plants to survive unfavorable environmental conditions (Rohde et al. 2000; Thomas et al. 1997). Bud dormancy also synchronizes shoot growth and influences plant architecture (Rohde et al. 2000). Phytohormones such as gibberellic acid (GA), abscisic acid (ABA), and ethylene, as well as environmental cues including day length, light quality, temperature, and nutrients regulate the cycle of bud dormancy (Rohde et al. 2000).

The genus *Populus* is native to only the northern hemisphere and includes cottonwoods, poplars, and aspens which are collectively termed poplars. The genus is comprised of approximately 30 species and is one of the fastest growing temperate zone trees (Taylor 2002). Poplars are deciduous or semi-evergreen and range from the tropics to the northern latitudinal limits of tree growth (Dickmann 2001). Poplars are dioecious and usually require about 5–7 years to flower after germination. The genome of poplar is relatively small, about 485 Mbp, consisting of 19 chromosomes (Taylor 2002; Wullschleger et al. 2002). The completion of the *Populus trichocarpa* genome sequence (Tuskan et al. 2006) led to the identification of more than 45,000 putative protein-coding genes and provides a valuable resource for studying functional genomics of poplar.

Populus trichocarpa, P. deltoides, P. alba, *P. tremula* and their hybrids have been used extensively by tree physiologists to study tree development and responses to abiotic and biotic stimuli. *Populus trichocarpa,* also known as black cottonwood, Western Balsam

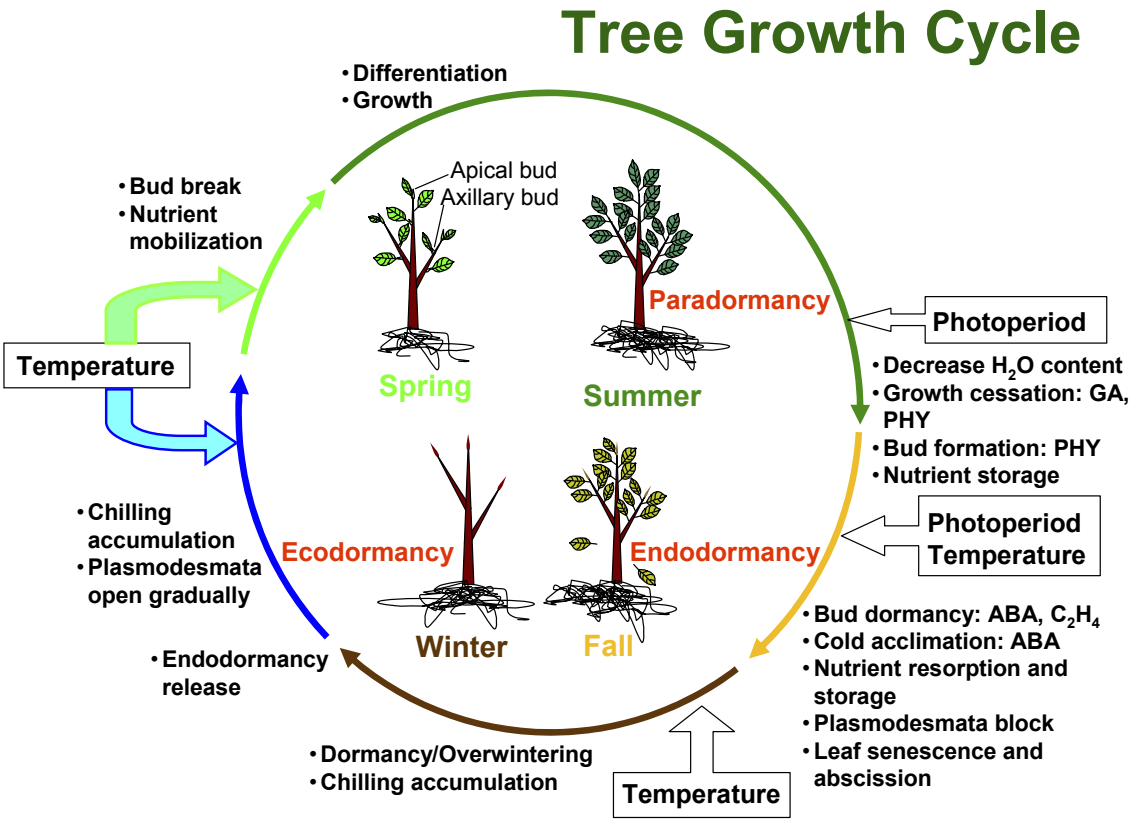


Figure 1-1. Annual growth cycle of temperate zone trees.
In summer the growing shoot apex inhibits axillary bud growth and is an example of paradormancy. Shortening photoperiods from summer to fall initiated bud formation. Continued short days in the fall combined with lower temperatures trigger continued bud development and dormancy. Also occurring during the fall is shoot growth cessation, reduced water content, and nutrient resorption and storage. Apical bud dormancy induced during this period is an example of endodormancy. From fall to winter, prolonged low temperature exposure releases bud dormancy but low temperatures are not favorable for bud break and shoot elongation and is an example of ecodormancy. In the spring properly chilled buds resume growth.

poplar, or California poplar is native to Northwestern America (British Columbia, Alaska, Washington, Oregon, and mountains in California). *Populus deltoides* is known as eastern cottonwood and is native throughout the southernmost part of eastern Canada to northeastern Mexico. *Populus alba*, white poplar, is a European species most closely related to the aspens (*Populus* sect. *Populus*) and ranges from Spain and Morocco through central Europe (north to Germany and Poland) to central Asia. *Populus tremula* is known as common aspen or Eurasian aspen and is naturally found throughout the northern areas including the boreal forests of Europe and Asia.

Apical bud formation, development, and dormancy

The definition of dormancy has been debated over the years. A working definition was proposed at the XXII International Horticultural Congress/83rd ASHS Annual Meeting in Davis, CA in August 1986 that defined dormancy as "the temporary suspension of visible growth of any plant structure containing a meristem" (Lang 1987). Recently, a new definition "the inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favorable conditions" has been proposed (Rohde et al. 2007a). Dormancy can be classified into three types: endodormancy, paradormancy, and ecodormancy (Lang 1987) (Figure 1-1). Endodormancy involves growth control that is mediated by an endogenous signal within the affected dormant structure. Typically endodormancy requires prolonged exposure to chilling temperatures to overcome dormancy. The formation of the apical bud and dormancy induction can be viewed as a morphological manifestation of endodormancy mediated by the apical meristem within the bud or apices (Rohde et al. 2000). Paradormancy involves a signal originating in a structure within the plant but different from the affected dormant structure, with apical dominance being a classical example of paradormancy. Ecodormancy occurs when environmental conditions are unsuitable for growth.

Vegetative buds play a major role in woody plant dormancy. Usually located at the apices of a stem, the apical bud is produced by the shoot apical meristem and consists of an unextended shoot, leaves and stipules encased by two or more pairs of protective bud scales

(Rohde et al. 2002) (Figure 1-2C). In plants, the activity of the bud and/or shoot apices determines the timing and extent of annual growth and dormancy. The shoot apical meristem (SAM) is formed during embryogenesis. In angiosperms, the SAM consists of a central zone in 3 layers (tunica (L1, L2) and corpus (L3)), the surrounding peripheral zone where organ primordia are initiated and rapid cell division occurs, and the rib meristem which gives rise to the central tissues of the shoot axis (Haecker et al. 2001; Rohde et al. 2000). Growth of the shoot apex involves expansion of leaf primordia formed through organogenesis at the SAM, elongation of internodes, initiation and activation of vascular cambium and initiation of axillary buds (Rohde et al. 2000). These processes stop in an organized way that is mediated by SAM during dormancy induction. This leads to the cessation of internode elongation, the formation of bud scales and stipules from leaf primordia, and the development of a vegetative bud (Rohde et al. 2000). The typical vegetative bud of *Populus trichocarpa* contains 6–9 leaf primordia encased by bud scales and/or stipules (Figure 1-2C) (Rohde et al. 2000). Primordia committed before the onset of short days (SD) develop into leaves and the last committed primordium is the last leaf to mature. Primordia initiated after the critical daylength change their morphogenetic fate to form bud scales or stipules (Rohde et al. 2002).

Photoperiod and vegetative bud development and dormancy

Bud development and dormancy are not rapid processes and it can take several weeks to months for the transition from active summer growth to quiescent winter dormancy to occur. Photoperiod is the primary environmental signal controlling the cessation of growth and the development of dormancy of many tree species including poplar (Perry 1971). The use of photoperiod as a signal for bud set is widespread in trees and also occurs in gymnosperms such as *Picea abies* (Norway spruce) (Clapham et al. 2001). Bud formation is prerequisite to dormancy induction and it occurs before the cessation of active vegetative growth and leaf abscission (Rohde et al. 2002).

Growth consists of cell division and organogenesis in the meristem and cell elongation in subtending tissues. Shoot elongation in woody plants is modulated by multiple light signals, including irradiance, photoperiod, spectral composition, the ratio of red:

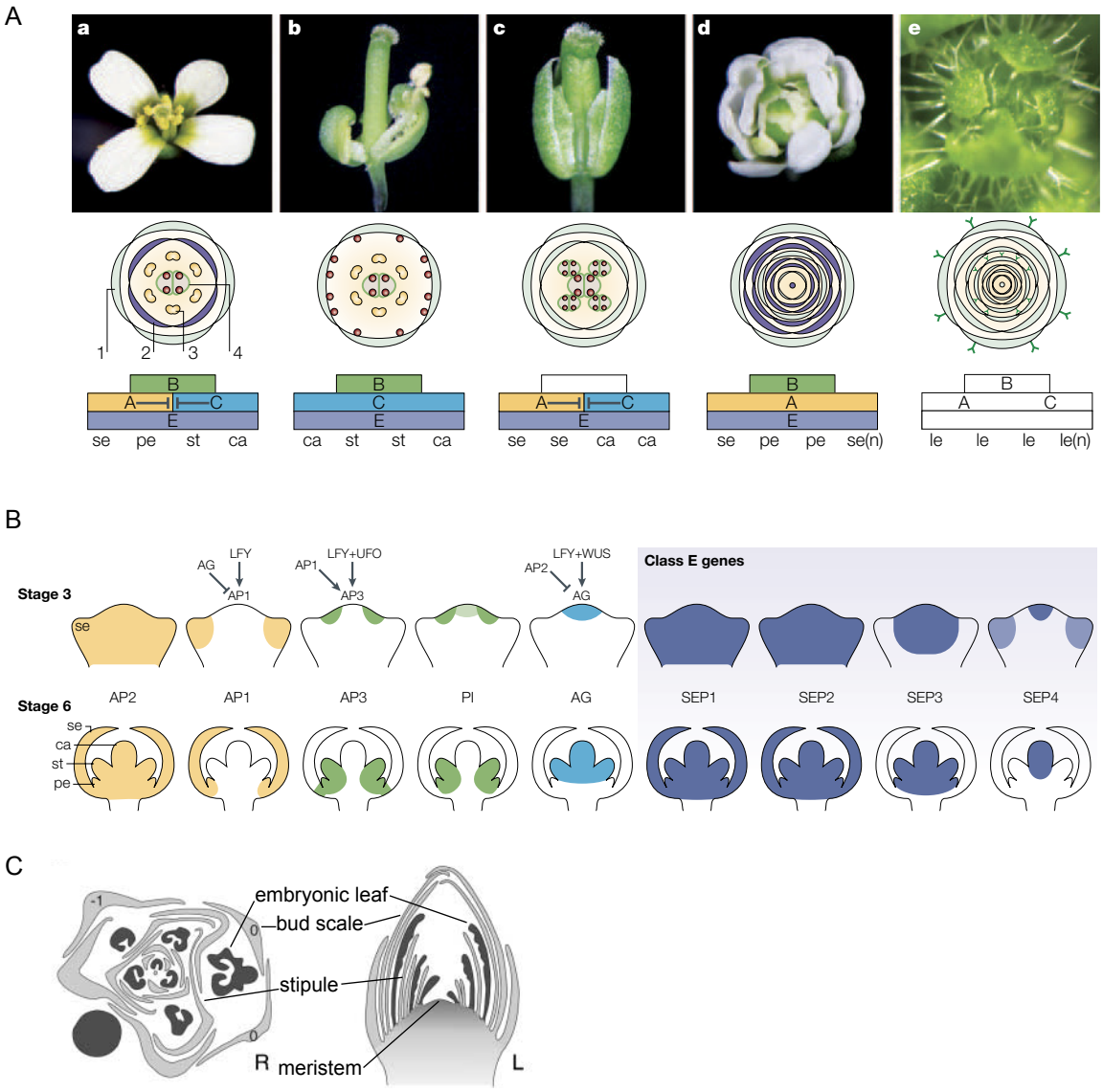


Figure 1-2. Comparison between *Arabidopsis* flower and poplar vegetative buds and associated expression of *Arabidopsis* floral organ identity genes.
(A) Phenotype of various mutants in the ABCE floral quartet model. *AP1*(A-class), *AP3* and *PI* (B-class), *AG* (C-class), and *SEP1–SEP4* (E-class) are type II MADS-box genes. The predicted composition of protein tetramer in whorl 1 is AP1-AP1-SEP-SEP, in whorl 2 is AP1-SEP-AP3-PI, in whorl 3 is AG-SEP-AP3-PI, and in whorl 4 is AG-AG-SEP-SEP. *AG, AGAMOUS; AP1, APETALA 1; AP3, APETALA 3; PI, PISTILLATA; SEP, SEPALLATA*. se = sepal, pe = petal, st = stamen, and ca = carpel. a is a wild type flower, b is an *ap2* flower, c is a *pi/ap3* flower, d is an *agamous* mutant flower, e is a leaf-like organs. (Krizek et al. 2005)
(B) Spatial expression patterns of the *Arabidopsis* floral organ identity genes during flower development. *AP1, AP3, PI, AG*, and *SEP1–SEP4* are MADS-box genes. *AP2, APETALA2; LFY, LEAFY; UFO, UNUSUAL FLORAL ORGANs; WUS, WUSCHEL*. (Krizek et al. 2005)
(C) Morphology of poplar vegetative bud after bud set (R: radial orientation, L: longitudinal orientation) (Rohde et al. 2002)

far-red (R:FR) wavelengths, and end-of-day FR treatment (Olsen et al. 1997; Olsen et al. 2002). The first visible sign of dormancy is the cessation of elongation growth, which is primarily based on the perception of daylength in many deciduous trees (Rohde et al. 2000). The photoreceptors phytochromes and cryptochromes are the key receptors receiving light signals.

Photoperiod measurement involves the use of the circadian clock which computes the daily durations of light and darkness by comparing the external light conditions with an endogenous oscillator (Thomas et al. 1997). The circadian clock synchronizes the internal biological processes of an organism through resetting rhythms by light and/or temperature signals from the environment. Plant circadian rhythms in nature are entrained to 24 hours by the day/night cycle, and this entrained process ensures that rhythmic processes occur at an appropriate time of day (Eriksson et al. 2003). The circadian clock contributes to plant physiology by regulating the phase of entrained rhythms. There are three elements in the circadian system: input, central oscillators, and output. The input elements receive and transmit the environmental signals to central oscillators, and oscillators generate rhythms to regulate gene expression through multiple output pathways (Alabadi et al. 2001).

Photoperiodism is defined as the responses to the length of the day that enable living organisms to acclimatize to seasonal changes in their environment, and it is a special case in which a circadian rhythm is combined with light signaling (Thomas et al. 1997). The photoperiod sensor system allows plants to respond to the annual cycle of day length which leads to the formation of flowers, tubers, or frost-tolerant buds at appropriate seasons. The circadian clock stands at the interface between external and endogenous regulators. Light-signaling pathways from both red/far-red (R_{660}/FR_{730}) absorbing phytochromes (PhyA, PhyB, PhyD, and PhyE) and blue/UV-A absorbing cryptochromes (CRY1 and CRY2) regulate clock components to achieve entrainment (Thomas et al. 1997). The role of phytochrome in photoperiod growth cessation and cold acclimation is supported by observations that overexpressing oat *PHYA* in a hybrid aspen changes critical daylength for these responses and end-of-day FR treatment restores wildtype-like daylength response (Olsen et al. 1997);

(Olsen et al. 2002). In addition, *PHYB2* and *ABI1B* that have been found to be coincident with QTLs (quantitative trait locus) affecting bud set and bud flush in poplar (Frewen et al. 2000) support the importance of phytochromes and photoperiod in regulating tree dormancy.

The first consistent model of the *Arabidopsis* circadian oscillator (Alabadi et al. 2001) suggested that the circadian clock system is comprised of three main players: the genes encoding Myb-related transcription factors *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*), *LHY* (*LATE ELONGATED HYPOCOTYL*) and a pseudo-response regulator *TOC1* (*TIMING OF CAB EXPRESSION 1*; also known as *Arabidopsis PRR1*). Both LHY and CCA1 proteins negatively regulate *TOC1* expression by binding to a region in the *TOC1* promoter which is critical for its clock regulation, but TOC1 appears to be involved in the positive regulation of *LHY* and *CCA1* expression (Alabadi et al. 2001). The loss-of-function or overexpression of *TOC1,* double mutants of *LHY* and *CCA1,* or any combination of these mutations cause arrhythmia under constant (free-running) environments (Alabadi et al. 2002; Makino et al. 2002; Mas et al. 2003; Mizoguchi et al. 2002). The rhythmic output from the oscillator controls a large number of physiological processes in plants. Flowering and hypocotyl elongation are the two processes that have been studied most intensively.

Arabidopsis is classified as a LD plant since it flowers more rapidly during LD than SD. Several photoperiod pathway mutants (*cryptochrome 2* (*cry2*), *phytochrome A* (*phyA*), *gigantea* (*gi*), *constans* (*co*), *flowering locus t* (*ft*), and *flowering locus d* (*fd*)) have been identified which flower later than wildtype *Arabidopsis* in LD but no in SD which have been proposed to belong to a single regulatory pathway (Turck et al. 2008). *CONSTANS* (*CO*) is a transcriptional activator for *Flowering Locus T* (*FT*) which accelerates flowering time in long days and involves the peak of *CO* expression 10-12 hours after dawn that remains high throughout the night until the following dawn (Blazquez 2005; Corbesier et al. 2006; Turck et al. 2008; Yanovsky et al. 2002). *CO* mRNA abundance remains high at the end of LD and the CO protein is stabilized toward the end of LD through the action of photoreceptors Cry2, Cry1, and PhyA, whereas the photoreceptor PhyB destabilizes the CO protein during early morning (Corbesier et al. 2006; Turck et al. 2008). CO protein is rapidly degraded

by a proteasome-dependent mechanism at darkness, so the accumulation of CO protein is dependent on the coincidence of light and mRNA expression and as a consequence fails to accumulate in SD (Corbesier et al. 2006; Turck et al. 2008). Because CO is a transcriptional activator, the expression of *FT* is a direct result of CO protein accumulation toward the end of LD. The circadian defect in *toc1-1* plants causes an early flowering phenotype when *toc1-1* plants are grown under SD of 24 hours (8 hours light/16 hours dark), but not under a SD of 21 hours (endogenous period , 7 hours light/14 hours dark) (Yanovsky et al. 2002). Late flowering in *lhy* (*LATE ELONGATED HYPOCOTYL*) and *gi-3* (*GIGANTEA*) correlates with reduced *CO* levels, whereas early flowering in *elf3-1* (*EARLY FLOWERING 3*), 35S::*CO lhy*, 35S::*CO gi-3* and 35S::*CO* correlates with elevated *CO* expression (Suarez-Lopez et al. 2001). Overexpression of *FT* in *Arabidopsis* (Abe et al. 2005; Wigge et al. 2005) and overexpression of *PtFT1* (Bohlenius et al. 2006) or *PtFT2* (Hsu et al. 2006) in poplar results in precocious flowering. Plants overexpressing *PtFT1* also show no growth cessation under SD and *PtFT* RNAi plants set buds under LD (Bohlenius et al. 2006). These results support a model where clock control over the phase of *CO* expression is the key to photoperiodic control of flowering time whereby *CO* expression is coincident with light at the end of the day in LD which stimulates CO activity and induces expression of *FT*, whereas in SD, *CO* expression begins to peak after lights-off and *FT* levels remain low (Figure 1-3) (Blazquez 2005; Schultz et al. 2003; Yanovsky et al. 2002).

Chilling requirement and vegetative bud development and dormancy

Temperature and precipitation are the two predominant factors that shape vegetation and plant distribution on the earth. Since plants can not move, they have evolved responses to continuous climatic temperature change to optimize their probability of survival and reproduction. Either high or low temperature tolerance may define the geographic limits of plant distribution and their adaptation to their environment. Plant metabolic reactions are temperature dependent because enzyme activity (activation and denaturation), salt and gas solubility, and membrane permeability are temperature dependent (Graham et al. 1982; Rohde et al. 2000). Several studies have shown that the temperature dependence of growth

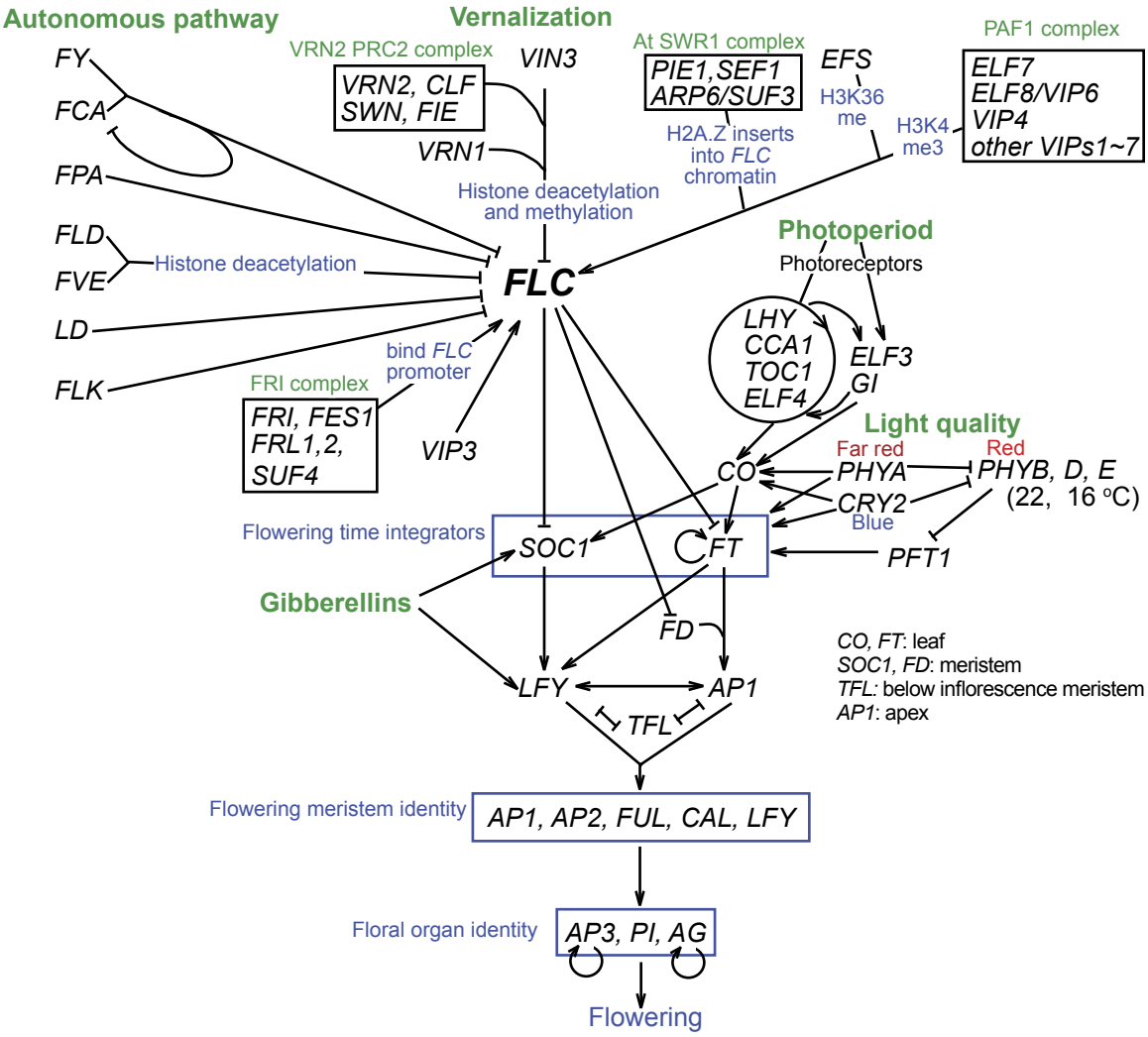


Figure 1-3. Pathways regulating flowering time in *Arabidopsis*.
The autonomous and vernalization pathways repress *FLC* expression. The *FRI* complex upregulates *FLC* expression. The *PAF1* complex mediates H3K4 trimethylation and activates *FLC* expression. *EFS* is a putative H3K36 methyltransferase. The histone variant H2A.Z is necessary for transcriptional activity of *FLC* and is inserted into *FLC* chromatin by the SWR1 complex. *VIN3/VRN2* polycomb repressive complex 2 (PRC2)*/VRN1* and *FLD/FVE* mediate histone deacetylation and methylation that repress *FLC* expression. *FLC* represses expression of flowering-time integrators, *SOC1* and *FT,* and *FD*. All flowering-time pathways regulate the expression of the flowering-time integrators, *SOC1* and *FT* which encode proteins that activate the flowering meristem identity genes (*AP1, AP2, FUL, CAL*, and *LFY)* that in turn upregulate the expression of other floral organ identity genes (*AP3, PI*, and *AG*) and convert the vegetative meristem to a floral fate. *AG, AGAMOUS; AP1, 2, 3, APETALA 1, 2, 3; ARP6, ACTIN RELATED PROTEIN6, SYNONYM SUF3; CAL, CAULIFLOWER; CCA1, CIRCADIAN CLOCK ASSOCIATED1; CLF, CURLY LEAF; CO, CONSTANS; CRY2, CRYPTOCHROME 2; EFS, EARLY FLOWERING IN SHORT DAYS; ELF3, 4, 7, 8, EARLY FLOWERING 3, 4, 7, 8; FES1, FRIGIDA ESSENTIAL1; FIE, FERTILIZATION INDEPENDENT ENDOSPERM; FLC, FLOWERING LOCUS C; FLD, FLOWERING LOCUS D; FLK, FLOWERING LOCUS K; FRI, FRIGIDA; FRL1, 2, FRIGIDA-LIKE1, 2; FT, FLOWERING LOCUS T; FUL, FRUITFUL;* H2A.Z, histone H2A variant*; LD, LUMINIDEPENDENS; LFY, LEAFY; LHY, LATE ELONGATED HYPOCOTYL; PFT1, PHYTOCHROME AND FLOWERING TIME1; PHYA, B, D, E, PHYTOCHROME A, B, D, E; PI, PISTILLATA; PIE1, PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1; SUF3, 4, SUPPRESSOR OF FRIGIDA3, 4; SWN, SWINGER; TFL, TERMINAL FLOWER; TOC1, TIMING OF CAB EXPRESSION 1; VIN3, VERNALIZATION INSENSITIVE 3; VIP1~7, VERNALIZATION INDEPENDENCE 1-7; VRN1, 2, VERNALIZATION 1, 2.* (from: Boss et al. 2004; Dennis et al. 2007; He et al. 2005; Henderson et al. 2004; Sablowski 2007).

rates is a function of the temperature dependencies of both metabolic rates and metabolic efficiency (Criddle et al. 1997). When considering daily variation in plant growth rate, the ultimate cause of high- and low-temperature growth limits is due to loss of substrate carbon conversion efficiency but not membrane phase transitions or enzyme denaturation (Criddle et al. 1997).

However, plants can not move or maintain a constant internal temperature, which make plants vulnerable to cold. Plants evolved two mechanisms, dormancy and freezing tolerance, to survive cold winters. Photoperiods shorter than certain critical daylength initiate dormancy, cold acclimation, and freezing tolerance, and a prolonged period of exposure to chilling temperature is required to release buds from dormancy (Rinne et al. 2001; Rohde et al. 2000). To maximize the growing period but also avoid possible frost damage, temperate zone plants have evolved a biological mechanism to measure the average length of winter by measuring prolonged exposure to chilling temperatures. The chilling requirement can be experimentally quantified by measuring when at least 50% of plants resume growth in permissive conditions after exposure to a given amount of chilling temperature (0–7 ^{o}C) (Rohde et al. 2000). The range of chilling temperature is species and genotype specific (Cesaraccio et al. 2004; Rohde et al. 2000).

Substantial progress has been made in understanding cold acclimation and vernalization, but little is known about the molecular basis of cold-mediated bud dormancy release in trees. During bud dormancy several events occur, including the formation of symplasmically isolated cells in the SAM through the deposition of 1,3-β-glucan plugs in the plasmodesmata (Rinne et al. 2001). It has be suggested that a component of chilling induced release of bud dormancy involves removal of these 1,3-β-glucan plugs from plasmodesmata, restoring symplasmic organization and cellular communication (Rinne et al. 2001). Several physiological and metabolic changes occur during chilling accumulation prior to bud burst, including releasing cellular water, increasing cytosolic ATP content, acidifying the cytoplasm, restoring membrane permeability through membrane composition and increased gluthatione levels and lipase activity (Rohde et al. 2000). After release of endodormancy but before

bud burst, several water-related events (resumption of xylem development and function and water transport) occur sequentially which results in increased water content in the of buds in maple, birch, alder, beech and ash during spring (de Fay et al. 2000).

A molecular comparison between *Populus* seasonal endodormancy, *Arabidopsis* paradormancy, *Arabidopsis* seed dormancy and potato tuber dormancy has been presented recently (Rohde et al. 2007a). A basic genetic understanding of the regulation of bud dormancy is still unclear even with decades of research in tree bud development and dormancy. Both vegetative and floral buds arise from the shoot apical meristem. Both vegetative bud development and dormancy and floral bud development are regulated by photoperiod and hormones. Dormant buds require chilling temperature to resume growth which is similar to vernalization induced flowering. The precocious flowering phenotype in juvenile poplar and induction of *PtAP1* expression by overexpression *PtFT1* (Bohlenius et al. 2006) and *PtFT2* (Hsu et al. 2006) in poplars is similar to early flowering when *FT* is overexpressed in *Arabidopsis* (Abe et al. 2005; Wigge et al. 2005). This suggests that both poplar and *Arabidopsis* use similar *CO/FT* regulons as a mediator to sense photoperiod change. Because of these morphological and genetic similarities, it is possible that analogous regulatory factors to flower initiation and development may govern vegetative bud development and dormancy. Since several key factors involved in vernalization, flower induction, and floral organ identification are members of type II MADS-box genes, it is possible that they may play a role in bud dormancy.

MADS-box genes

Transcription factors

Transcription factors are important regulators of cellular processes and mediate responses to biotic and abiotic stimuli. Transcription factors are typically components of regulatory complexes that regulate gene expression levels by binding to specific DNA sequences (*cis*-acting elements) to enhance or repress their transcription rates. Several large

gene families of transcription factors including AP2-EREBP, bHLH, bZIP, C2H2, MADS, MYB+MYB-related, and NAC exist in plants (Riano-Pachon et al. 2007).

Type I and type II MADS-box genes

MADS-box genes encode a family of transcription factors present in plants, animals, and fungi. MADS-box genes take their name from the *MINICHROMOSOME MAINTENANCE1 (MCM1)* genes in yeast, *AGAMOUS (AG)* genes in *Arabidopsis*, *DEFICIENS (DEF)* genes in *Antirrhinum* and serum response factor *(SRF)* in humans (Ng et al. 2001; Riechmann et al. 1997). MADS-box genes consist of type I (Mα, Mβ, Mδ, and Mρ classes), type II (A, AGL12, AGL15, ANR1, B-AP3, B-PI, Bs, C, D, E, F, FLC, G, SVP, and U classes), and MIKC* (S) genes (Becker et al. 2003; Kaufmann et al. 2005).

The evolutionary history of the MADS-box gene family in plants is characterized by duplication events and subsequent divergence and fixation (Alvarez-Buylla et al. 2000b; Litt et al. 2003). All MADS-box genes originated from a common ancestor and have diverged into two large groups, type I and type II, by gene duplication that occurred prior to divergence of plants and animals (Alvarez-Buylla et al. 2000b). The type II MADS-box genes comprise the MEF2-like genes in animals and fungi and the MIKC-type genes in plants. MIKC-type proteins contain 4 major domains: a highly conserved MADS-box domain of ~55–60 amino acids that includes the DNA-binding domain and dimerization motif, a less-well-conserved intervening (I) domain (~30 amino acids) that appears to be involved in DNA binding specificity and dimerization, a well-conserved keratin-like coiled-coil (K) domain (~70 amino acids) that contains a protein-protein interaction region, and a variable length C-terminal (C) domain that is involved in transcriptional activation, posttranslational modification, or protein-protein interaction (Alvarez-Buylla et al. 2000b; Hileman et al. 2006; Kaufmann et al. 2005; Nam et al. 2003; Vandenbussche et al. 2003). Type I MADS-box genes contain a MADS-domain but lack the K-domain and experienced faster birth-and-death evolution than type II MADS-box genes (Nam et al. 2004). The biological function of type I MADS-box genes are poorly understood due to lack of mutant phenotypes.

It has been suggested that the multimer formation with other MADS proteins through heterodimerization mediated by the K-domain may be crucial for the functional divergence of B class MADS-box genes (Hernandez-Hernandez et al. 2007). The C-terminal domain of B class proteins appears to be essential for ternary complex formation between A and B class proteins (Egea-Cortines et al. 1999; Tzeng et al. 2004; Vandenbussche et al. 2003). Several studies have demonstrated that MADS-box proteins bind to CArG-boxes, $CC(A/T)_6GG$ motif, of DNA as homo- and/or hetero-dimer and may also form high-order complexes with MADS-domain and non-MADS-domain proteins through K-domain or/and C-terminal to regulate the expression of their target gene (de Folter et al. 2005; Honma et al. 2001; Kaufmann et al. 2005; Theissen et al. 2001).

Functions of type II MADS-box genes

MADS-box genes are known to be involved in the regulation of diverse developmental processes in flowering plants including reproductive (flower, seed, fruit) and vegetative (root, leaf) development and differentiation. The most well studied are those MADS-box genes involved in several key steps of flower initiation and development, especially the genes involved in flowering time control and floral organ identification.

There are 108 MADS-box genes in *Arabidopsis thaliana* and more than 70 MADS-box genes in *Oryza sativa* (rice) (Becker et al. 2003; Nam et al. 2003; Parenicova et al. 2003). Type II MADS-box genes can be classified on the basis of their function, expression pattern, and phylogenetic analysis into at least 14 classes (A, PI, AP3, C, D, E, F, G, T/SVP, AGL12, ANR1, FLC, Bs, S) (Nam et al. 2003; Nam et al. 2004) or 13 clades (SQUA, DEF, GLO, AG, AGL2, TM3, AGL6, STMADS11, AGL12, AGL17, FLC, GGM13, AGL15) (Becker et al. 2003) (Table 1-1B). Many *Arabidopsis* loss-of-function mutants (*agamous, agl24, ap1, ap3, cal, pi, flc, ful, maf1, maf2, sep1, sep2, sep3, sep4, soc1, svp*, etc.) have been studied in detail to understand their regulatory roles. For example, the floral-quartet-model describes how combinations of class A, B, C, D and E genes form specific tetramers involved in the regulation of floral organ identification (sepals, petals, stamens, carpels, and ovules) (Theissen et al. 2001). Flowering-time genes such as *FLC, AGL24*, *SVP* and *SOC1* repress

Table 1-1. Summary of *Arabidopsis* and poplar MADS-box genes.

The sequences of 108 *Arabidopsis* MADS-box genes were retrieved from TAIR (http://www.arabidopsis.org/browse/genefamily/MADSlike.jsp) and their functional classes are based on recent studies (Theissen et al., 2000; Ng and Yanofsky, 2001; Becker and Theissen, 2003; Nam et al., 2003; Parenicova et al., 2003; Ratcliffe et al., 2003; Nam et al., 2004) and phylogenetic analysis in Figure 2-1A. The functions of each class are based on TAIR. The sequences of 104 poplar MADS-box genes were retrieved from JGI poplar database and the functional classes are based on the phylogenetic analysis in Figure 2-1B.

(A) Type I, S (MIKC*), and unknown MADS-box genes

(B) Type II MADS-box genes. Genes are highly similar to each other inside parentheses: (*PtA1, PtA5*), (*PtA2, PtA3*), (*PtANR1d, PtANR1f*), (*PtAP3a, PtAP3b*), (*PtPI1, PtPI2*), (*PtSVP4, PtSVP5*), and (*PtSVP6, PtSVP7*)

The abbreviation of classes are: *ABS: Arabidopsis BSister, AG: AGamous, AGL: AGamous-Like, ANR1: A NO3(-)-inducible Arabidopsis gene, AP: APetala, CAL: CAuLiflower, FLC: Flowering Locus C, FLM: Flowering Locus M, FUL: Fruitfull, MAF1: MADS Affecting Flowering 1, PI: PIstillata, SEP: SEPallata, SHP: SHatter Proof, SOC1: Suppressor of Overexpression of Constants 1, SVP: Short Vegetative Phase, TM3: Tomato MADS-box gene No. 3, TT16: Transparent Testa16.* AP_3 and *PI* have evolved into 2 different linkage clades with important difference in certain motif, so I separated them into 2 classes in all phylogenetic trees.

A

Classes / Clades	**66 Type I and 2 S (MICK*) *Arabidopsis* MADS-box genes**	***Populus trichocarpa* from JGI database (43 TYpe I, 2 S (MIKC*), and 2 unknown)**
S (MIKC*)	AGL66, 104	PtS1, 2, (3) (LG_II_6563226, LG_VII_4368520, (LG_VII_1917501, already assigned to PtSVP9 after sequence updated in JGI)). 4 possible pseudo-genes at LG_V without MADS, LG_V with 61 amino acids, scaffold205 with 65 amino acids, and scaffold1217 with 65 amino acids
Mα (TypeI)	AGL23, 28, 29, 39, 40, 55, 56, 57, 58, 59, 60, 61, 62, 64, 73, 74, 83, 84, 85, 88, 91, 97, 99, 100, 102?	PtMα1–24, (LG_I_16744493, LG_II_21635983, LG_II_4185298, LG_IV_10743581, LG_IV_10764691, LG_IX_5018359, LG_IX_5022979, LG_IX_7706698, LG_VI_17498731, LG_VII_1985244, LG_VII_1993865, LG_VIII_2138320, LG_XII_12294705, LG_XVI_11310110, LG_XVII_825799, scaf_12122_644, scaf_166_372387, scaf_252_64499, scaf_3668_1794, scaf_3897_4199, scaf_6288_1178, scaf_64_143598, scaf_64_205434, scaf_7800_1468) 2 possible pseudo-genes at LG_IX without MADS and scaffold185 without MADS
Mβ (TypeI)	AGL26, 43, 47, 49, 50, 51, 52, 53, 54, 75, 76, 77, 78, 81, 82, 89, 93, 98, 103, 105	PtMβ1–10 (LG_I_15434940, LG_I_24061915, LG_II_23650084, LG_VIII_914762, LG_X_16351213, LG_X_20796339, LG_XII_12289238, LG_XV_5832818, scaf_251_62892, scaf_86_81385)
Mγ (TypeI)	AGL34, 35, 36, 37 (PHE1), 38, 41, 45, 46, 48, 80, 86, 87, 90, 92, 95, 96	PtMγ1–6 (LG_VI_10148248, LG_XIII_1196601, LG_XIII_220100, LG_XVI_4720857, scaf_131_153574, scaf_41_1407398)
Mδ (TypeI)	AGL30, 65, 66, 67, 94	PtMδ1–3 (LG_VIII_5478843, LG_X_15317193, LG_XVIII_3434074)
Mu (TypeI)		PtMu1, Mu2 (LG_VII_1629954, LG_VII_1916636) 2 possible pseudo-genes at LG_I without MADS and LG_VII with 66 amino acids

Table 1-1. Summary of *Arabidopsis* and poplar MADS-box genes (continued)

B

Classes / Clades	40 *Arabidopsis* type II MADS-box genes and functions	*Populus trichocarpa* from JGI database (57 type II MADS-box genes)				
		Gene	Location	Gene model	Forward primer	Reverse primer
A / SQUA	**AGL7 (AP1), 8 (FUL), 10 (CAL), 79** Floral homeotic genes. Required for the specification of sepal and petal development, floral meristem and fruit valve identity	PtA1	LG_IV_13113961	eugene3.00041083	TGTTGGCTTTCTTGTTTTTCTCAC	CCGCAGCTCATTAAGGGATTC
		PtA2	LG_VIII_6135149	grail3.0010027201	ATGCCGAGGTTGCCTTGATC (or GATACTTGAACGCCACGAGAGG)	AGTGCCGGCTGTGCCAGT
		PtA3	LG_X_14488440	grail3.0006057701	TTGATACCGCCCTTAAACACATTC	GCTCCGTAACCTCCAAGGTGAAAAG
		PtA4	LG_XII_6043656	grail3.0042013901	GCAAATGATGATCCCGAAAACCA	AACAGTGCGTTGGCTCGATGTTG
		PtA5	scaffold_155_266786	estExt_Genewise1_v1.C_1550090	CACCATACCATCACAGAAATAGAA	TAGAGAAAACAATCACAGCGACAT
AGL12	**AGL12** Preferentially expressed in root tissues such as atrichoblast, primary root differentiation zone, and root stele, and still found expression in few cells type in flower and shoots	PtAGL12a	LG_XIX_8821601	fgenesh1_pm.C_LG_XIX000160	AGGTACATGTTCGGAGCAAGAGC	GGTGCGGAAAATCAGAGGTTACT
		PtAGL12b	scaffold_41_1912703	eugene3.00410181	GCAAGCGCCGATCTGGTCT	TTTCAGCAGCACGCCCTCCA
AGL15	**AGL15, 18** AGL15 is preferentially expressed during embryogenesis, also expressed in leaf primordia, shoot apical meristems and young floral buds. AGL18 is expressed in endosperm and developing gametophytes.	PtAGL15a	LG_VI_3223689	fgenesh1_pm.C_LG_VI000173	CGGCCAAATAATCGCAAACC	CACCCTGAAGCCACCTGACT
		PtAGL15b	LG_IX_6992103	gw1.IX.3465.1	CCCCCACGCAAATAAAAGATA	GCTTGTGGCGCCATTGTCTA
ANR1 / AGL17	**AGL16, 17, 21, 44 (ANR1)** Root development: AGL16 expresses in leaf, root and stem, with higher RNA accumulation in guard cells and trichomes. AGL17 and 21 are preferentially expressed in root tissues. AGL44 is involved in lateral root formation	PtANR1a	LG_I_27950423	fgenesh1_pg.C_LG_I002719	GCATGGTGGCCGTTAGGAA	GTGGCCGGGAAGCTCTGTTTG
		PtANR1b	LG_I_27954924	gw1.I.4342.1	ATGGGGAGAGGGAAGATTGT	CACATTCCGTTCTCCATAAACTAG
		PtANR1c	LG_I_27979078	fgenesh1_pg.C_LG_I002721	AAAGGTGTTCGAATTAAAAAGGAA	CACATTCCGTTCTCCATAAACCTT
		PtANR1d	LG_II_8019018	fgenesh1_pg.C_LG_II001009	CACCGGCAAGCTCTATGATTTCTC	GCTGCAGTTATGGGGGACAAAAG
		PtANR1e	LG_IX_5310307	fgenesh1_pm.C_LG_IX000350	CTCCAGCACAGGCAAACTCTATGA	TTTCCTGGTGCCTCGATGTTATGA
		PtANR1f	LG_V_6895746	fgenesh1_pm.C_LG_V000234	GGGGAGGGGGAAGATAGAG	ATTTTCATGACAAATTAGGTTTGC
		2 possible pseudo-genes at LG_I with 57 amino acids and LG_II with 60 amino acids				

B

Table 1-1. Summary of *Arabidopsis* and poplar MADS-box genes (continued)

Classes / Clades	**40 *Arabidopsis* type II MADS-box genes and functions**	***Populus trichocarpa* from JGI database (57 type II MADS-box genes)**				
		Gene	Location	Gene model	Forward primer	Reverse primer
B (PI,AP3) / DEF,GLO	**AP3, PI** Petal and stamen development: Floral homeotic gene. Required for the specification of petal and stamen identities. Express in floral meristem and root	PtAP3a	LG_II_1844015	fgenesh1_pm.C_LG_II000127	CTAGCCCCTCCACTACAACGAA	GATCCATTGCTTCTAGTTCCACTTG
		PtAP3b	LG_VII_11390510	estExt_fgenesh1_pm_v1.C_LG_VII0384	AGTACATTAGCCCCTCCACATC	TGCTGCCATACACATTACCC
		PtAP3c	scaffold_57_1085666	eugene3.00570129	AGTACATTAGCCCCTCCACAA	CCACCTCACTACGTTCTTCATTAT
		PtPI1	LG_II_5439585	estExt_Genewise1_v1.C_LG_II0439	ACTGGCCTTGCTGCTGTCCGTAAA	CGGGCTAAGTGGTTCTCGCAAAG
		PtPI2	LG_V_12019318	eugene3.00050898	TCACAGTTTTATGCGATGCTC	GTTACTATAAAATATTGTACTGGC
		3 possible pseudo-genes in AP3 class at LG_V without MADS, LG_X with 53 amino acid, and LG_XIII with 16 amino acids				
Bs / GGM13	**ABS (AGL32, TT16)** Seed coat development: Regulate proanthocyanidin biosynthesis and control of cell shape in the inner-most cell layer of the seed coat. Necessary to determine the identity of the endothelial layer within the ovule.	PtBs1	LG_V_2481438	gw1.V.3278.1	AGTACCATCTTCAACGTCCAGAGG	GCGGCACGATGCTCCCTTG
		PtBs2	LG_VII_6070786	eugene3.00070739	GCGCCGCTATACAGGTGAAGATAC	AGCGCTACACGATGCTCCTGAAT
		PtBs3	LG_XII_13913993	eugene3.00121259	AGGCAGCTGCAGCAATGGTGAA	ACGGTAAGGCTGGAACTGTGGAG
		PtBs4	LG_XV_9186485	eugene3.00151043	AGTACCATCTTCAACGTCCAGAGG	AATCTTGAAGGTTGGGCTGTGTAG
CD / AG	**AG, AGL1 (SHP1), 5 (SHP2), 11 (STK)** AG is floral homeotic genes and specifies floral meristem and carpel and stamen identity. SHPs are required for carpet, ovule, and fruit development and control dehiscence zone differentiation and promote the lignification of adjacent cells. STK regulates ovule development.	PtCD1	LG_IV_3040968	estExt_fgenesh1_pm_v1.C_LG_IV0101	AGATCAGATGGCCCTTCAGTTAG	AGGCCCTCCGCTACTTCAG
		PtCD2	LG_XI_5382211	eugene3.00110505	GATGCCGGGAGGTGTCAACTTC	GCCGCTGCTGCTACTGAAACC
		PtCD3	LG_XIX_8868352	eugene3.00190689	AGCCGTGGCCGTCTCTATG	TCAAAATACCACTTGCACACCAG
		PtCD4	scaffold_41_1645679	estExt_Genewise1_v1.C_410455	CGTGAGTAATCTGTCGGTGAA	GATGGAGAATCTTCTTGTTATAAG
E / AGL2	**AGL2 (SEP1), 3 (SEP4), 4 (SEP2), 9 (SEP3)** Involved in the determination of flower meristem and organ identity and the development of sepals, petals, stamens and carpels. SEP4 expresses in all major organs above ground	PtE1	LG_I_4214308	grail3.0008011201	CAAGTGCAGAAGATGTGGAGTACG	GGGCCAGCAGTGACCATTG
		PtE2	LG_III_15152248	grail3.0047013501	TATGGGGAGAGGTAGGGTTGAGTT	GTTGAGCTTGTTGTCGAGAAAAAC
		PtE3	LG_IV_13174266	grail3.0139000303	AAAAACAAAAGAAAAGGAAAGATA	TTGCCACGGTTAGAGAAGATG
		PtE4	LG_VIII_6125364	gw1.VIII.2550.1	GGGCCAGTCTGAAAAGGAGACG	TGAACCCGTTAACATTTTGAGAT
		PtE5	scaffold_155_252286	eugene3.01550021	CAGCTATGGGGCAGAAGAAGTCA	GCGGCATGTGTTGCAGTTATCT
F / TM3	**AGL14, 19, 20 (SOC1), 42, 71, 72** SOC1 expresses in leaf, controls flowering and is required for CO to promote flowering. AGL14, 19, 42 express in root tissues. AGL19 also expresses in leaf and flower.	PtF1	LG_I_8446428	eugene3.00010979	TTGAGAAGCTAAAGGGCGAGGAG	GCAGGCCGTGTCTCTGGAGTC
		PtF2	LG_II_11472544	grail3.0033033501	CACCATCCGAGCTAGAAAGAATCA	CTTGCGCAAATGCAATACAGTTAA
		PtF3	LG_III_11436854	eugene3.00030922	GAGAGCCAAGGACGTAGGAATCAG	TGGGGCAAGCCGTGTCTCT
		PtF4	LG_XII_13348211	eugene3.00121185	TCTTTCCCGTTATACCGTTTTGAT	TTGGCCATGTTTGCTGATTCTTGT
		PtF5	LG_XIV_1766124	estExt_Genewise1_v1.C_LG_XIV0941	ATCCGTGCGAGAAAGAATCAGGTT	CGCAGGTGCAACATAGCATTAGC
		1 possible pseudo-gene at LG_I with 61 amino acid only				

Table 1-1. Summary of *Arabidopsis* and poplar MADS-box genes (continued)

B

Classes / Clades	**40 Type II *Arabidopsis* genes and functions**	***Populus trichocarpa* from JGI database (57 type II MADS-box genes)**				
		Gene	Location	Gene model	Forward primer	Reverse primer
FLC / FLC	**AGL25 (FLC, FLF), 27 (MAF1, FLM), 31 (MAF2), 68 (FCL1, MAF5), 69 (MAF4, FCL2), 70 (MAF3)** All involve in regulation of flowering. FLC is a repressor of floral transition and its expression is downregulated during cold treatment. Vernalization, FRI and the autonomous pathway all influence the state of FLC chromatin. FLC interacts with SOC1 and FT chromatin. AGL27, AGL69 are negative regulator of flowering. AGL68 is upregulated during vernalization. AGL31, 68, 69 have several alternatively spliced forms.	PtFLC1	LG_III_15165199	grail3.0047013602	GGCCAAAATCCTGAAACGTCATG	TTGCGCATCCGATCCTCATAACA
		PtFLC2	LG_III_15178184	grail3.0047013701	TCTCTAGCGTTGGCAGTACAACC	TGCCATTATAGCCGCCTCCAG
		PtFLC3	scaffold_690_1864	grail3.0690000103	AGCTAAAGCGAATCGAAAAGAAG	CCTGAAGAGAGAAGCTGCGTC
		PtFLC4	LG_I_4198539	gw1.I.7772.1 (MADS) + fgenesh1_ pg.C_LG_I000499 (K)	GACGTCCAAGTAGCTCTCCTCAC	GATCCACCTTGCCGTTAT
		PtFLC5	scaffold_690_14046	grail3.0690000201		
		1 possible pseudo-gene at scaffold4342 with 69 amino acids without MADS-domain. PtFLC1 might be the allele of PtFLC3 and PtFLC2 might be allele of PtFLC5				
G / AGL6	**AGL6, 13** Regulate development of both flowers and vegetative organs. Expresses in vegetative and also in 4 whorls of flower (AGL6) and ovule (AGL13).	PtG1	LG_XII_13390265	fgenesh1_pg.C_LG_XII001247	AGGTTAAGGGCAAAATGTGAGACT	TGGGAGACGCAGAACAACTT
		PtG2	LG_XIV_1752699	fgenesh1_pm.C_LG_XIV000068	TGCCCTTATCATCTTCTCCAGTCG	CTTTTTGCGCAGGTCTTCCATTT
		PtG3	LG_XV_9758746	eugene3.00151130	CAAGGTTAAGGGCAAGATGTGAGA	TTGCGTAACTCTTCCACCCTATCA
T, SVP / STMADS11	**AGL22 (SVP), 24** SVP is a floral repressor which functions within the thermosensory pathway and represses FT expression via direct binding to the CArG III motif in the FT promoter. AGL24 is a flower promoter. Both strongly express in your leaves and SAM. AGL24 also expresses in floral meristem and organs.	PtSVP1	LG_II_7637335	grail3.0003092401	CAGCTAAGGCGGATGAGAGGTG	TCCAGCGATAGCAGATCTGTTCC
		PtSVP2	LG_V_7689040	grail3.0083000102	CAACTAAGGCGAATGAGAGGAGAA	GAGAGGTGTCGGAGCTATCATCC
		PtSVP3	LG_VII_11836798	estExt_Genewise1_v1.C_LG_VII4000	GAAGTTGCGGAGAAAAGCCATCAG	ATCTCCCTGCGATCTCCCATTCAG
		PtSVP4	LG_VII_1898517	grail3.0058021902	GCATGGGGCAAGTGATTGAAAGG	GGTAGCCCCTAGTTTGAGAAAGGCAC
		PtSVP5	LG_VII_1942427	gw1.VII.1215.1	AACCCGTGAACTGAGCCAGG	CAATCGCCGGTTCTCTTCTACTAA
		PtSVP6	LG_XVII_577724	eugene3.00170054	AGGCGAACAAGGGGAGAAGACC	TGTCGTAGTCCTGACGAGGATTT
		PtSVP7	LG_XVII_614596	eugene3.00170057	AGGCGAACAAGGGGAGAAGACC	TTGTCGTAGTCCTGACGAGGATAA
		PtSVP8	scaffold_4049_2200	eugene3.40490001	ATGGCTCGCAAGAAAATCC	TAAGAAAGAGAAAGGGAGCAGAAC
		PtSVP9	LG_VII_1917501	fgenesh1_pg.C_LG_VII000296	N/A	N/A
Unknown	AGL33, 63, (102?)	PtU1	LG_XII_11588108	fgenesh1_pg.C_LG_XII001001	TGGGGAGAGGGAAAGTGGA	TCATGGGAGGAAGAAGAACTACTG
		PtU2	LG_XV_7284364	eugene3.00150771	CCAAGCGGAAAGTTTTACCAGTT	TGAAGGGCTCTTTGCTCGCTC

or promote the floral transition mediated by internal or environmental factors (plant age, day-length, cold) (Hartmann et al. 2000; Hepworth et al. 2002; Michaels et al. 2003a; Sheldon et al. 2002; Sheldon et al. 2000). MADS-box genes have also been shown to be involved in regulating embryo, pollen, endosperm, seed, fruit, root, guard cell, and leaf development (Alvarez-Buylla et al. 2000a; Ng et al. 2001; Theissen 2001; Theissen et al. 2000). Table 1-1 summarizes the functional classes/clades of 108 *Arabidopsis* MADS-box genes found in TAIR (The *Arabidopsis* Information Resource) and recent studies (Becker et al. 2003; Nam et al. 2003; Nam et al. 2004; Ng et al. 2001; Parenicova et al. 2003; Ratcliffe et al. 2003; Theissen et al. 2000) by phylogenetic analysis.

Extensive studies have focused on revealing the regulatory roles of MADS-box genes in plant growth and development. *Arabidopsis* and rice are two species that have been studied the most. Poplar has recently joined this group after the publication of the genome sequence in 2004. The functional conservation of MADS-box genes across species could provide a starting point towards revealing possible regulatory networks in trees. Reconstructing a robust tree of MADS-box genes from representative angiosperms will help to make rational assumptions about their functions. Along with examining these relationships is the caveat that orthologous MADS-box genes do not necessarily have analogous developmental roles (Causier et al. 2005; Kramer et al. 2004) and paralogous MADS-box genes may evolve to equivalent developmental functions (Causier et al. 2005). Sub- and/or neo-functionalization might be maintained and novel or ancestral roles might be swapped between these genes during the events of gene duplication and diversification (Irish et al. 2005; Moore et al. 2005). Due to the redundancy, subfunctionalization, neofunctionalization, and gene function swapping during the gene evolution, a shift from analyzing individual gene functions towards studying MADS-box gene function at the subfamily level has been proposed (Rijpkema et al. 2007).

Development of flower and apical bud

If the hypothesis that analogous regulatory factors to flower initiation and development in *Arabidopsis* may govern vegetative bud development and dormancy in poplar is true then it is important to understand what genes are involved in flower development and how they regulate this process. From this, candidate genes can be selected for study of the poplar homologs. Several key factors involved in vernalization, flower induction, and floral organ identification are members of type II MADS-box genes. Little is known about the molecular and genetic regulation in vegetative apical bud dormancy, then the regulatory mechanism governed the initiation and development of a similar tissue will be used as a starting point. Therefore the focus of this study is mainly on applying the knowledge about the genes regulating floral bud formation and the signals induced transition from vegetative to inflorescence meristem in *Arabidopsis* to discover the possibility that the poplar homologs of these genes are involved in apical bud formation and dormancy release.

ABCE floral quartet model

An important developmental switch in a plant life cycle is the transition from a vegetative to inflorescence meristem. Photoperiod and temperature are the key environmental cues that determine the timing of this transition. Using the term "short-day, long-day, and day-neutral" before plants describe the effect of photoperiod on the timing of this transition. Most floral organs of dicot flowers are arranged in 4 whorls. The floral-quartet-model describes how different combinations of A, B, C, D and E class MADS-box proteins form various tetramers which are involved in the regulation of floral organ identification (sepals, petals, stamens, carpels, ovules) (Figure 1-2A) (Honma et al. 2001; Jack 2004; Krizek et al. 2005; Ng et al. 2001; Theissen et al. 2001). The expression patterns of these genes support this quartet model (Figure 1-2B) (Krizek et al. 2005). In *Arabidopsis*, A class genes include *AP1, CAL/AGL10* (*CAULIFLOWER*) and *FUL/AGL8* (*FRUITFULL*) genes and B class genes include *PI* and *AP3* genes. Mutations in the *AP1* gene disturb floral meristem specification and sepal and petal development (Bowman et al. 1993). The *ap1 cal* double mutant displays a massive proliferation of shoot-like meristems in place of flowers (Bowman

et al. 1993). The *Arabidopsis* mutant *ful-1* showed a primary defect within the valves of fruit which fail to elongate and differentiate, and a reduction in the number of internal cell layers in cauline leaves (Gu et al. 1998). This suggests that *FUL/AGL8* is required for the normal pattern of cell division, expansion, and differentiation during morphogenesis of the silique and leaf development (Becker et al. 2003; Gu et al. 1998). The *ap1 cal ful* triple mutants show an extreme enhancement of the *ap1 cal* phenotype and never flower under standard growth conditions (Ferrandiz et al. 2000; Ng et al. 2001). This indicates partially functional redundancy between these 3 A class genes (*AP1, CAL*, and *FUL*). Mutations in either *Arabidopsis AP3* or *PI*, B class genes, cause homeotic transformation of petals to sepals and of stamens to carpels (Bowman et al. 1991; Goto et al. 1994; Jack et al. 1992). Constitutive *AP3* expression in *Arabidopsis* results in the replacement of carpels by stamens (Jack et al. 1994). The ectopic expression of either *ABCE* genes alone does not convert vegetative leaves into floral organs, but leaves in the triple transgenic plants with constitutive expression of *PI+AP3+AP1* or *PI+AP3+SEP3* are transformed into petaloid organs and those with constitutive expression of *PI+AP3+SEP3+AG* are transformed into staminoid organs (Honma et al. 2001; Jack 2004). This suggests a quartet ABCE model where these type II MADS-box proteins function together in tetramer complexes (Honma et al. 2001; Jack 2004; Krizek et al. 2005; Theissen et al. 2001).

A review paper (Cseke et al. 2004) has summarized past studies in expression patterns of A, B, C, D, E, F, and SVP classes of MADS-box genes in trees such as poplar, apple, birch, *Eucalyptus*, pine, and spruce along with results from transformation of some of these genes into *Arabidopsis* and tobacco. Typically, the A, B, C, D, and E classes of genes in trees are expressed preferentially in flower, fruits and/or cones (Cseke et al. 2004) as expected from the ABCE floral quartet model. This suggests that gene functions have been conserved in flower related MADS-box genes among species during evolution.

A morphological comparison between vegetative bud (scales, stipules, embryonic leaves) (Rohde et al. 2002) and floral organs (sepals, petals, stamens, carpels) (Krizek et al. 2005) shows the analogous morphological structure, especially between an *agamous* flower

or mutant flower with leaf-like organs of *Arabidopsis* and vegetative bud of poplar (Figure 1-2). An *agamous* mutant flower consists of sepals in the first whorl, petals in the second and third whorls, and reiterations of this pattern in interior whorls (Krizek et al. 2005). A mutant flower with leaf-like organs occurs in a quadruple mutant (*sep1 sep2 sep3 sep4*) that lacks class E activity which results in loss of floral determinacy (Ditta et al. 2004).

Flowering time control and *FLC* class genes

Arabidopsis flowering is regulated by four major pathways: GA, photoperiod, autonomous, and vernalization (Figure 1-3) (Boss et al. 2004; Dennis et al. 2007; He et al. 2005; Henderson et al. 2004; Sablowski 2007). The hormone GA promotes flowering by upregulating the expression of *SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1)* and *LFY (LEAFY)*. Long day photoperiod promotes flowering in *Arabidopsis*, a LD plant, by activating *CO (CONSTANS)* which results in upregulation of the flowering time integrators genes (*FT* and *SOC1*) (Abe et al. 2005; He et al. 2005; Wigge et al. 2005). The autonomous pathway which has no involvement in either the photoperiodic or GA pathway promotes flowering constitutively by epigenetic downregulation of *FLC* (Simpson 2004). Vernalization promotes flowering by repressing *FRI* (*FRIGIDA*)-mediated *FLC* expression after exposure to a prolonged periods of cold. *FLC* is a point of integration for the vernalization and autonomous pathways and is upregulated by *FRI* and *PAF1* complexes. Light quality can also repress flowering through the red light photoreceptors *PHYB, PHYD*, and *PHYE* and promote flowering by *PHYA* and the blue/UV light receptor *CRY2* (Boss et al. 2004). Flowering time integrators (*SOC1* and *FT*) activate floral meristem identity genes (*AP1, AP2, FUL, CAL*, and *LFY*) which results in the activation of other floral organ identity genes (*AP3, PI*, and *AG*) (Sablowski 2007).

Plants have evolved the ability to sense the coming of winter by photoperiod change and measure the completion of the winter season. *FLC*, a type II MADS-box gene, is a floral repressor which quantitatively represses flowering by repressing the flowering time integrators genes (*SOC1* and *FT*) and *FD*. A 600–800 kDa multimeric protein complex with more than one FLC polypeptide binds directly to a region containing CArG boxes within the

first intron of *FT* and promoter region of the *SOC1* and *FD*. This represses the expression of *FT*, *SOC1* and *FD* (Helliwell et al. 2006; Searle et al. 2006). A complex of two or four MADS-box proteins is expected to be approximately 50–60 kDa or 100–120 kDa (Helliwell et al. 2006). The levels of *FLC* mRNA are positively regulated by *FRI* and negatively regulated by vernalization (Michaels et al. 1999) and autonomous pathways. *FLC* expression is predominately in shoot and root apices and vasculature, and it is also detectable in leaves and stems (Michaels et al. 1999; Michaels et al. 2000; Sheldon et al. 1999). Overexpression of *FLC* is sufficient to delay flowering even without an active *FRI* allele (Michaels et al. 1999).

Many combinations of different allelic variants at *FRI* (at least 20 non-functional haplotypes and 31 polymorphysims) and *FLC* (at least 11 haplotypes, 65 SNPs and 11 indels) are major determinants of natural variation in flowering time of *Arabidopsis* (Caicedo et al. 2004; Gazzani et al. 2003; Johanson et al. 2000; Michaels et al. 2003b; Shindo et al. 2005; Werner et al. 2005). Most of polymorphisms of *FLC* are located in the ~3.5 kb first intron (Caicedo et al. 2004) which contains key *cis*-regulatory elements and is the target of chromatin modification (Bastow et al. 2004; Sheldon et al. 2002; Sung et al. 2004b). Usually the rapid cycling, early flowering, *Arabidopsis* accessions have either a natural-mutant non-functional *fri* allele or a weak or non-functional *FLC* allele, whereas the winter-annual, late flowering, accessions have dominant alleles of *FRI* and *FLC* (Caicedo et al. 2004; Shindo et al. 2005; Werner et al. 2005). A mutation in *FLC* upstream regulators changes *FLC* expression and flowering time. For example, the autonomous pathway mutant *fca* changes a rapid cycling *fri FLC* genotype to a winter annual genotype and a mutation in a vernalization gene such as *vrn* converts a winter annual *FRI FLC* genotype to vernalization insensitive genotype (Henderson et al. 2004).

There are five homologs of *FLC* in *Arabidopsis, MAF1–4 (MADS AFFECTING FLOWERING1–4*) are flower repressors and *MAF5* is upregulated during vernalization (Ratcliffe et al. 2003; Ratcliffe et al. 2001). Overexpression *MAF1–MAF5* delays flowering (Ratcliffe et al. 2003; Ratcliffe et al. 2001; Scortecci et al. 2001). Overexpression of *MAF1*

(or *maf1* mutant) modifies flowering time in a comparable manner to *FLC* overexpression (or *flc* mutant) and it does not affect *FLC* expression level (Ratcliffe et al. 2001; Scortecci et al. 2001). Overexpression of *MAF2* results in a range of flowering times but only late flowering phenotype was consistent between T1 and T2 generations (Ratcliffe et al. 2003). Plants overexpressing *MAF2* flowered late and become insensitive to vernalization similar to plants overexpressing *FLC* or *MAF1*; however overexpression does not affect *FLC* expression, but does repress *SOC1* expression (Ratcliffe et al. 2003). This suggests that *MAF1* and *MAF2* act downstream of *FLC* or in a different pathway.

Vernalization and epigenetics

In *Arabidopsis,* vernalization results in changes in the chromatin structure of the flowering repressor *FLC* to switch it to the mitotically stable repressed state (Sung et al. 2004a). The 6 kb region including promoter, exon 1, intron 1, and exon 2 are the *cis*-acting elements which regulate *FLC* expression and vernalization-mediated *FLC* repression (Sheldon et al. 2002). Epigenetic silencing of genes is a process mediated by several modifications (mono-, di-, or tri-methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation) of DNA and histone and forms a histone code on the N-terminal tails of histones H3 and H4 (Turner 2002). Acetylation of H3 and H4 and methylation of H3K4 (histone 3 lysine 4), H3K36, and H3K79 are correlated with active transcription, while H3K9, H3K27, and H3K20 methylations are imprints for silenced chromatin (He et al. 2005; Turner 2002). H3K4 di-/tri-methylation is rich in euchromatin in plants and H3K9 dimethylation is a marker for heterochromatin (He et al. 2005; Turner 2002).

Vernalization results in *FLC* repression only when *VERNALIZATION INSENSITIVE 3* (*VIN3*) is induced after a period of cold exposure (Sung et al. 2004b). *VIN3* encodes a protein associated with histone deacetylation which consists of a plant homeodomain (PHD), a component of chromatin-remodelling complexes, and fibronectin type III repeats which are involved in protein-protein interactions (Sung et al. 2004b). In *vin3* mutants, the prolonged cold mediated decrease in histone acetylation and increase in H3K9 and

H3K27 methylation does not occur, resulting in vernalization insensitive *FLC* (Sung et al. 2004b). In *vrn1* and *vrn2* mutants, *FLC* mRNA level increases after returning plants to warm environments (Gendall et al. 2001; Levy et al. 2002; Sung et al. 2004a). This suggests that the *VERNALIZATION* (*VRN*) genes, *VRN1* and *VRN2*, are involved in the downregulation of *FLC* expression by stably maintaining *FLC* repression after a cold treatment (Gendall et al. 2001; Horvath et al. 2003; Levy et al. 2002; Sung et al. 2004a). *VRN2* encodes a nuclear-localized zinc finger protein similar to *Drosophila* polycomb (*PcG*) which is a component of chromatin remodeling complex responsible for epigenetic-like regulation of body development (Gendall et al. 2001; Horvath et al. 2003). *VRN1* encodes a plant specific DNA-binding protein with two B3 domains (Levy et al. 2002). Chromatin immunoprecipitation (ChIP) studies demonstrated that vernalization increases dimethylation of H3K9 and H3K27 within the *FLC* locus, but dimethylation was absent at H3K27 in the *vrn2* mutant and at H3K9 in both *vrn1* and *vrn2* mutants (Bastow et al. 2004; Sung et al. 2004b). Therefore, in the case of *FLC*, epigenetic memory of winter in plants is mediated by a histone code which specifies a silent chromatin state (histone deacetylation and methylation, H3K9 and H3K27 methylation) through chromatin modifications (Bastow et al. 2004; He et al. 2005). Vernalization is also dominant over the *FLC* activation by *FRI* or lesions in autonomous pathway genes (He et al. 2005).

Alternative splicing in plants

Alternative splicing (AS)

Most eukaryotic genes contain one or more introns which are spliced out from pre-mRNA by spliceosomes to produce functional mRNA that is translated into protein. One extreme example of RNA splicing is human *dystrophin* gene which is 2500 kb long with more than 80 introns while it's mRNA is only 14 kb long (Hartwell et al. 2004). Plants typically have shorter genes and a fewer number of exons and introns per gene with the introns being smaller with a higher AT content compared to human genes (Reddy 2007). Monocots and dicots may also differ in splicing since rice tends to have introns longer that

are with a higher AT content and exons with lower AT content compared to *Arabidopsis* (Reddy 2007).

There are 4 types of conserved short sequences within the pre-mRNA involved in the specificity of splicing mediated by spliceosomes (Figure 1-4). These include: (a) splice donor at the 5' site of the intron with a GU dinucleotide flanked by a few purines (Pu, i.e. A or G) that is recognized by the U1 small nuclear ribonucleoprotein particle (snRNP), (b) splice acceptor at the 3'site of the intron with an AG dinucleotide that is recognized by the U2 auxillary factor 35 (U2AF35), (c) the branch site UACUAAC in yeast and vertebrates and CURAY in plant (R is purines (A, G) and Y is pyrimidine (C, U)) which is located within the intron about 30 nucleotides upstream of the polypyrimidine tract and is recognized by U2 snRNP, and (d) a polypyrimidine tract (12–14 pyrimidines (Py, i.e. C or U)) or several U-rich sequences between the 3' splice site and branch site which is recognized by U2AF65 (Hartwell et al. 2004; Reddy 2007; Simpson et al. 2002). Splicing is catalyzed by the spliceosome which consists of 5 small nuclear RNAs (snRNAs) and nearly 300 proteins (Reddy 2007).

Two types of spliceosomes include: (a) the major U2-type spliceosome that consists of U1, U2, U4, U5, and U6 snRNPs and catalyzes the removal of introns with canonical (GT-AG) splice sites, and (b) the minor U12-type splicesome consisting of U11, U12, U4atac, U5, and U6atac snRNPs and is responsible for the recognition of non-canonical splice sites (Reddy 2007). Each U-type snRNP contains one snRNA and several proteins and each spliceosome contains many non-snRNP proteins (Reddy 2007).

Two models, the exon definition and intron definition, have been proposed to explain splice site recognition (Reddy 2007). There are 4 groups of exonic and intronic *cis*-acting regulatory sequences of 4–18 nucleotides, the exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing suppressors (ISSs), which facilitate the splice site selection during constitutive and alternative splicing (Reddy 2007). In the exon definition model, splicing regulators such as serine/arginine-rich (SR) and other proteins bind to ESE and recruit U1 snRNP to the 5' splice site

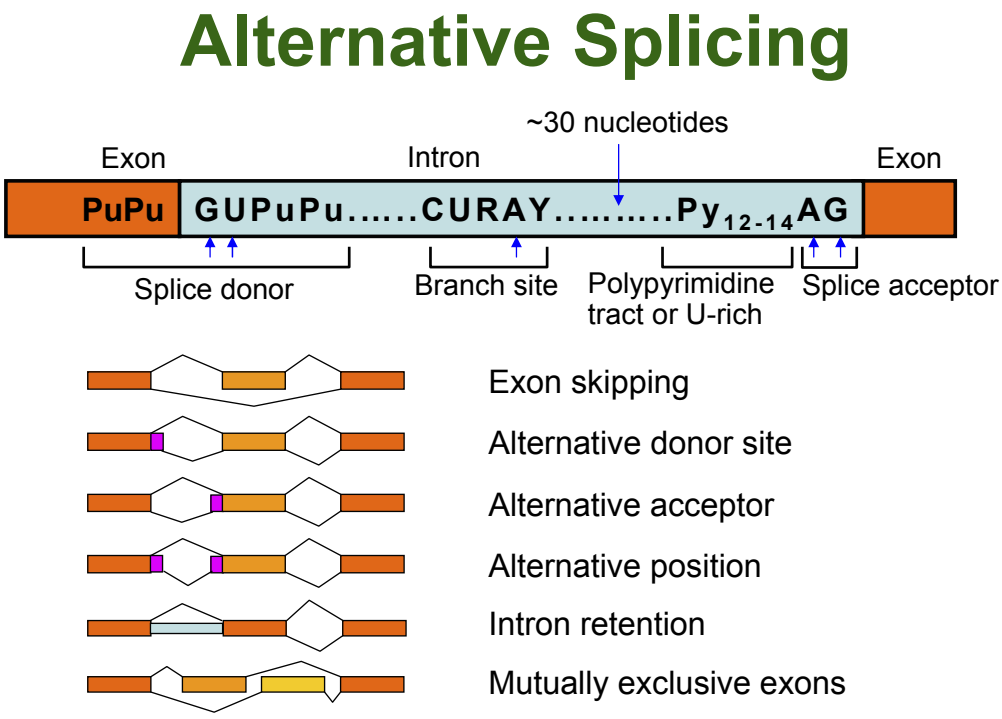


Figure 1-4. Characteristics of alternative splicing.
4 types of conserved short sequences within the pre-mRNA help to ensure the specificity of splicing mediated by spliceosomes including the splice donor at the 5' site of intron with GU dinucleotide flanked by few purines (Pu, i.e. A or G), splice acceptor at the 3'site of intron with AG dinucleotide, branch site (UACUAAC in yeast and vertebrates and CURAY in plant, R is purines (A, G) and Y is pyrimidine (C, U)) within the intron, and polypyrimidine tract or several U-rich sequences. 6 types of AS events occur, exon skip, alternative donor (5' splice site), alternative acceptor (3' splice site), alternative position, intron retention, and mutually exclusive. (from: Black 2003; Hartwell et al. 2004; Simpson et al. 2002)

and U2AF to the 3' splice site. This results in the binding of U2 snRNP to branch point and the assemble of splicing machinery. In the intron definition model, splicing regulators such as SR proteins bind to ISR (intronic splicing regulator) and recruit U1 snRNP and U2AF to the 5' and 3' splice sites, resulting in the binding of U2 snRNP to the branch point and the assembling of splicing machinery. The exon definition model is favored in organisms such as humans and other vertebrates with large introns separated by relatively small exons. It has been suggested that the intron definition model is favored in organisms such as plants with small introns (Reddy 2007).

Variable splicing patterns can produce multiple transcripts from a single gene and increase protein diversity. Alternative splicing of pre-mRNA is an important post-transcriptional regulatory mechanism that affects mRNA stability and increases the transcriptome and proteome complexity within and between cells and tissues (Reddy 2007; Wang et al. 2006). Alternative splicing has been studied extensively in mammalian systems. One of the well-known examples is sex determination in *Drosophila* which depends on a regulated series of RNA splicing events of *sex-lethal (Sxl), transformer (tra*), and *doublesex (dsx)* genes (Alberts et al. 1994; Black 2003). Alternative splicing generates two or more mRNAs from the same pre-mRNA by using different combinations of splicing mechanisms. Six types of alternative splicing have been observed, including exon skipping (or cassette exon), alternative donor site (or alternative 5' splice site), alternative acceptor (or alternative 3' splice site), alternative position, intron retention, and mutually exclusive exons (Black 2003; Hartwell et al. 2004; Reddy 2007; Wang et al. 2006). Most AS events are mutually independent. For example , 67% of the *Arabidopsis* and 60% of the rice identified alternatively spliced genes have only single AS event while 10.6% of the *Arabidopsis* and 7.4% of the rice have multiple events within the same transcript (Wang et al. 2006). This suggests that multiple AS events in these genes are coordinated.

It has been estimated from EST and/or cDNA comparisons that 35–60% human genes are alternatively spliced, while estimates from splicing-sensitive microarrays are as high as 70%. Over half (58%) of alternative splicing in humans involves exon skipping and only

5% is intron retention (Reddy 2007; Wang et al. 2006). Using data from 7 different plant species (rice, wheat, maize, barley, sorghum, soybean, and *Arabidopsis*), it was estimated that 36.9% of plants genes were alternatively spliced (Chen et al. 2007). From *Arabidopsis* and rice EST/cDNA sequences, it has been shown that at least 21.8% of the *Arabidopsis* and 21.2% of the rice genes have alternative splicing events and over half (56.1% for *Arabidopsis* and 53.5% for rice) involved intron retention while only 8.1% (*Arabidopsis*) and 13.8% (rice) of the alternative splicing events were exon skipping. This study also found that 41.7% of *Arabidopsis* AS genes are also alternatively spliced in rice (Wang et al. 2006). This suggests that human genes favor the exon definition model and plants favor the intron definition model in splice site recognition, possibly due to the larger intron size in humans compared to plants. This alternative splicing mechanism might be conserved among plant species. The lower frequency of AS events in plants might also be due to the reduced number of ESTs/cDNAs used in the study. About 70–88% of the human and 78.4% of the *Arabidopsis* AS events occur in protein coding regions and affect the encoded protein (Modrek et al. 2002; Reddy 2007). Altered reading frames occurred in 35% of the human, 43% of *Arabidopsis*, and 36% of rice AS events possibly producing premature termination codons (PTCs) which may be the targets of a mRNA surveillance mechanism, nonsense-mediated mRNA decay (NMD) (Wang et al. 2006). PTCs is defined as an in-frame stop codon that resides >50 bp upstream of the 3' most exon-exon junction (Lewis et al. 2003).

Biological roles

Several observations suggest that splice variants may have biological roles (Reddy 2007). These include: (a) AS is predominant in some gene families and not evenly distributed in most intron-containing genes, (b) many AS events occur in genes that encode multidomain proteins, (c) AS in plants is tissue-specific and also regulated by developmental stages, stresses and hormones, (d) the position of AS introns is conserved among distantly related plant species, (e) many splicing variants with retained introns are not removed by RNA surveillance mechanisms and are recruited for translation, (f) a biological function (photosynthesis, defense response, flowering, and stress response) of AS

variants from several genes have been identified, and (g) various biotic (viral and bacterial pathogens) and abiotic stresses (heat, cold, and heavy metals) affect alternative splicing of pre-mRNA in plants. Examples of AS in higher plants include: Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase in spinach and *Arabidopsis* (Werneke et al. 1989), diacylglycerol kinase in tomato (Snedden et al. 2000), N resistance gene in tobacco (Dinesh-Kumar et al. 2000; Reddy 2007), chloroplast ascorbate peroxidase in spinach (Yoshimura et al. 1999), autoregulation of 4 splicing variants of flowering time control gene *FCA* in *Arabidopsis* (Macknight et al. 2002; Quesada et al. 2003), a stress and hormone regulated conserved family of splicing regulators - serine/argining-rich SR proteins (Palusa et al. 2007), cold stress upregulated nuclear protein and pre-mRNA splicing factor *STABILIZED1* in *Arabidopsis* (Lee et al. 2006), and temperature-sensitive splicing of mutant *apetala3-1* gene in *Arabidopsis* (Sablowski et al. 1998).

Members of the SR protein family are highly conserved with one or two RNA recognition motifs (RRMs) and a protein-protein interaction arginine/serine-rich (RS) domain (Palusa et al. 2007). SR proteins are splicing regulators which bind exonic splicing enhancers or silencers (ESE/ESS) by RRMs and recruit other spliceosomal proteins through the RS domain to weak splice sites and promote splicing (Palusa et al. 2007; Reddy 2007). There are 10, 19, and 24 SR genes identified in human, *Arabidopsis*, and rice respectively (Palusa et al. 2007; Reddy 2007). In *Arabidopsis*, 15 out of 19 SR genes produce 95 transcripts due to extensive alternative splicing and temperature stress (cold and heat) which dramatically altered AS patterns of several SR genes while hormones only affected AS patterns of 3 SR genes (Palusa et al. 2007). This suggests that temperature and hormone stresses influence alternative splicing of SR genes which alters the splicing of other pre-mRNAs (Reddy 2007). For example, ectopic expression of *SR30* in *Arabidopsis* resulted in a large down-regulation of full-length endogenous *SR1/SR34* mRNA and showed pleiotropic changes in morphology and development including delayed transition from vegetative to flowering (Lopato et al. 1999).

Another example is with the *ap3-1* mutant of *Arabidopsis* which when grown at 16°C has nearly normal petals and stamens, but when grown at 28°C develops sepaloid organs in the second whorl and carpelloid organs instead of stamens (Sablowski et al. 1998). This phenotype is similar to a loss-of-function *ap3* mutation (Sablowski et al. 1998). It has been suggested that this temperature-dependent phenotype of *ap3-1* mutant is caused by a temperature-sensitive splicing defect since the mutation changes the nucleotide from A to U at the end of the *AP3* exon 5 which weakens the interaction with U1 snRNA and increases exon 5 skip (Sablowski 2007).

AS and flowering time control: *FCA* and *FLC*

One well-studied alternative splicing event involved in flowering time regulation is with *Arabidopsis FCA* (*FLOWERING TIME CONTROL PROTEIN A*). *FCA* regulates flowering time together with *FY* (*FLOWERING LOCUS Y*) by repressing *FLC* expression through the autonomous pathway (Figure 1-3). *FCA* contains 20 introns and encodes a nuclear protein containing an RRM-type RNA-binding domain and WW (tryptophan-tryptophan) protein interaction domain (Macknight et al. 1997; Macknight et al. 2002; Quesada et al. 2003). FCA's WW domain is suggested to bind the consensus Pro-Pro-Leu-Pro sequences of FY, a RNA 3' end-processing factor which facilitates polyadenylation at the RNA 3' end (Simpson et al. 2003). The *Arabidopsis FCA* transcript is alternatively processed at introns 3 and 13 resulting in 4 transcripts (α, β, γ, δ), with only the γ-transcript encoding a functional protein (Eckardt 2002; Macknight et al. 1997; Macknight et al. 2002). *FCA* also negatively regulates its own expression spatially and temporally by promoting cleavage and polyadenylation within intron 3 by FCAγ-FY to generate a major non-functional transcript *FCAβ* (Quesada et al. 2003). This autoregulation of *FCA* expression normally controls the levels of active *FCA* transcript γ and prevents precocious flowering (Quesada et al. 2003). FCA is an ABA receptor and FCA binding with ABA prevents the interaction with FY required to autoregulate full-length *FCA*γ and reduce *FLC* expression, thereby maintaining FLC-mediated flowering repression (Finkelstein 2006; Razem et al. 2006). Three alternatively spliced variants (α, β, γ) were identified in rice *OsFCA*, a homolog of

Arabidopsis FCA (Lee et al. 2005). Overexpression *OsFCA* in a *fca-1* mutant *Arabidopsis* showed weak upregulation in *SOC1* and slightly earlier flowering, but it did not affect *FCA* autoregulation and the expression of *FLC* and *FCA* variants (α, β, γ) (Lee et al. 2005).

Twenty-seven *Arabidopsis* MADS-box genes, or 25% of all *Arabidopsis* MADS-box genes, in the A, AGL15, ANR1, Bs, CD, FLC, E, F, Mα, Mγ, and Mδ classes with alternative splicing events were found in 3 databases (Alternative Splicing in Plants (ASIP) (http://www.plantgdb.org/ASIP/), Alternative Splicing and TRanscription Archives (ASTRA) (http://alterna.cbrc.jp/), and National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez)) and are summarized (Table 1-2).

Alternative splicing has been reported for *FLC* (Caicedo et al. 2004; Lempe et al. 2005; Werner et al. 2005) and *MAF1–MAF5* (Ratcliffe et al. 2003; Ratcliffe et al. 2001; Scortecci et al. 2001) genes. At least three *MAF1/AGL27/FLM* (196, 192, and 173 amino acids), four *MAF2/AGL31* (196, 145, 145 and 80 amino acids), five *MAF3/AGL70* (196, 185, 118, 77 and 77 amino acids), five *MAF4/AGL69* (200, 136, 63, 66, and 69 amino acids), and two *MAF5/AGl68* (198 and 184 amino acids) splice variants have been identified (Ratcliffe et al. 2003; Ratcliffe et al. 2001). For the case with *FLC*, some of the alternatively spliced variants encode a truncated protein without C-terminal or without I + K domains (Lempe et al. 2005; Werner et al. 2005). There is one *FLC* splicing variant consisting of MADS-domain, C-terminal, and partial intron 1 and 6 that was detected after 15 days of vernalization but not at normal growth temperatures (Caicedo et al. 2004). There is no functional consequence yet ascribed to splice variation among *Arabidopsis FLC*-like genes. *FLC* homologs have also been identified in *Brassica* (Schranz et al. 2002) and sugar beet (Reeves et al. 2007). The sugar beet *FLC* homolog, *BvFL1*, is downregulated by cold and produces 4 alternatively spliced variants with all variants delaying flowering in the transgenic *Arabidopsis* null mutant *flc-3* (Reeves et al. 2007).

Table 1-2. Alternative splicing of MADS-box genes of *Arabidopsis*.
Twenty-seven *Arabidopsis* MADS-box genes, in A, AGL15, ANR1, Bs, CD, FLC, E, F, Mα, Mγ, and Mδ classes with alternatively splicing events were retrieved from 3 database (ASIP, ASTRA, and NCBI) on Feb. 21, 2008. Gene names are based on the *Arabidopsis* Information Resource (TAIR) (http://arabidopsis.org/).

Locus ID	Gene	Class	Type(s) of alternative splicing	Source
Arabidopsis type II MADS-box genes				
At5g60910	AGL8/FUL	A	5UTRs(1)	ASTRA, NCBI
At5g13790	AGL15	AGL15	IntronR(3)	ASIP
At3g57390	AGL18	AGL15	IntronR(1)	ASIP, ASTRA, NCBI
At3g57230	AGL16	ANR1	IntronR(1)	ASIP, ASTRA, NCBI
At2g14210	ANR1/AGL44	ANR1		ASTRA
At5g23260	ABS/AGL32/TT16	Bs	AltA(2)	ASIP, ASTRA, NCBI
At3g58780	AGL1/SHP1	CD	AltA(1)	NCBI
At4g09960	AGL11/STK	CD	ExonS(1)	ASIP, ASTRA, NCBI
At2g42830	AGL5/SHP2	CD	AltD(2)	ASIP, ASTRA
At2g03710	AGL3/SEP4	E	AltA(2);IntronR(1)	ASIP, ASTRA, NCBI
At1g24260	AGL9/SEP3	E	AltD(2)	ASIP
At5g62165	AGL42	F	IntronR(1)	ASIP, ASTRA, NCBI
At5g51870	AGL71	F	IntronR(1)	ASIP, ASTRA, NCBI
At5g51860	AGL72	F	AltA(1)	ASTRA, NCBI
At5g10140	FLC/AGL25	FLC	ExonS;AltA;AtlD	NCBI and papers
At1g77080	MAF1/AGL27	FLC	AltA(2);ExonS(3);IntronR(4)	ASIP, ASTRA, NCBI
At5g65050	MAF2/AGL31	FLC	AltA(2);ExonS(3);IntronR(2)	ASIP, ASTRA, NCBI
At5g65060	MAF3/AGL70	FLC	AltA(3);ExonS(1);IntronR(3)	ASIP, ASTRA, NCBI
At5g65070	MAF4/AGL69	FLC	AltA(2);AltP(4);ExonS(1);IntronR(2)	ASIP, ASTRA
At5g65080	MAF5/AGL68	FLC	ExonS(1)	ASIP, ASTRA, NCBI
Arabidopsis type I MADS-box genes				
At1g29960	AGL64/T1P2.16	Mα		ASTRA
At1g48150	AGL74	Mα	AltD(2)	ASIP
At1g18750	AGL65	Mδ	IntronR(1)	ASIP, NCBI
At5g26650	AGL36	Mγ		ASTRA
At2g26880	AGL41/F12C20.8	Mγ	IntronR(1)	ASIP
At3g05860	AGL45	Mγ	IntronR(1)	ASIP, ASTRA, NCBI
At1g22590	AGL87/F12K8.7	Mγ	5'UTRs(1)	ASTRA, NCBI

ASIP (Alternative Splicing in Plants, http://www.plantgdb.org/ASIP/)

ASTRA (Alternative Splicing and Transcription Archives, http://alterna.cbrc.jp/)

NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez)

IntronR = intron retention, ExonS = exon skip, 5'UTRs = 5'UTR splice

AltD = alternative donor site, AtlA = alternative acceptor site, AltP = alternative position

Questions

Little is known about the regulatory factors that govern bud dormancy in woody plants even after decades of study. What genes are involved in the regulation of bud initiation, development, dormancy and the release from dormancy? Do analogous regulatory pathways control flowering and bud dormancy? Do type II MADS-box genes regulate diverse morphological development in poplars? This study focused on the relationship between type II MADS-box genes and plant dormancy with an in-depth study of *PtFLC2*. Here, I present the first analyses of expression of type II MADS-box genes in poplar vegetative and floral tissues and shoot apices throughout bud development and dormancy. DNA sequencing results will help to clarify their exon-intron structures and possible alternatively spliced transcripts. Functional studies of *PtFLC2* and its alternatively spliced variants examined their role in the regulation of bud development and cold-mediated dormancy release in poplar and the possible conservation of function by investigating flowering time control in *Arabidopsis*.

Chapter 2: Molecular and Phylogenetic Analyses of the Type II MADS-box Gene Family in Poplar

Abstract

MADS-box genes encode a group of transcription factors that regulate the development of vegetative and reproductive organs in plants. Little is known about the role of MADS-box genes in tree development. To advance the understanding of MADS-box genes in tree vegetative bud development and dormancy, 57 putative type II MADS-box genes representing 14 functional classes were identified from the JGI *Populus trichocarpa* genome database. DNA sequencing of cDNAs for the Poplar type II MADS-box genes revealed that a significant number of these genes possess different intron-exon structures than those predicted in the JGI Poplar genome database. Furthermore, splicing variants were detected for approximately 20% of the type II MADS-box genes. Using RT-PCR analysis, transcripts of all 14 classes were detected in vegetative (shoot apices, leaf, bark, xylem, and root) and/or floral tissues. These results indicate that these genes may play specific regulatory roles in specific developmental process in poplar. Such knowledge could be useful for selecting candidate genes for functional studies in poplar development.

Introduction

Transcription factors are important regulators of cellular processes and mediate responses to biotic and abiotic stimuli. MADS-box genes encode a family of transcription factors present in plants, animals, and fungi. MADS-box genes are known to be involved in the regulation of diverse developmental processes in flowering plants including reproductive (flower, seed, fruit) and vegetative (root, leaf) development and differentiation (Table 1-1B). Many *Arabidopsis* loss-of-function mutants (*agamous, agl24, ap1, ap3, cal, pi, flc, ful, maf1, maf2, sep1, sep2, sep3, sep4, soc1, svp*, etc.) have been studied in detail to understand their regulatory roles. For example, floral homeotic genes such as *AP1, PI, AP3, AG*, and *SEP1–4*

identify floral organs (Ng et al. 2001; Theissen 2001; Theissen et al. 2000), some MADS-box genes govern reproductive development (Ng et al. 2001; Theissen 2001), and flowering-time genes such as *FLC, AGL24*, *SVP* and *SOC1* repress or promote the floral transition mediated by internal or environmental factors (plant age, day-length, cold) (Hartmann et al. 2000; Sheldon et al. 2002; Sheldon et al. 2000). MADS-box genes have also been shown to be involved in regulating embryo, pollen, endosperm, seed, fruit, root, guard cells, and leaf development (Alvarez-Buylla et al. 2000a; Theissen et al. 2000). The floral-quartet-model describes how combinations of class A, B, C, D and E genes form specific tetramers involved in the regulation of floral organ identification (sepals, petals, stamens, carpels, ovules) (Figure 1-2A) (Krizek et al. 2005; Theissen et al. 2001).

The evolutionary history of the MADS-box gene family in plants is characterized by duplication events and subsequent divergence and fixation (Alvarez-Buylla et al. 2000b; Litt et al. 2003). All MADS-box genes originated from a common ancestor and have diverged into two large groups, type I and type II, by gene duplication that occurred prior to divergence of plants and animals (Alvarez-Buylla et al. 2000b). The type II MADS-box genes comprise the MEF2-like genes in animals and fungi and the MIKC-type genes in plants. MIKC-type proteins contain 4 major domains: a highly conserved MADS-box domain of ~55–60 amino acids which includes the DNA-binding domain and dimerization motif, a less-well-conserved intervening (I) domain (~30 amino acids) that appears to be involved in DNA binding specificity and dimerization, a well-conserved keratin-like coiled-coil (K) domain (~70 amino acids) which contains a protein-protein interaction region, and a variable length C-terminal (C) domain which is involved in transcriptional activation, posttranslational modification, or protein-protein interaction (Alvarez-Buylla et al. 2000b; Hileman et al. 2006; Kaufmann et al. 2005; Nam et al. 2003; Vandenbussche et al. 2003). Type I MADS-box genes contain MADS-domain but lack the K-domain and experienced faster birth-and-death evolution than type II MADS-box genes (Nam et al. 2004). The biological functions of type I MADS-box genes are poorly understood due to lack of mutant phenotypes.

It has been suggested that the multimer formation with other MADS-box proteins through heterodimerization mediated by the K-domain may be crucial for the functional divergence of B MADS-box genes (Hernandez-Hernandez et al. 2007). The C-terminal domain of B class proteins appears to be essential for ternary complex formation between A and B class proteins (Egea-Cortines et al. 1999; Tzeng et al. 2004; Vandenbussche et al. 2003). Studies have demonstrated that MADS-box proteins bind to CArG-boxes of DNA as homo- and heterodimer and may also form high-order complexes with MADS-box and non-MADS-box proteins through K-domain or/and C-terminal (de Folter et al. 2005; Honma et al. 2001; Kaufmann et al. 2005; Theissen et al. 2001).

There are 108 MADS-box genes identified in *Arabidopsis thaliana* and more than 70 MADS-box genes identified in *Oryza sativa* (rice) (Becker et al. 2003; Nam et al. 2003; Parenicova et al. 2003). Type II MADS-box genes can be classified on the basis of their function, expression pattern, and phylogenetic analysis into at least 14 classes (A, PI, AP3, C, D, E, F, G, T/SVP, AGL12, ANR1, FLC, Bs, S) (Nam et al. 2003; Nam et al. 2004) or 13 clades (SQUA, DEF, GLO, AG, AGL2, TM3, AGL6, STMADS11, AGL12, AGL17, FLC, GGM13, AGL15) (Becker et al. 2003). The functional classes/clades of 108 *Arabidopsis* MADS-box genes found in TAIR (The *Arabidopsis* Information Resource) and recent studies (Becker et al. 2003; Nam et al. 2003; Nam et al. 2004; Ng et al. 2001; Parenicova et al. 2003; Ratcliffe et al. 2003; Theissen et al. 2000) by phylogenetic analysis are summarized in Table 1-1. Extensive studies have focused on revealing the regulatory roles of MADS-box genes in plant growth and development. *Arabidopsis* and rice are the two species that have been, in the past, studied the most. Poplar has recently joined this group after the publication of the genome sequences in 2004. The functional conservation of MADS-box genes across species could provide a starting point towards revealing possible regulatory networks in trees. Reconstructing a robust tree of MADS-box genes from representative angiosperms will help to make rational assumptions about their functions. Along with examining these relationships is the caveat that orthologous MADS-box genes do not necessarily have analogous developmental roles (Causier et al. 2005; Kramer et al. 2004) and paralogous

MADS-box genes may evolve to equivalent developmental functions (Causier et al. 2005). Sub- and/or neo-functionalization might be maintained and novel or ancestral roles might be swapped between these genes during the events of gene duplication and diversification (Irish et al. 2005; Moore et al. 2005). Due to the redundancy, subfunctionalization (partitioning of ancestral function between duplicates), neofunctionalization (gain of new function), and gene function swapping during the gene evolution, a shift from analyzing individual gene functions towards studying MADS-box gene function at the subfamily level has been proposed (Rijpkema et al. 2007).

The annual growth cycle of temperate trees is characterized by active shoot growth during spring and summer followed by a quiescent or dormant vegetative phase. The length of the growth period between spring bud break and fall bud set impacts tree productivity and wood quality. Dormancy is a survival strategy that enables plants to survive unfavorable environmental conditions (Rohde et al. 2000; Thomas et al. 1997). Bud dormancy also synchronizes shoot growth and influences plant architecture (Rohde et al. 2000). Dormancy is defined as "the temporary suspension of visible growth of any plant structure containing a meristem" (Lang 1987). Dormancy can be divided into three types: endodormancy, paradormancy, and ecodormancy (Lang 1987). Endodormancy involves growth control mediated by an endogenous signal within the affected dormant structure. Typically endodormancy requires exposure to chilling temperatures to overcome dormancy. The formation of the apical bud and dormancy induction can be viewed as a morphological manifestation of endodormancy mediated by the apical meristem within the bud or apices (Rohde et al. 2000). Paradormancy involves a signal originating in a structure within the plant but different from the affected dormant structure. Apical dominance is a classical example of paradormancy. Ecodormancy occurs when environmental conditions are unsuitable for growth. Photoperiod, light quality, temperature and phyto-hormones are important regulators of bud formation and dormancy in trees (Horvath et al. 2003; Rohde et al. 2007b). Poplar apical bud initiation and development is induced by exposure to short day

photoperiods (Ruttink et al. 2007). Exposure to prolonged chilling temperatures is required in order for bud burst and shoot regrowth to occur (Rohde et al. 2007b).

Here, I present the analyses of expression of type II MADS-box genes in various vegetative tissues (shoot apices, leaf, bark, xylem, root) of three different genotypes: *Populus trichocarpa* Nisqually-1, *Populus deltoides* x *trichocarpa* clone 545-4183, and *Populus* alba x *tremula* clone 717-1B4 as well as in reproductive tissue of *Populus* alba x *tremula* clone 717-1B4. In addition the expression of type II MADS-box genes was also monitored throughout bud development and dormancy in apical buds of these three different genotypes. Sequence analysis of the type II MADS-box gene transcripts provided additional data to clarify their exon-intron structures and assist in gene annotation. It also indicated that a number of the type II MADS-box gene transcripts are alternatively spliced. Such knowledge could be useful for selecting candidate genes for functional studies in poplar development.

Results

Gene identification and nomenclature

From the JGI *Populus trichocarpa v1.0* genome database (http://genome.jgi-psf.org/cgi-bin/searchGM?db=Poptr1), 883 unique gene models representing 119 unique genes were retrieved by searching sequences similar to 19 *Arabidopsis* MADS-box genes (4 type I, 1 S (MIKC*), and 14 type II) and filtering by gene model and scaffold location. Those gene models with predicted protein sequences less than 70 amino acids or lacking the MADS-domain were removed from the analysis. Using phylogenetic analysis with 32 *Arabidopsis* and 13 rice type I and II MADS-box genes, 2 S-like, 45 type I-like and 57 type II-like MADS-box genes were identified in the poplar genome. These genes grouped into S-like, 4 type I and 14 type II functional classes (Table 1-1, Figure 2-1B). The 108 *Arabidopsis* MADS-box genes and 104 functional poplar MADS-box genes plus 15 possible pseudo-genes are summarized in Table 1-1. Each poplar gene was assigned a name based on similarity to the functional class and poplar genome linkage group or scaffold location. For example, there are 5 genes in class A located on linkage group (LG) IV, VIII, X, XII and

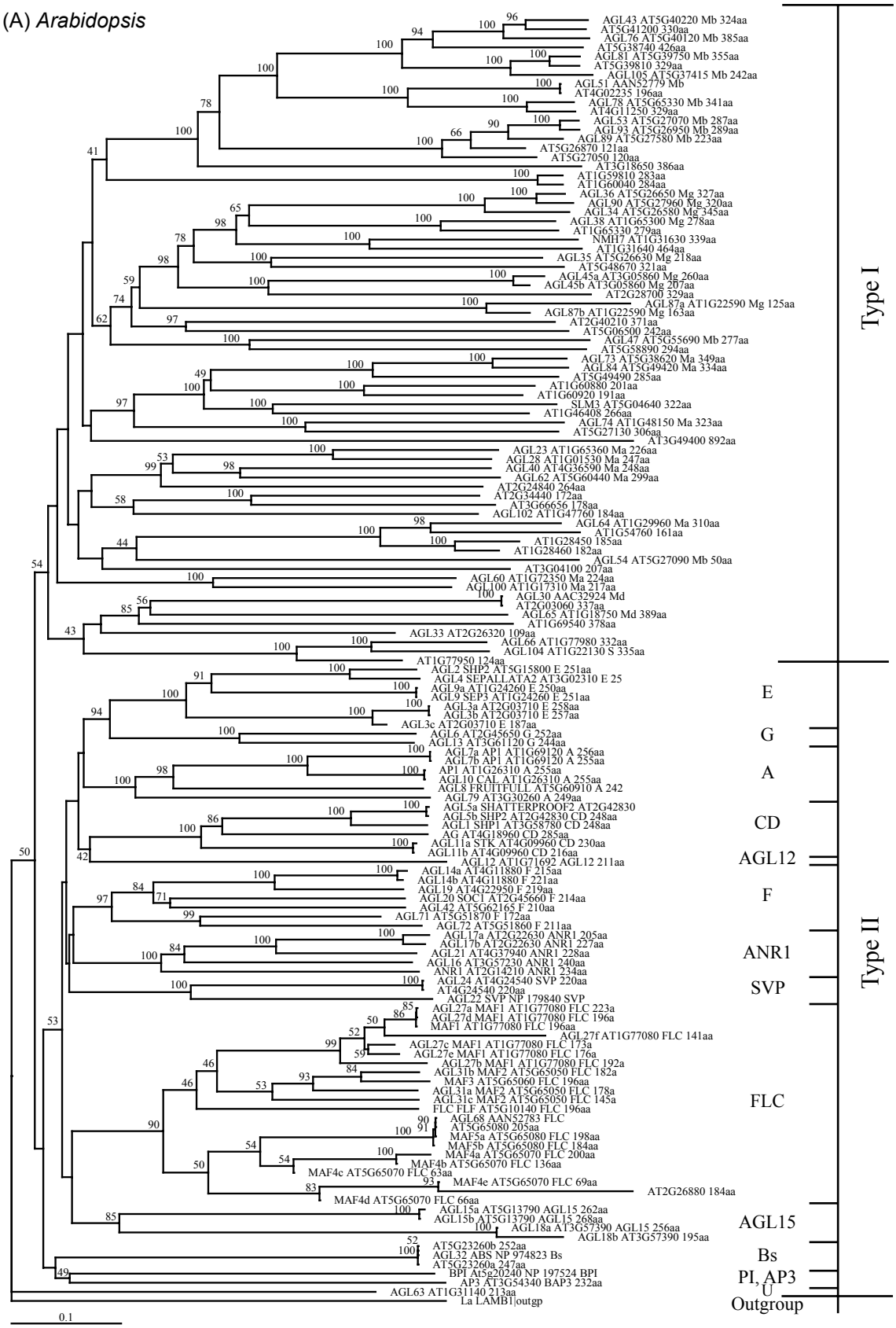


Figure 2-1. Phylogeny of (A) *Arabidopsis* and (B) *Populus trichocarpa* MADS-box proteins (NJ, 1000 bootstrap)

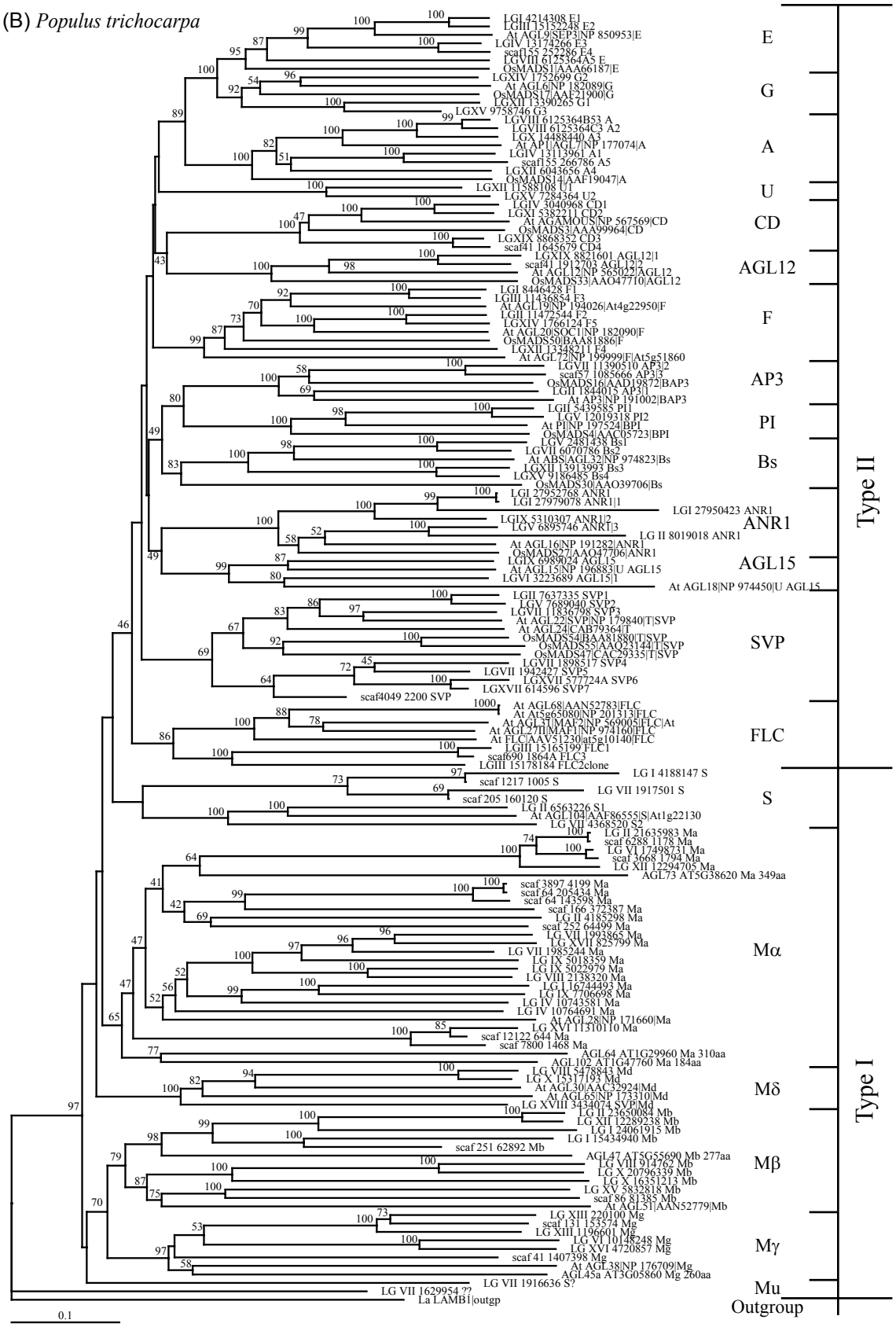


Figure 2-1. Phylogeny of (A) *Arabidopsis* and (B) *Populus trichocarpa* MADS-box proteins (NJ, 1000 bootstrap)

scaffold 155, so they were named as *PtA1, PtA2, PtA3, PtA4*, and *PtA5* respectively. Pt is the abbreviation of *Populus trichocarpa*. Two genes located on linkage group VII (nucleotide location 1629954 and 1916636) were not assigned to any of the functional classes with this analysis (Figure 2-1B) and are designated as PtMu. Two genes located on LG XII with nucleotide location 11588108 and LG XV with nucleotide location 7284364 were grouped with all other type II MADS-box genes with 46.4% bootstrap support but were not assigned to any of the functional classes in type II and are designated as *PtU*. Gene functions of *Arabidopsis* type II MADS-box genes and gene annotations, gene models used, their scaffold locations and PCR primer sequences of the putative poplar type II MADS-box genes are also included in Table 1-1.

Gene structure and splicing variants of poplar type II MADS-box genes

Gene specific primers were designed to analyze gene expression of 55 type II MADS-box genes in poplars except *PtFLC5* and *PtSVP9*. Specific primer combination could not be designed for *PtFLC5* because of its similarity to *PtFLC2* and the incomplete sequence present in the JGI database. To verify the fidelity of amplifications, PCR products from each primer combination for each gene were cloned and sequenced. From sequence analyses, new primers were designed when the original primer combination did not distinguish similar genes. Three genes (*PtANR1b, PtBs1*, and *PtBs4*) failed to produce PCR products whose sequences corresponded to the predicted sequences in the JGI database. When a discrepancy was found with the JGI database, such as different 5' and 3' intron splicing sites, number of introns and exons, start codon location, or open reading frame prediction, a new gene name with "_M" after original name was used to address this modified coding sequence and protein sequence. Where splicing variants were found, a new name with "as1", "as2", etc. was used to distinguish this splicing variant from the predicted sequence. In total, the DNA sequence of the transcript for 16 genes differed (28.1% of the genes) from the JGI predicted transcript sequence (Table 2-1). Splicing variants were detected for 11 genes (20%) (Table 2-2, Figure 2-2). Because the primer combinations were not designed to amplify the entire coding sequences, it is likely that the actual percentage of splicing variants may be higher than 20%.

Table 2-1. Locations and alignment differences in intron-exon structure between predicted JGI genes and cloned cDNA sequences.

5'UTR and 3'UTR, 5'and 3' untranslated region; 5'-S, 5'-splice site; 3'-S, 3'-splice site; 5'- and 3'-S, 5'- and 3'-splice sites; IR, intron retention; +Exon, add exon; Indel, indel within exon; N/A, not available due to lack of cloned sequence; no JGI, no sequences in JGI poplar database; ~: no sequence; -#-: many nucleotides skipped.

Gene	5'UTR	Start codon	5'-S	3'-S	5'- and 3'-S	IR	+Exon	Indel	3'UTR	Difference
PtA3	N/A	0	0	2	0	0	0	0	N/A	15 bp
PtA5	1	0	0	0	0	0	0	0	N/A	27 bp
PtAGL15b	N/A	0	1	0	0	1	0	0	N/A	262 bp
PtANR1a	N/A	0	1	2	0	0	0	1	N/A	141 bp
PtANR1d	1	1	0	0	0	1	1	0	N/A	98 bp
PtANR1e	N/A	0	1	0	0	0	0	0	N/A	15 bp
PtCD1	N/A	0	0	1	0	0	0	1	N/A	12 bp
PtE4	N/A	0	0	1	0	0	0	0	N/A	3 bp
PtFLC2	N/A	0	0	0	0	0	2	1	1	61 bp
PtFLC3	N/A	0	0	1	0	0	0	0	1	
PtFLC4	1	1	0	0	0	0	0	0	0	
PtFLC5	N/A	0	0	0	0	0	2	0	no JGI	
PtG1	N/A	0	1	0	0	0	1	0	N/A	18 bp
PtG2	N/A	0	1	0	0	0	0	0	N/A	3 bp
PtSVP2	N/A	0	0	1	0	0	0	0	N/A	10 bp
PtSVP7	N/A	0	0	0	0	0	0	0	1	200 bp

Gene	The alignment of differences between JGI poplar database and cloned partical cDNA sequences
PtA3 (Nisqually-1)	JGI: CATGTTAGTGAAGCAG~~~~~~~~~~AAGGAGAAGAATGATAAA-#-GCTTAAACATCGGT~~~~~~CACCAGGAAGAAGCTCC Cloned:CATGTTAGTGAAGCAGATCAAGGAGAAGGAGAAGAATGATAAA-#-GCTTAAACATCGGTGGCAGTCACCAGGAAGAAGCTCC
PtA5 (Nisqually-1)	JGI: CACCATACCATCACAGAAATAGAAACTT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ATGGGGAGAGGTAGGGTTCAGTTGAAGA Cloned:CACCATACCATCACAGAAATAGAAACTTTGAAGAAAAGAGTGAAAAATAATAAAGATGGGGAGAGGTAGGGTTCAGTTGAAGA
PtAGL15b (Nisqually-1)	JGI: CCACGCAAATAAAAGAT~~~~~~~~~~~~~~~~~~~~AAACCAGATTTGAAAGAG-#-CGCAGACAG~~~~~~~~~ATTGAGGA Cloned:CCACGCAAATAAAAGATGTACTAT-#-CTTCTTCAGAAACCAGATTTGAAAGAG-#-CGCAGACAGGCAAGGATTATTGAGGA
PtANR1a (717-1B4)	JGI: GCAGCCAGTCACC-#-TGAAGATTTA-#-CCTAGTTGTG-#-CATCGGTAAAGCAAT-#-AAAGCATGC-#-CACAGGAACAA Cloned:GCAG~~~~TCACC-#-TGAAG~~~~~~~~~~~~~~TTGTG-#-CATCG~~~~~GCAAT-#-AAAG~~~~~~~~~~~~~~GATCAA
PtANR1d (Nisqually-1)	JGI: exon 1 (fgenesh1_pg.C_LG_II001009)+GAAAAAG~~~~~#~~~~~GGCAA-#-TTGGG~~~~~~#~~~~~~AGTC Cloned:change exon 1 to grail3.0003096101+GAAAAAGGACC-#-AAAGGGCAA-#-TTGGGGTACA-#-ACCAGAGTC
PtANR1e (Nisqually-1)	JGI: AAAACCAGCTGGAGATGAGTTTGAAAGGA~~~~~~~~~~~~~~~~GAACAAATTTTAACTGATGAAATTAAAGACCTGAACAGA Cloned:AAAACCAGCTGGAGATGAGTTTGAAAGGAGTTCGAATGAAAAAGGAACAAATTTTAACTGATGAAATTAAAGACCTGAACAGA
PtCD1 (717-1B4)	JGI: GATGGCCCTTCAGTTAGT~~~~~~~~~~CCAAGTGCAGCAGTTT-#-CTACATGTATGCTAAAAAA~~CCTGAAGTAGCGGAG Cloned:GATGGCCCTTCAGTTAGTTTAATAATCTCCAAGGGCAACAGTTT-#-CTACATGTATGCTAAAAAAAACCTGAAGTAGCGGAG
PtE4 (Nisqually-1)	JGI: GGGCCAGTCTGAAAAGGAGACGCAGCAGAACAACTACCAGGAATACTTGAAGCTAAAAACGAGAGTGGACGTGCTACAGCGTT Cloned:GGGCCAGTCTGAAAAGGAGACGCAG~~~AACAACTACCAGGAATACTTGAAGCTAAAAACGAGAGTGGACGTGCTACAGCGTT
PtFLC2 (Nisqually-1)	JGI: ATCATGCAGAG~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GAACCTTGAA-#-GAAAAGATGGTTGAAAGAAGAAAACCA Cloned:ATCATGCAGAGGTATATTGTGGTA-#-TACTAATGGTTGAAAGGAACCTTGAA-#-GAAAAGATG~TTGAAAGAAGAAAACCA
PtFLC3 (Nisqually-1)	If relocated exon 7 and 3'UTR based on PtFLC1, there will be only 8 bp difference between transcripts of PtFLC1 (1021 bp) and PtFLC3. Those 8 bp contains 4 SNP in coding sequence and 4 in the last 7 nts at the end of 3'UTR. If it is true, there will be 2 AS.
PtFLC4 (Nisqually-1)	Cloned:gw1.I.7772.1 as exon 1 for MADS + fgenesh1_pg.C_LG_I000499 as rest of exons (I,K,C) Detail sequence and exon-intron structure is shown in figure 3-1
PtFLC5 (Nisqually-1)	Completely reconstruct exon-intron structure based on PtFLC2 Detail sequence and exon-intron structure is shown in figure 3-1
PtG1 (Nisqually-1)	JGI: AGGAAGGTACATACATTTACACGCATTATG~~~~~~GAACAAGAGCT Cloned:AGGAAG~~~~~~~~~~~~~~~~~~~~~~~~~ACACAACTGATGTTTGATAAAATGGAAGAGTTACGCCTAAAGGAACAAGAGCT
PtG2 (Nisqually-1)	JGI: AAGGCAAAGTATGAATCCCTTCAACGCACTCAAAGGTTGCATTTGCTTGGGGAGGATCTTGGGCCCCTGAATGTGAAAGAGCT Cloned:AAGGCAAAGTATGAATCCCTTCAACGCACTCAAAG~~~GCATTTGCTTGGGGAGGATCTTGGGCCCCTGAATGTGAAAGAGCT
PtSVP2 (Nisqually-1)	JGI: TTTGGAAGAGAATAAGCATTTGAAACAGAAA~~~~~~~~~~ATGACAACCATTTGTAAGGGAAAGAGACCTGCCCTTGTTGAT Cloned:TTTGGAAGAGAATAAGCATTTGAAACAGAAATTGTATGCAGATGACAACCATTTGTAAGGGAAAGAGACCTGCCCTTGTTGAT
PtSVP7 (Nisqually-1)	JGI: GAACCAGCGATTGAAGCAGAGA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~TTAATGAATTTATCAAAGGGACAAGGGC Cloned:GAACCAGCGATTGACGCAGAGAGTGAGACTATGAGACCTA-#-TTTATTCGGCAGTTAATGAATTTATCAAAGGGACAAGGGC

Table 2-2. Summary of alternative splicing (AS) events in poplar type II MADS-box genes. Alt. 5'-S, alternative 5'-splice site; Alt. 3'-S, alternative 3'-splice site; Alt. position, alternative 5'- and 3'-splice sites; IR, intron retention; ES, exon skip

Gene	Alt. 5'-S	Alt. 3'-S	Alt. position	IR	ES	No. of AS
PtA2	0	0	0	0	1	2
PtAGL12a	0	0	1	0	0	2
PtANR1a	1	3	0	0	0	2
PtANR1c	0	0	0	3	0	2
PtAP3b	0	0	0	1	0	2
PtCD4	0	0	0	3	0	2
PtFLC1	0	0	0	0	1	2
PtFLC2	1	2	0	4	12	13
PtFLC4	0	0	0	1	0	2
PtSVP3	1	0	0	0	0	2
PtSVP7	0	0	0	2	0	3

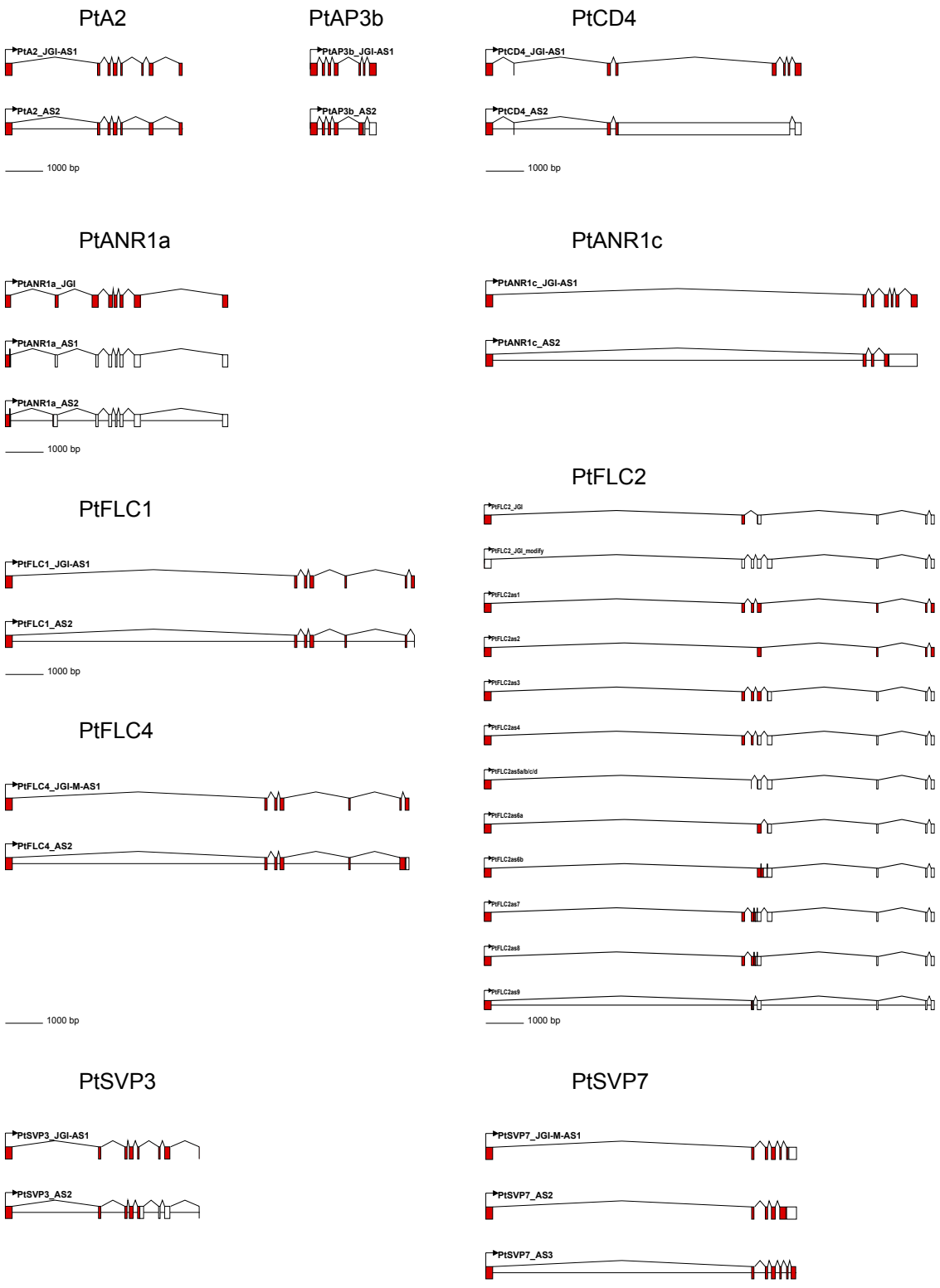


Figure 2-2. Structure of alternatively spliced variants of poplar type II MADS-box genes. Coding exons are represented as red filled boxes, non-coding exons due to early stop codon are open boxes, introns are shown as solid lines, and splicing events are represented by angled lines.

Phylogenetic analysis of the *Populus trichocarpa* MADS-box gene family

Because of these differences between the type II MADS-box proteins predicted in the JGI database and the sequences generated through cDNA cloning and sequencing, a comparison of the phylogenetic relationships between JGI predicted sequences and those generated in this study were compared. The alignments of predicted and sequence-generated type II MADS-box proteins were performed using MAFFT 6 (E-INS-i) and MAFFT 6 (L-INS-i) (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh et al. 2005b; Katoh et al. 2002). RevTrans 1.4 was used to align coding sequences based on MAFFT aligned sequences. Most of the gaps were removed and the majority of the conserved sequences used for PAUP* and PhyML analyses included MADS and K domains. Character number was reduced from 1811 to 993 and 534 for MAFFT (E-INS-i) aligned DNA sequences after removing gaps. All 1811, 993 and 534 characters of MAFFT (E-INS-i) aligned DNA sequences were tested to see if removing gaps would affect the analysis. The analysis with 1811 characters was unable to generate sufficient bootstrap values to support grouping to functional classes due to the high percentage of gaps in the aligned sequences. There was minor difference in the deep branch of tree topology when using 993 and 534 characters. All the phylogenetic analyses of MAFFT (E-INS-i) and MAFFT (L-INS-i) aligned DNA and protein sequences with parsimony, distance and likelihood methods confirmed that all the modified sequences and splicing variants were still grouped in the same class with their predicted sequence in JGI database. The majority of the differences revealed in intron-exon sites or splicing variants occurred in regions outside of the MADS-box domain with a few genes where the location of the start codon differed. The results of these phylogenetic analyses are summarized in Figure 2-3. All trees were rooted with the *LAMB1* gene of clubmoss. The trees shown in Figure 2-3 are ML (maximum likelihood) trees with branch length and bootstrap supports shown at appropriate nodes. Only bootstrap supports higher than 50% were shown.

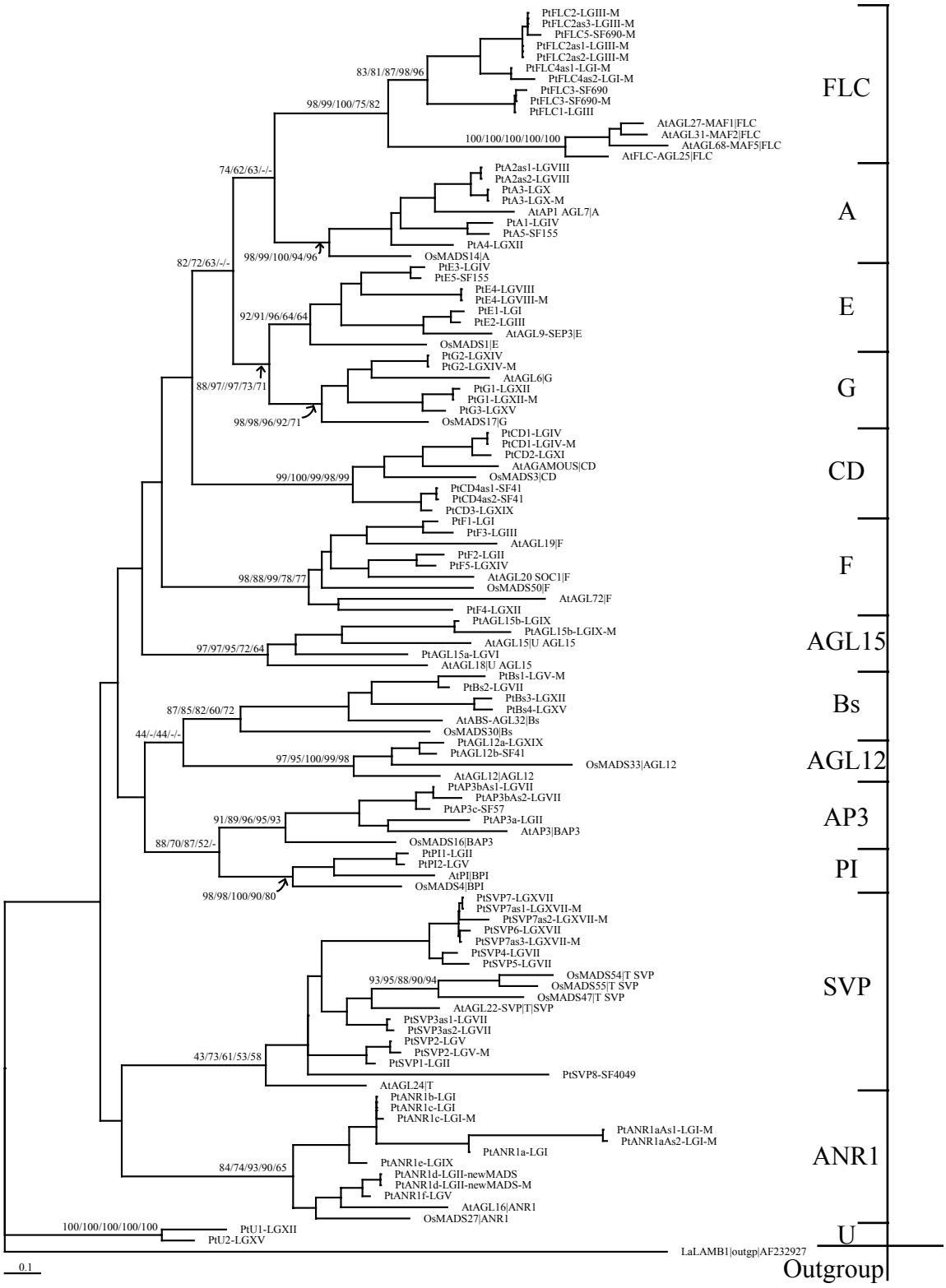


Figure 2-3. Summary of phylogenetic trees of poplar type II MADS-box genes.
(A) DNA coding sequences are from JGI prediction and modifications from this study described in table 3. Only bootstrap values >40 of each class were displayed: ML(GTR+G)-MAFFTE-113CDS-993char /537char / MAFFTL-113CDS-543char / MP-MAFFTE-124CDS-537char / MAFFTL-124CDS-543char.

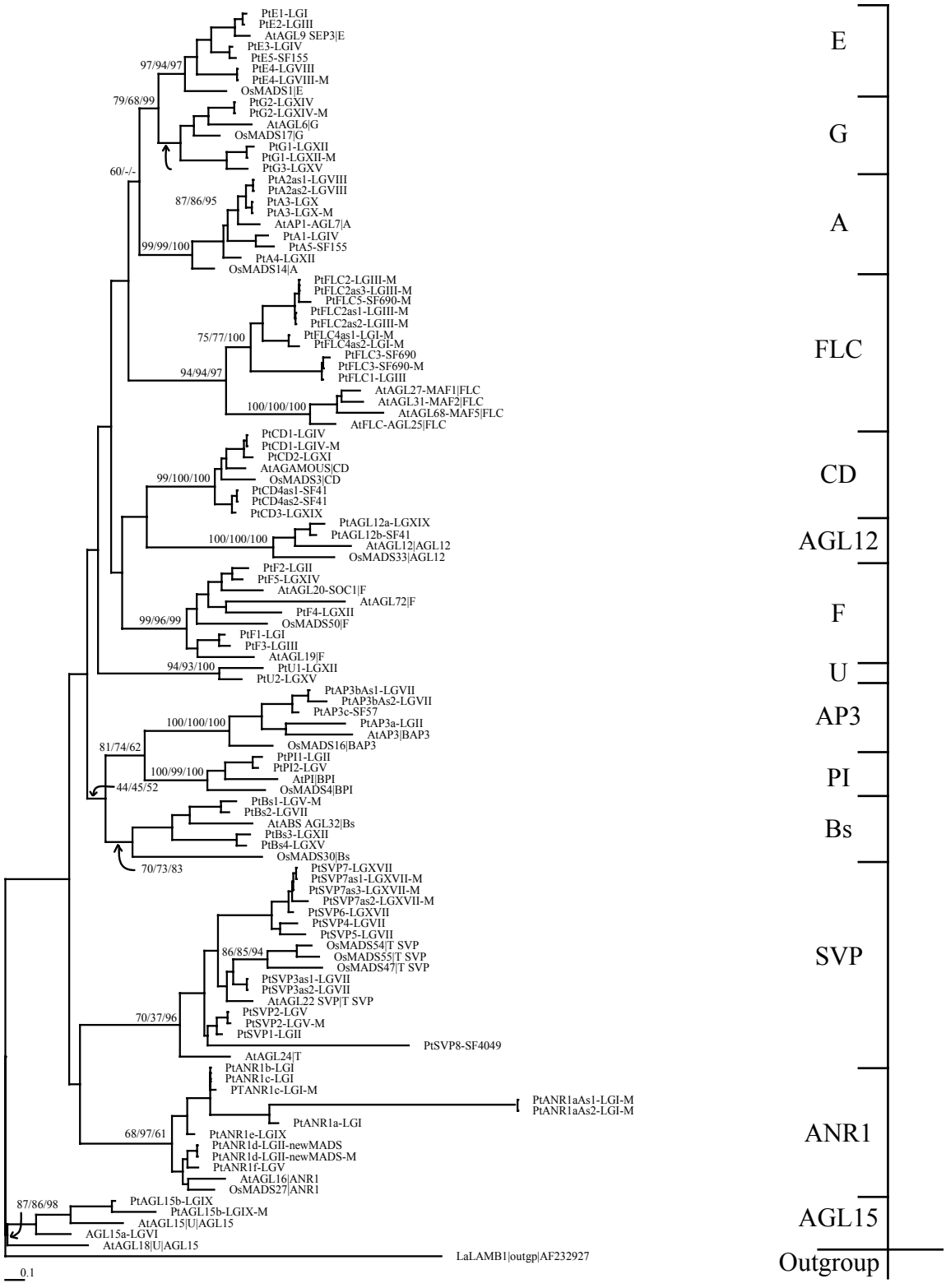


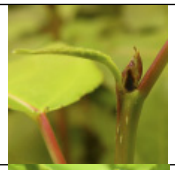
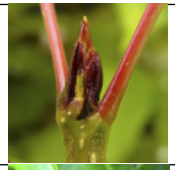
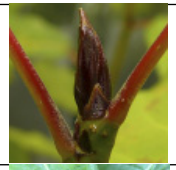
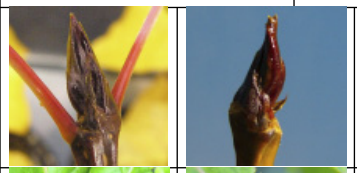
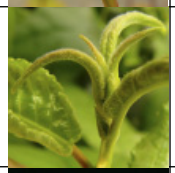
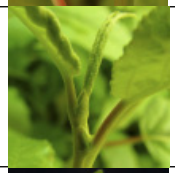
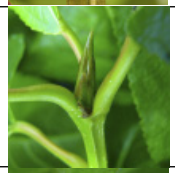
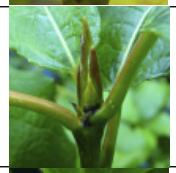
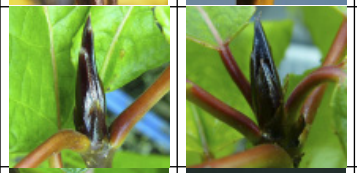
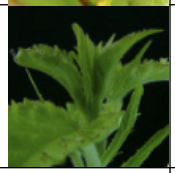
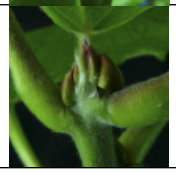
Figure 2-3. Summary of phylogenetic trees of poplar type II MADS-box genes (continued)
(B) Protein sequences are from JGI prediction and modifications from this study described in table 3. Only bootstrap values >50 of each class were displayed: ML (JTT+G)-MAFFTL-113aa-181char / ML (JTT+G)-MAFFTE-113aa-331char / MP-MAFFTE-124aa-281char.

Expression of type II MADS-box gene in various tissues and dormant buds of poplars

Gene specific primers were designed to analyze gene expression of 55 poplar type II MADS-box genes in different tissues (shoot apices, leaf, bark, xylem, root, and flower) and during experimentally induced bud dormancy and release (Table 2-3) in various genotypes of poplar (Nisqually-1, 717-1B4, and 545-4183). The majority of the primer combinations resulted in either a single PCR product or 2–3 products that may represent alternatively spliced products. Some primer combinations including *PtAGL15a, PtBs3, PtCD1, PtCD2, PtCD3, PtFLC2*, and *PtFLC3* produced a single PCR product at lower PCR cycle numbers but additional products could be detected with increased amplification cycles. In addition, differences in the number of PCR products for *PtSVP7* primer number 2 (*PtSVP72*) varied among genotypes with Nisqually-1 and 545-4183 producing single products while multiple bands were observed in the 717-1B4 genotype. Several genes, including *PtAGL15b, PtANR1a, PtFLC1, PtSVP71* (*PtSVP7* primer number 1) resulted in complicated multiple bands. Expression patterns of *PtAGL15b* and *PtANR1a* were unclear, so their expression data was excluded in the analysis. The specificity of amplification for *PtANR1b*, *PtBs1*, and *PtBs4* could not be confirmed by sequencing, so their expression data was excluded from the analysis.

Expression of type II MADS-box genes were summarized to floral organ related genes, vegetative tissue related genes, and flowering time related genes (Figure 2-4, Table 2-4). Several *PtCD* and *PtE* genes show no or weak expression with high PCR cycles in shoot apices in LD and SD induced dormancy, which indicates that shoot apices and apical bud might be an *agmous* leaf-like organ (Figure 1-2A). Expression of several type II MADS-box genes was detected in all tissues including both vegetative and floral tissues. This included *PtA1, PtF1, PtF2, PtF3, PtF4, PtF5, PtFLC1as1, PtFLC2as1, PtFLC3, PtG1, PtG3, PtSVP2, PtSVP3, PtSVP5, PtSPV6, PtSVP7as1, PtSVP7as3*, *PtSVP8*, and *PtU2*. Genes in *F, FLC* and *SVP* classes are involved in flowering time control and the transition from vegetative to flower in *Arabidopsis* (Table 1-1). *PtSVP1* was detected in all vegetative tissues but not in floral tissue. Several genes including *PtAGL12a, PtAGL12b, PtANR1cas2,*

Table 2-3. Dormancy development of *Populus trichocarpa* Nisqually-1, *P. alba* x *tremula* 717-1B4, and *P. deltoides* x *trichocarpa* 545-4183

Dormancy stage	Active growth	Bud Initiation	Bud Maturation	Dormancy Induction	Endodormancy	Chilling Accumulation	Bud break
Experimental Induction	LD (16 hrs light) @ 18 °C	1-3 weeks SD (8 hrs light) @ 18 °C	3-6 weeks SD @ 18 °C	6-8 weeks SD @ 18 °C	8-12 weeks SD @ 10/4 °C (day/night)	12-18 weeks SD @ 10/4 °C or DD @ 4 °C	2-6 weeks LD @ 18 °C
Features	- Active growth	- Reduced stem elongation and leaf production - Bud scale initiation and growth - Enclosure internal bud structures - Shoot growth resumes in LD	- Reduced water content - Increased bud dry weight - Accumulation of storage reserves - Shoot growth resumes after 2-4 weeks of LD	- Reduced water content - Increased bud dry weight - Acquisition of desiccation tolerance - Buds of 717 & Nisqually-1 resume growth after 3-8 weeks of LD	- Reduced water content - Increased bud dry weight - Leaf turns yellow - Meristem quiescence - Only axillary buds resume growth after 4-6 months of LD - Bud break does not occur in LD (> 6 months)	- Defoliation - Number of days required for bud break and shoot growth resume in LD decrease with increasing chilling hours accumulated - Bud water content increases	- Bud breaks and shoot resumes growth
P. deltoides x *trichocarpa* 545-4183							
P. trichocarpa Nisqually-1							
P. alba x *tremula* 717-1B4							
SD (weeks)	SD0	SD3	SD6	SD8	SDC12	SDC14	SDC18	LD

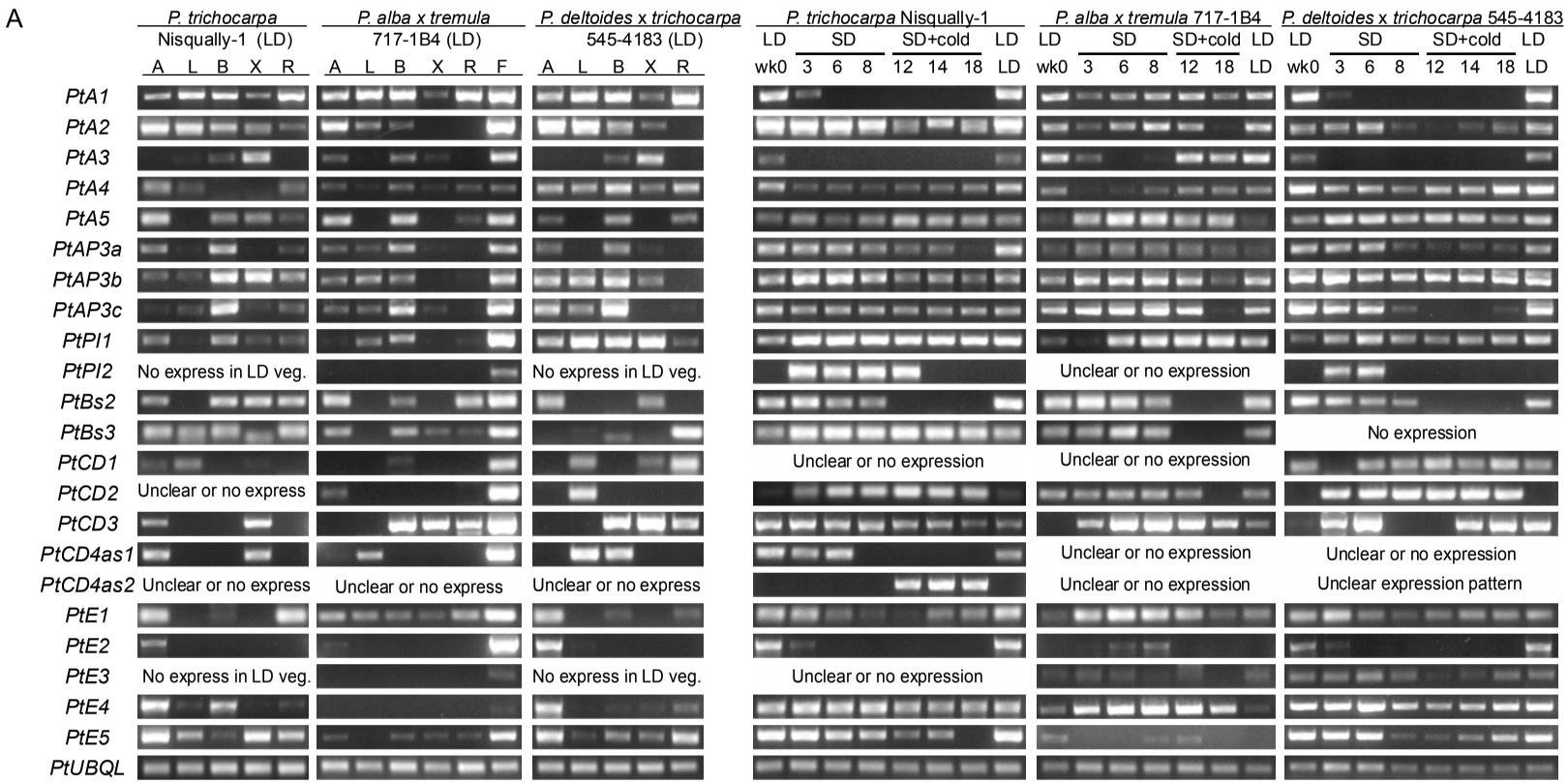


Figure 2-4. Expression of poplar type II MADS-box genes.
(A) Floral organ related genes from the *PtA, PtAP3, PtPI, PtBs, PtCD*, and *PtE* classes.
(B) Vegetative tissue related genes from the *PtAGL12, PtAGL15, PtANR1, PtG*, and *PtU* classes.
(C) Flowering time related genes from the *PtF, PtFLC*, and *PtSVP* classes.
For tissues, shoot apex (A), leaf (L), bark (B), xylem (X), and root (R), total RNA from 3 different genotypes *Populus trichocarpa* Nisqually-1, *Populus deltoides* x *trichocarpa* clone 545-4183, and *Populus alba* x *tremula* clone 717-1B4, and reproductive tissue (F) of *Populus alba* x *tremula* clone 717-1B4 were used for RT-PCR using gene specific primers. Gene expression in shoot apex (or apical bud) during bud development and dormancy of *Populus trichocarpa* Nisqually-1, *Populus deltoides* x *trichocarpa* clone 545, and *Populus alba* x *tremula* clone 717-1B4 used total RNA from apices or buds and RT-PCR with gene specific primers. PCR products were cloned and verified by DNA sequencing. Unclear or no expression indicates that no definitive product could be obtain even with extensive PCR cycles (>40). No expression in LD vegetative tissue indicates that no PCR product could be detected. *PtUBQL* was used as internal control. PCR conditions are listed in Table A-1.

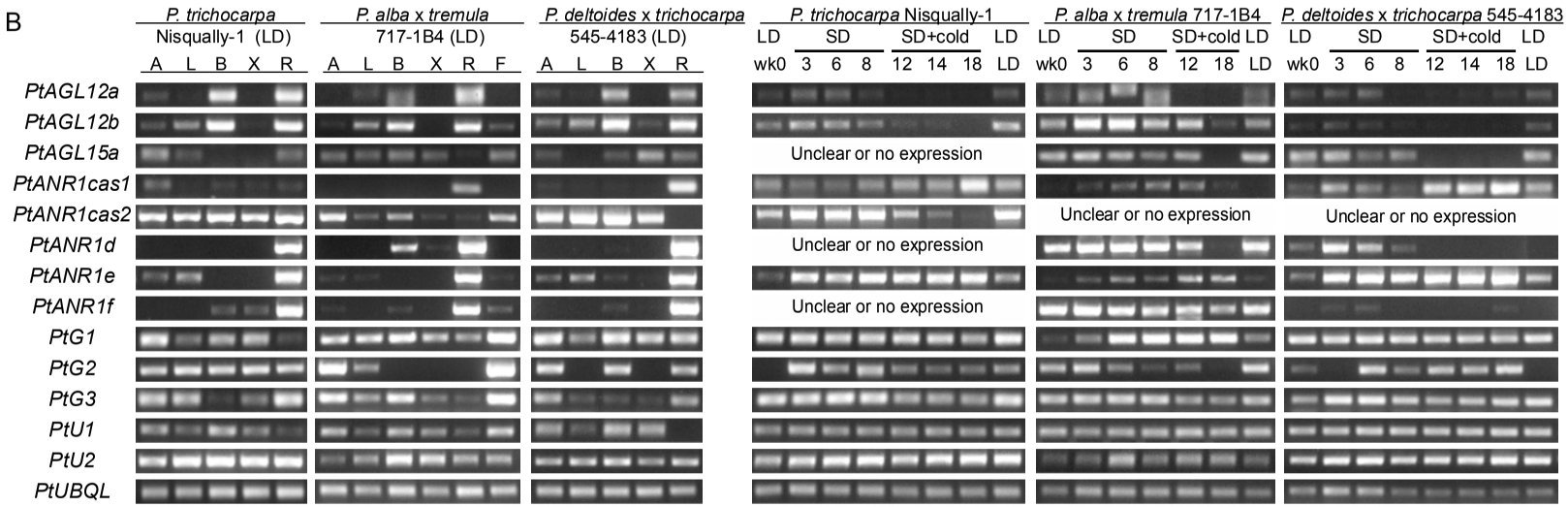


Figure 2-4. Expression of poplar type II MADS-box genes. (continued)

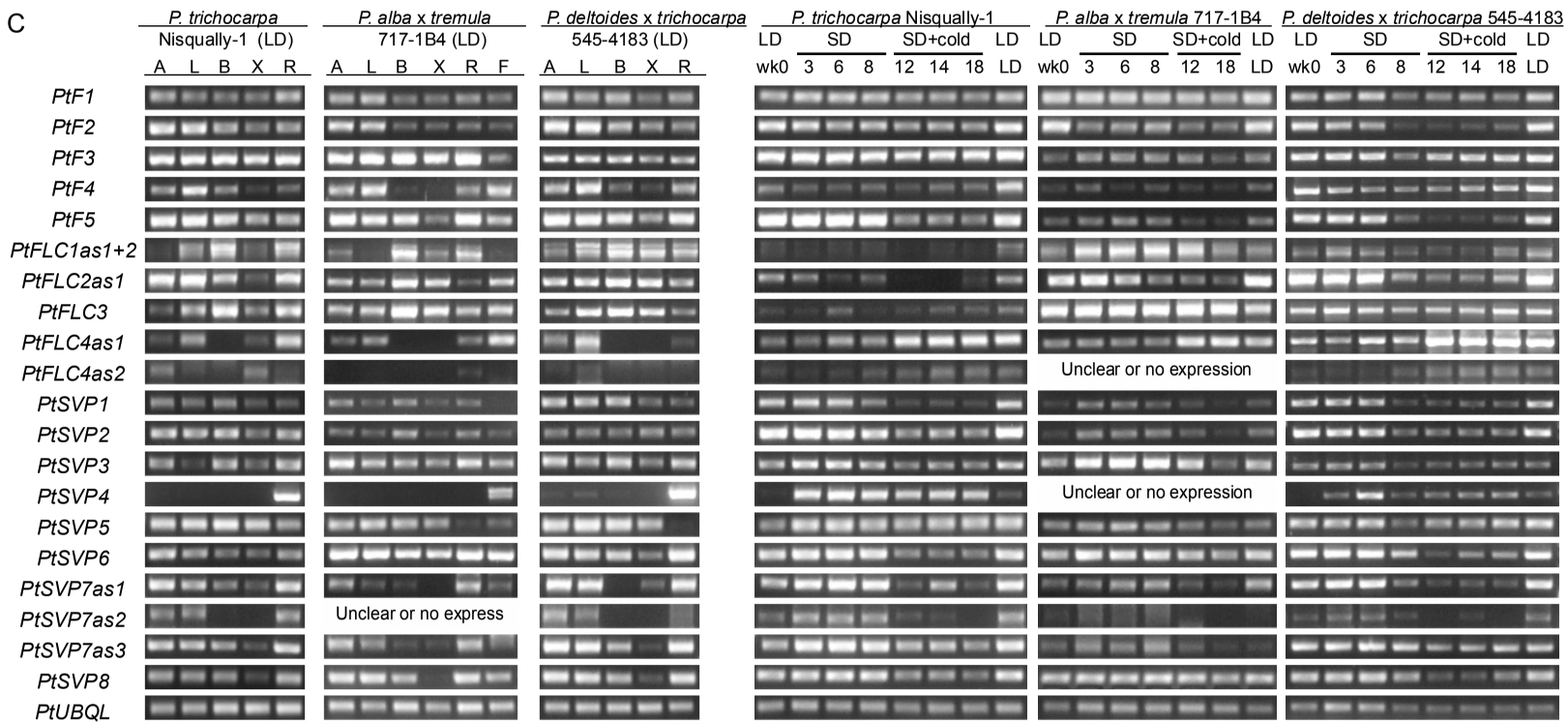


Figure 2-4. Expression of poplar type II MADS-box genes. (continued)

Table 2-4. Summary of expression of poplar type II MADS-box genes.
Expression was assayed by RT-PCR using gene specific primers for RNA from different tissues (shoot apex, leaf, bark, xylem, root, and floral bud) and during experimentally induced bud development, dormancy and release in various genotypes of poplar (Nisqually-1, 717-1B4, and 545-4183). Expression in floral buds are compared only to other tissues in 717-1B4 genotype.

Gene	Difference among genotypes	All tissues in LD	2 major expression tissues in LD				Apex during dormancy	
			Floral bud	Apex or leaf	Xylem or bark	Root	Down-regulation	Up-regulation
Floral organs related genes								
PtA1	Still express in 717 during dormancy	v	v			v	v	
PtA2			++	v			v	
PtA3	Upregulate in 717 by cold		++		v			
PtA4			v			v	by SD	by cold
PtA5	Upregulate in Nisqually by SD		v		bark		by cold	by SD
PtAP3a			++		bark		v	
PtAP3b			++		bark		v	
PtAP3c			++		bark		v	
PtPI1	Down by cold in 545		++		bark			by SD
PtPI2	Express in Nisqually and 545 in SD	not in veg.	++				by cold	by SD
PtBs2			++	apex			v	
PtBs3	No express in apex of 545		++			v	v	
PtCD1	High express in root of 545		++					545
PtCD2	Express in leaf of 545		++					v
PtCD3	Transient in apex of 545 in SD		++		v		Nisqually	717, 545 by SD
PtCD4as1	No express in apex of 717 and 545		++	v			Nisqually	
PtCD4as2	Express in apex of Niqually by cold							Nisqually
PtE1	Up by SD and down by cold in 717		++	apex			Nisqually, 545	717
PtE2			++	apex			v	
PtE3	No express in Nisqually in dormancy	not in veg.	v				717, 545	
PtE4	Upregulate in apex of 717 in SD		v	apex			Nisqually, 545	717
PtE5			++	apex			Nisqually, 545	
Vegetative related genes								
PtAGL12a					bark	v	v	
PtAGL12b					bark	v	v	
PtAGL15a			v	v			v	
PtANR1cas1						v		
PtANR1cas2	Dormancy expression in Nisqually		v	v	v		by cold	by SD
PtANR1d	Dormancy expression in 717 & 545					v	by cold	by SD
PtANR1e				v		v		v
PtANR1f	Dormancy expression in 717					v	717	
PtG1	No change in Nisqually & 545 in dormancy	v	++	v				717
PtG2	Different expression in dormancy		++	v			Nisqually, 717	545
PtG3		v	++	v			v	
PtU1			v		v			
PtU2		v		v	v			
Flowering time related genes								
PtF1		v					v	
PtF2		v					v	
PtF3		v					v	
PtF4		v					v	
PtF5		v					v	
PtFLC1as1		v	no				v	
PtFLC2as1		v					v	
PtFLC3		v						
PtFLC4as1			v					v
PtFLC4as2	No expression in dormancy of 717	none						v
PtSVP1		v	no	v	bark		v	
PtSVP2		v		v	v		v	
PtSVP3		v		v	v		by cold	by SD
PtSVP4	No express in vegetative tissues in 717		v			v	by cold	by SD
PtSVP5		v		v	v		v	
PtSVP6		v	v	v		v	v	
PtSVP7as1		v		v		v	by cold	by SD
PtSVP7as2	No express in 717 LD tissues			v		v	v	
PtSVP7as3		v		v		v	by cold	by SD
PtSVP8		v		v		v	v	

PtANR1d, PtANR1e, and *PtANR1f* were expressed mainly in root tissue which is similar to the expression pattern of those genes in *AGL12* and *ANR1* classes of *Arabidopsis*. *PtA1, PtA4, PtBs3, PtSVP4, PtSVP6, PtSVP7as1, PtSVP7as2, PtSVP7as3,* and *PtSVP8* showed relatively abundant expression in roots.

Several genes including *PtA1, PtA2, PtA3, PtA5, PtAP3a, PtAP3b, PtAP3c, PtPI1, PtBs2, PtBs3, PtCD1, PtCD2, PtCD3, PtCD4as1, PtE1, PtE2, PtE5, PtFLC4as1, PtG1, PtG2*, and *PtG3* were expressed predominately in floral buds. Some of them also showed relatively higher expression in shoot apices or leaf among all vegetative tissues. Genes in *A, AP3, PI, C,* and *E* classes are involved in floral organ identification in *Arabidopsis* (Table 1-1). Genes in *Bs* and *D* classes are involved in the formation of ovule and seed in *Arabidopsis* (Table 1-1).

Most genes show differential expression in the dormancy cycle. Several genes including *PtA1, PtA2, PtAGL12a, PtAGL12b, PtAGL15a, PtANR1cas2, PtAP3a, PtAP3b, PtAP3c, PtBs2, PtBs3, PtCD3* (Nisqually-1)*, PtCD4as1* (Nisqually-1)*, PtE1* (Nisqually-1, 545-4183)*, PtE2, PtE3* (717-1B4, 545-4183), *PtE4* (Nisqually-1, 545-4183), *PtE5* (Nisqually-1, 545-4183)*, PtF1, PtF2, PtF3, PtF4, PtF5, PtFLC1as1, PtFLC2as1, PtG3, PtSVP1, PtSVP2, PtSVP5, PtSVP6, PtSVP7as1, PtSVP7as2, PtSVP7as3,* and *PtSVP8* showed reduced expression in terminal bud during the dormancy cycle. Expression of some genes including *PtA5, PtANR1cas2, PtANR1d, PtANR1e, PtPI1, PtPI2, PtCD1* (545-4183)*, PtCD2, PtCD3 (*717-1B4, 545-4183*), PtCD4as2* (Nisqually-1)*, PtE1* (717-1B4)*, PtE4* (717-1B4)*, PtFLC4as1, PtFLC4as2*, a *PtG1* (717-1B4)*, PtSVP3, PtSVP4, PtSVP7as1,* and *PtSVP7as3* were induced by SD and/or SD with cold. They might be involved in the regulation of bud formation. Expression of some of the above genes (*PtA5, PtANR1cas2, PtANR1d, PtPI2, PtSVP3, PtSVP4, PtSVP7as1,* and *PtSVP7as3*) was down-regulated by cold.

Dormancy cycle and chilling requirement

Vegetative bud formation and dormancy development can be experimentally induced in poplar. Examples of the experimental treatments as well as associated physiological and developmental events for *Populus trichocarpa* clone Nisqually-1, *Populus alba* x *tremula*

clone 717-1B4, and *Populus deltoides* x *trichocarpa* clone 545-4183 are summarized in Table 2-3.

A number of experiments were performed from 2003 to 2007 to quantify the role of photoperiod and chilling temperatures in dormancy development and release for different poplar genotypes. Only the experiments with expression pattern of type II MADS-box genes during dormancy summarized in Table 2-4 and Figure 2-4 were presented in Table 2-5A. The "days to terminal or axillary bud burst" of 60% plants is labeled as >150 days for all those plants unable to regrow after 150 days.

Eight weeks of SD is sufficient for 545-4183 genotype to enter deep dormancy, but Nisqually-1 and 717-1B4 genotypes require extended SD to reach deep dormancy. The chilling requirement has been satisfied around 1,200–1,400 hours for Nisqually-1 and 545-4183 genotypes and ~1,000 chilling hours for 717-1B4 genotypes.

The temperature was held at 18°C for most dormancy measurements in the LD controlled environment chamber, but the temperature was 18–36°C for a few experiments of 545-4183 and 717-1B4 genotypes regrown in a LD greenhouse. The higher temperatures were caused by the inability of our controlled greenhouse to maintain the temperature below 25°C during hot summer afternoons. It is clear that the higher regrowth temperatures shorten the length of days required to bud burst at all treatments even when the chilling requirement is not satisfied (Table 2-5B).

Discussion

Annotation of the poplar MADS-box gene family

The annotation of genes has progressed rapidly since the publication of the genome database of *Populus trichocarpa* Nisqually-1 in 2004 (Tuskan et al. 2006), but a significant number of genes still remain unclassified . Using 3 methods of phylogenetic analyses (parsimony, distance, and likelihood) and 2 different kinds of sequences (DNA coding and protein) to identify poplar homologs of *Arabidopsis* and rice MADS-box genes assign these homologs to a specific functional class. In total, 119 unique poplar MADS-box genes were

Table 2-5. Dormancy quantification in *Populus trichocarpa* clone Nisqually-1, *Populus alba* x *tremula* clone 717-1B4, and *Populus deltoides* x *trichocarpa* clone 545-4183.
(A) Comparison among 3 genotypes
(B) Comparison between different regrowth temperatures
The "days to terminal or axillary bud burst" of 60% plants is labeled as >150 days for all those plants unable to regrow after 150 days.

A

Treatment	SD (wks)	3	6	8	10	12	14	16	18	20
	chilling (hrs)			0	224	448	672	896	1120	1344
Genotype	Bud	Days to terminal bud (TB) and axillary bud (AB) burst of 60% plants								
Nisqually-1	TB	6	10	22	>150	>150	81	41	29	27
Nisqually-1	AB	8	10	15	20	59	17	20	24	20
717-1B4	TB	6	9	19	>150[a]	118[a]	33[a]	26[a]	N/A	15[a]
717-1B4	AB	6	12	37	107[a]	107[a]	24[a]	21[a]	N/A	13[a]
545-4183	TB	6	48	>150	>150	>150	111	34	27	18
545-4183	AB	6	24	>150	>150	127	48	31	31	19

B

Treatment	SD (wks)	8	10	12	14	16	18
	chilling (hrs)	0	224	448	672	896	1120
Genotype	Regrow temp (°C)	Days to terminal bud (TB) burst of all plants					
717-1B4	18	63	>150[a]	>135[a]	45[a]	26[a]	N/A
717-1B4	18-36	19	38[a]	23[a]	12[a]	8[a]	N/A
545-4183	18	>150	>150	>150	>111	64	50
545-4183	18-36	42	63	96	68	40	33

[a] The treatment for *Populus alba* x *tremula* clone 717-1B4 is 10.3, 12.4, 14.7, 17 and 21 weeks of SD instead of 10, 12, 14, 16, and 20 weeks of SD, and 256, 496, 752, 1024, and 1360 chilling hours instead of 224, 448, 672, 896, and 1344 chilling hours.

retrieved and 15 of the 119 either laced a predicted MADS-domain or encoded short protein sequences (predicted protein is less than 70 amino acids) and were classified as pseudo-genes.

Sequence analysis of coding sequences obtained by RT-PCR and sequencing revealed that 28.1% of the predicted open reading frames of the type II MADS-box genes in the JGI database were incorrect (Table 2-1). It could be due to the lack of EST for those particular genes which would have assisted annotation and prediction. It is possible that this number under estimates the number of incorrect gene model predictions since the primers used to amplify the MADS-box gene transcripts were not designed to amplify the full-length coding sequence.

Gene expression analysis showed that 92.9% (52 of 56) of poplar type II MADS-box genes are transcribed, with *PtANR1b*, *PtBs1*, and *PtBs4* being the exceptions under the conditions used in this study. It is possible that not all these 52 expressed genes are functional because the expression patterns of 2 genes, *PtAGL15b* and *PtANR1a*, were unclear. It is also possible that the remaining genes are expressed in other tissues of different developmental stages (i.e seed or seedlings) that were not included in this study.

Initially 10 possible *PtSVP* genes were identified which is similar to the recent report (Zhu et al. 2007). Eventually, two of these genes were removed from *PtSVP* class. The 1st one with gene model eugene3.00070315 (LG_VII_1928153) predicts a 66 amino acid protein which was neither grouped with the *PtSVP* class nor other type I and II MADS-box genes in some different alignments and phylogenetic analyses. This gene was subsequently classified as a pseudo-gene in Mu group. The 2nd one with gene model fgenesh1_pg.C_LG_VII000296 (LG_VII_1917501) predicts a protein encoded by 5 exons which is 1–3 exons less than all other *PtSVP* genes. Furthermore this gene model lacked clear K and MADS domains. ClustalX alignments and NJ analyses with 1000 bootstrap on 119, 110, and 106 poplar, 32 *Arabidopsis*, and 13 rice MADS-box proteins sequences assigned this gene to S class with 110 and 106 poplar sequences and to SVP class with 119 poplar sequences. MAFFT alignment and NJ analyses with 1000 bootstrap replications assigned this gene to the

S class with 110 poplar sequences and to the SVP class with 119 and 106 poplar sequences. Subsequently this gene was assigned to the S class. The gene location and sequence of fgenesh1_pg.C_LG_VII000296 has been updated to linkage group VII (LG_VII) 1917576–1928422 with 7 exons, MADS and K domains which is similar to other *PtSVP* genes and is now designated this gene *PtSVP9* and a location of LG_VII_1917576 (Table 1-1).

There are also discrepancies between the JGI gene model prediction for 6 *PtFLC* genes and the cDNA sequencing data produced from this study. This included exon exclusion for the gene predictions for *PtFLC2*, the wrong exon-intron prediction for *PtFLC3*, the incorrect start codon and gene location for *PtFLC4*, and huge gaps in the DNA sequences for *PtFLC5* and *PtFLC6* (Table 2-1). It is important to generate more ESTs and full length cDNA sequences of poplar MADS-box genes to correct these deficiencies with the JGI gene predictions and enable researchers to annotate each gene more accurately.

Phylogenetic and functional classification of poplar MADS-box genes

Both DNA coding sequences and protein sequences were used to reconstruct phylogeny of the combination of *Arabidopsis*, rice and *Populus* genes since it has been suggested that protein sequences may give more robust estimates than DNA sequences in the study of long-term evolution (Glazko et al. 2003) and the evolution pattern of protein sequences is much simpler than DNA sequences (Nam et al. 2003). The bootstrap supports of the deepest branches are lower than 50%; therefore duplication orders of these classes can not be resolved. AP3 and PI, E and G, A and FLC, and E, G, A, FLC genes form monophyletic groups with high bootstrap supports for some analyses. The bootstrap supports are greater than 70 for genes of the AP3 and PI classes, 80 for genes of E and G classes, 60 for genes of A and FLC classes, and 70 for genes of E, G, A, and FLC classes in DNA ML trees. The bootstrap supports of DNA sequences with 993 characters are better than DNA sequences with 543 or 537 characters, and bootstrap support of DNA sequences with 543 or 537 characters is better than all protein sequences. DNA sequence alignment based on MAFFT aligned protein sequences also gives better resolution and a more robust result in phylogenetic analysis of poplar type II MADS-box genes (Figure 2-3). The phylogenetic

analyses also suggest that the maximum likelihood method is more robust than maximum parsimony and distance methods.

For all phylogenetic analyses of DNA sequences, the rice A, E, G, Bs, AP3, and PI genes were located at the base of their classes. MP (maximum parsimony) trees of DNA sequences showed that rice CD and AGL12 genes were also located at the base of their classes. For phylogenetic analyses of protein sequences, rice A, AGL12, AP3, PI, Bs and E genes were located at the base of their class in ML trees while rice A, AGL12, PI, Bs, CD, and E genes were located at the base of their class in MP trees. All 3 rice SVP genes from both DNA and protein analyses were grouped with *Arabidopsis* and poplar SVP genes as a separated monophyletic clade. This suggests that gene duplications within these classes (A, AGL12, AP3, PI, Bs, CD, E, and SVP) may have happened after the divergence of monocots and dicots. There is no *FLC*-like gene identified in rice. This suggests that the FLC class evolved in dicots only after the divergence of monocots and dicots. All analyses showed that 5 *Arabidopsis FLC* class genes and 5 poplar *FLC* class genes were grouped together as 2 separated monophyletic clades with very strong bootstrap support. The bootstrap supports of *FLC* genes were greater than 70 for all *FLC* genes, 100 for *Arabidopsis* FLC genes, and greater than 70 for poplar *FLC* genes. This suggests that additional gene duplication within *FLC* class may have occurred from a common ancestor after the speciation between *Arabidopsis* and poplar. All these classes mentioned above are involved in floral organ identification and flowering time regulation except AGL12. A detailed analysis including more genes of these classes in rice, *Arabidopsis* and other species should be performed to confirm this.

Alternative splicing in poplar type II MADS-box genes

Twenty-seven *Arabidopsis* MADS-box genes (i.e. 25% of all *Arabidopsis* MADS-box genes) in the A, AGL15, ANR1, Bs, CD, FLC, E, F, M_{α}, M_{γ}, and M_{δ} classes appear to have alternative splicing events based on data in three databases (Alternative Splicing in Plants (ASIP) (http://www.plantgdb.org/ASIP/), Alternative Splicing and TRanscription Archives (ASTRA) (http://alterna.cbrc.jp/), and National Center for Biotechnology Information

(NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez)) and are summarized (Table 1-2). In poplar, splicing variants were detected in 11 genes (~20%) in the PtA, PtANR1, PtAP3, PtCD, PtFLC, and PtSVP classes of 57 poplar type II MADS-box genes (Table 2-2, Figure 2-2). It has been suggested that 35–60% (ESTs/cDNAs) or 70% (splicing sensitive microarry) of human genes are alternatively spliced (Reddy 2007; Wang et al. 2006), and 36.9% of genes of 7 plants (rice, wheat, maize, barley, sorghum, soybean, and *Arabidopsis*) were estimated to undergo alternative splicing (Chen et al. 2007). Several observations have also suggested that alternatively spliced variants are predominant in some gene families and many AS events occur in genes that encode multidomain proteins (Reddy 2007). It is likely that the estimate of alternative splicing in the MADS-box gene family of both *Arabidopsis* and poplar is underestimated due to the lower number of cDNAs/ESTs used to identify splicing variants. Alternatively spliced events have been observed in genes of A (*FUL* in *Arabidopsis* and *PtA2* in poplar), AP3 (*ap3-1* mutant in *Arabidopsis* (Sablowski et al. 1998) and *PtAP3b* in poplar), ANR1 (*ANR1* in *Arabidopsis* and *PtANR1a* and *PtANR1c* in poplar), CD (*AGL1, 5, 11* in *Arabidopsis* and *PtCD4* in poplar), and FLC (*FLC* and *MAF1–5* in *Arabidopsis* and *PtFLC1, 2, 4* in poplar) classes in both *Arabidopsis* and poplar. All of them except the ANR1 class are genes involved in the identification of floral organs or flowering time regulation. It suggests that flowering plants might utilize the alternative splicing process to increase protein diversity instead of undergoing more gene duplications in order to regulate the transition from vegetative to flowering phase and the development of reproductive organs.

Expression of poplar type II MADS-box genes

The functions and expression patterns of *Arabidopsis* type II MARD-box genes are summarized in Table 1-1B. Overall genes in A, B (AP3, PI), Bs, C, D, and E classes express typically in floral organs, genes in AGL12 and ANR1 express preferentially in root tissues, genes in FLC and SVP classes express predominately in young leaves and vegetative meristem, and genes in AGL15, F, and G express in both floral and vegetative tissues. Beside this, the following expressions are also significant (Becker et al. 2003): *SEP4* in all major plant organs above ground, G class genes in all 4 whorls or the ovule, AGL12 in a few cell

types in flowers and shoots, and AGL24 in the floral meristem. A review paper (Cseke et al. 2004) has summarized past studies in expression patterns of A, B, C, D, E, F, and SVP classes of MADS-box genes in trees such as poplar, apple, birch, *Eucalyptus*, pine, spruce, etc. with some transformation on *Arabidopsis* and tobacco. Basically those A, B, C, D, and E classes of genes in trees are expressed preferentially in flower, fruits and/or cones (Cseke et al. 2004) similar to the suggestion in the ABCE floral quartet model. The expression of poplar type II MADS-box genes (Table 2-4 and Figure 2-4) is highly similar to that in *Arabidopsis* and other trees. For example, the major expression organs are floral organs for genes in A, B (AP3, PI), Bs, C, D, and E classes, root tissues for genes in AGL12 and ANR1, leaves and vegetative meristem for some genes in FLC and SVP, and both floral and vegetative tissues for genes in AGL15, F, and G. In addition, expression of PtAGL12b is detectable in spring floral buds, all three *PtG* genes strongly express in floral buds, and 4 *PtE* genes are detectable in vegetative tissues. The most significant difference between *Arabidopsis* and poplar are the expression of genes in SVP and FLC classes. For genes in SVP and FLC classes in *Arabidopsis*, only *AGL24*, a flower promoter in the SVP class, expresses in the floral meristem and organs, but almost all *PtFLC* and *PtSVP* genes are expressed in floral buds. These results suggest that gene functions have been conserved in type II MADS-box genes among species during evolution and *PtFLC* and *PtSVP* genes may have diverse regulatory roles to genes in the FLC and SVP classes in *Arabidopsis*.

The expression pattern of a number of *PtA, PtAP3, PtPI, PtC, PtD*, and *PtE* genes indicated these genes may involve in the development of both vegetative and floral buds. In 717-1B4 poplars, *PtPI2, PtE2, PtE3, PtE4*, and *PtSVP4* were expressed only in floral tissues which suggests that they may only regulate floral organ identification but are not involved in the development of vegetative tissues in 717-1B4. The expression of *PtPI2, PtE2, PtE4*, and *PtSVP4* are different between 717-1B4 poplars and Nisqually-1 and 545-4183 poplars in various tissues and experimentally induced dormancy. For example, *PtPI2* was found in SD induced terminal buds in Nisqually-1 and 545-4183 poplars, *PtE2* was found in LD shoot apices in Nisqually-1 and 545-4183 poplars, *PtE4* was found in some other vegetative

tissues in Nisqually-1 and 545-4183 poplars, and *PtSVP4* was found in root tissues in Nisqually-1 and 545-4183. The more similar expression pattern between Nisqually-1 and 545-4183 poplars than 717-1B4 might be due to the fact that *Populus trichocarpa* is one of the parents of the hybrid 545-4183 poplars. Most of the differences in expression pattern among genotypes exist in experimentally induced dormancy and release (Table 2-4). The number of potential MADS-box proteins can be increased by alternative splicing variants as demonstrated here in several members of multiple classes (Table 2-2). For example, *PtCD4as1* and *PtCD4as2* have opposite expression patterns in Nisqually-1 during dormancy.

The expression of *PtPI2, PtE2, PtE4,* and *PtSVP4* in shoot apices (and leaf) indicates that they may be involved in the regulation of shoot apical development in Nisqually-1 and 545-4183 poplars. Floral bud samples for Nisqually-1 and 545-4183 poplars were not available, so it could not be determined whether there was any gene which mainly expresses in floral organ of these 2 genotypes. Several *PtCD* and *PtE* genes were found to have little if any expression in shoot apices in LD or during SD induced dormancy even when PCR cycles were extended to more than 40 PCR cycles. This suggests that the vegetative shoot apex and apical bud have a gene expression profile similar to an *agamous* and/or leaf-like organ (Figure 1-2A).

Materials and Methods

Sequence collection from databases and phylogenetic analysis

Three species of angiosperms (*Magnoliophyta*) were selected to conduct this study. In the eudicotyledons, *Populus*, model organism for trees, and the herbaceous plant *Arabidopsis thaliana*, a model organism for plants, were selected. In the *Liliopsida* (monocotyledons), *Oryza sativa* (rice) was selected. One hundred forty *Arabidopsis* MADS-box proteins including some alternatively spliced variants were first aligned by neighbor-joining (NJ) in Clustal X (Figure 2-1A) to select one gene from each MADS-box class as a reference sequence. This reference gene was then used to search MADS-box genes in the JGI *Populus trichocarpa v1.0* genome database (http://genome.jgi-psf.org/cgi-

bin/searchGM?db=Poptr1). One hundred nineteen putative poplar MADS-box genes were obtained through searching gene model hits with Smith-Waterman criteria by locus IDs of 19 *Arabidopsis* type I and II MADS-box genes (*AP1* (AT1G69120), *AP3* (AT3G54340), *PI* (AT5G20240), *ABS* (AT5G23260), *AG* (AT4G18960), *SEP1* (AT5G15800), *SOC1/AGL20* (AT2G45660), *AGL6* (AT2G45650), *AGL24* (AT4G24540), *FLC* (AT5G10140), *AGL12* (AT1G71692), *AGL15* (AT5G13790), *ANR1* (AT2G14210), *U/AGL33* (AT2G26320), S-*AGL66* (AT1G77980), Mα-*AGL28* (AT1G01530), Mβ-*AGL47* (AT5G55690), Mγ-*AGL38* (AT1G65300), and Mδ-*AGL65* (AT1G18750)) from the JGI poplar database. After removing short sequences (i.e. predicted proteins less than 70 amino acids long) and filtering by gene model, scaffold location and phylogenetic alignment with *Arabidopsis* and rice type I and type II MADS-box genes, 2 MIKC* (i.e. S class) genes, 2 unknown genes, and 43 type I and 57 type II MADS-box genes were identified. These type I and II MADS-box genes were grouped into 4 and 15 functional classes respectively (Figure 2-1B). The MADS-box genes of *Arabidopsis* and rice were selected based on previous studies (Becker et al. 2003; Nam et al. 2003; Nam et al. 2004; Ng et al. 2001; Parenicova et al. 2003; Ratcliffe et al. 2003; Theissen et al. 2000) and information from TAIR (the *Arabidopsis* Information Resource, http://www.arabidopsis.org/index.jsp). Protein and DNA coding sequences of *Arabidopsis* and rice were obtained from TAIR and GenBank. Thirty-two representative *Arabidopsis* MADS-box genes (21 type II classes, 10 type I classes, and 1 S class) and 13 rice type II MADS-box genes were used to categorize functional classes of poplar MADS-box genes by ClustalX NJ tree (BLOSUM, PAM, and Gonnet protein weight matrix) with 1000 bootstrap replicates . The clubmoss (*Lycopodium annotinum*) gene *LAMB1* (GenBank AF232927), a distant related type I MADS-box gene, was used as the outgroup to root the tree.

Each poplar gene was named based on its functional class and scaffold location. Each type II poplar MADS-box gene was cloned and sequenced and then compared to the predicted gene model in the JGI database. Genes with sequencing discrepancies to the JGI database were included in further phylogenetic analysis to confirm that those modifications would not affect their functional class.

Both DNA and proteins sequences were used to reconstruct phylogenies of *Arabidopsis*, rice and *Populus* genes. It has been suggested that protein sequences appear to give more robust estimates than DNA sequences in the study of long-term evolution (Glazko et al. 2003). RevTrans 1.4 (http://www.cbs.dtu.dk/services/RevTrans/) (Wernersson et al. 2003) provides multiple alignment of coding DNA based on aligned amino acid sequences. This method gives better signal-to-noise ratio because DNA only contains 4 different bases while proteins are built from 20 amino acids; hence unrelated DNA sequences typically display ~25% identity over their entire length (Wernersson et al. 2003). MAFFT 6 (L-INS-i) (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh et al. 2005b; Katoh et al. 2002) is faster of the two most accurate multiple protein sequence alignment programs among the nine programs (Clustal W, T-Coffee, MAFFT (FFT-NS-2, L-INS-i), Muscle, POA, ProbCons, Dialign-T, Dialign2.2, and Kalign) tested (Katoh et al. 2005a; Katoh et al. 2005b; Nuin et al. 2006). RevTrans was used to translate all coding sequences to protein sequences. Three options, L-INS-i, G-INS-i and E-INS-i, are available in MAFFT, and their characteristics are described below based on the manual in the website of MAFFT 6. MAFFT (E-INS-i) is applicable to sequences containing several conserved motifs embedded in long unalignable regions and aligns locally with Smith-Waterman algorithm with generalized affine gap costs (Altschul). MAFFT (L-INS-i) assumes that input sequences only have one alignable domain and aligns locally with Smith-Waterman algorithm with affine gap costs (Gotoh). MAFFT (G-INS-i) is the most suitable option because it assumes that entire regions can be aligned and aligns globally with a Needleman-Wunsch algorithm. Unfortunately MAFFT (G-INS-i) is unavailable on web, so both MAFFT (E-INS-i) and MAFFT (L-INS-i) were selected. MAFFT (L-INS-i and E-INS-i) aligned protein sequences were used to reconstruct phylogeny of protein sequences using PAUP* v4.0 beta and PhyML v2.4.4 (Guindon et al. 2003; Guindon et al. 2005). RevTrans aligned coding sequences based on MAFFT aligned protein sequences. These RevTrans aligned coding sequences were used to reconstruct phylogeny of DNA sequences with PAUP* and PhyML.

BioEdit 7.0.5.3 was used to edit the aligned sequences and remove less or un-informative gaps. The character numbers were shortened from 1811 to 534 in DNA sequences and 637 to 281 in protein sequences for MAFFT (E-INS-i) aligned sequences. The character numbers were shortened from 2139 to 543 in DNA sequences and 713 to 310 in protein sequences for MAFFT (L-INS-i) aligned sequences. PAUP* and PhyML were used to reconstruct phylogeny for both DNA and protein sequences. The maximum parsimony (MP) tree and minimum evolution distance (ME) trees with bootstrap analysis by PAUP* and the maximum likelihood (ML) tree with bootstrap analysis by PhyML were reconstructed. 534/543 nucleotide sites (MAFFTE/MAFFTL) and 281/310 amino acid sites (MAFFTE/ MAFFTL) of 124 sequences were selected for the analysis by PAUP*. After removing 11 *Arabidopsis* type I MADS-box genes, 534/543 nucleotide sites (MAFFTE/MAFFTL) and 179/181 amino acid sites (MAFFTE/MAFFTL) of 113 sequences were selected for the analysis by PhyML.

For DNA sequences, the ModelTest 3.7 (Posada et al. 2004; Posada et al. 1998) was used to test the best fit DNA substitution model. Although GTR+I+G is the best model in ModelTest results for both MAFFT (E-INS-i) and MAFFT (L-INS-i) aligned coding sequences, GTR+G was used in likelihood analysis by PhyML to save about 70% time and HKY+G was used to generate a starting tree to be used in parsimony analysis by PAUP* to save on computational time. The difference of likelihood scores (-lnL) and AIC (Akaike information criterion) between GTR+I+G and GTR+G models and between GTR+I+G and HKY+G models in the ModelTest analysis were less than 0.03% and 0.16%. Using PAUP*, a NJ tree with HKY model and gamma distribution (HKY+G) with shape parameter = 1.142176 (MAFFT-E) and 1.089986 (MAFFT-L) (estimate from ModelTest) were first constructed as a starting tree for parsimony method. In parsimony analysis by PAUP*, the equal weight, equal weight without the 3rd codon, weight1 (weight the first codons with value 2), weight2 (weight T-ratio (transversion/transition) = 2), and weight3 (weight the first codons with value 2 and T-ratio = 2) parsimony trees were compared to each other. The tree-bisection-reconnection (TBR) and random addition was used in a heuristic

search for parsimony. A 50% majority-rule consensus tree was retained in each analysis. A likelihood score (-lnL) was calculated on the 50% consensus tree by the GTR+G model with empirical base frequencies, estimated gamma shape parameter and substitution rates. Bootstrap bipartition with 100 replicates of heuristic search (simple sequence addition and nearest-neighbor interchange (NNI) swapping) were used to evaluate support values of tree topology. Using PhyML, an optimized BioNJ distance-based tree was used as a starting tree for likelihood analysis. The GTR model, empirical base frequencies, estimated gamma shape parameter, estimated substitution rates, and 4 substitution rate categories were applied to calculate best tree and likelihood score. Bootstrap bipartition with 100 replicates were used to evaluate support values of tree topology.

For protein sequences, the ProtTest1.3 (Abascal et al. 2005) was used to test the best fit of protein evolution model, i.e. amino acid replacement. JTT+I+G is the best model based on AIC (Akaike Information Criterion), AIC-3, BIC-1 (Bayesian Information Criterion), BIC-2, BIC-3, and –lnL (Maximum Likelihood score) evaluation frameworks. Using PAUP*, a NJ tree was first constructed to serve as a starting tree. The nearest-neighbor interchange (NNI) and tree bisection-reconnection (TBR) swapping were used in a heuristic search in the parsimony and distance methods, respectively. A 50% majority-rule consensus tree was retained in each analysis. The distance score and tree length were calculated. Bootstrap bipartition with 500 and 100 replicates of heuristic search (simple sequence addition and NNI swapping) were used to evaluate support values of tree topology in distance and parsimony analyses. The computer program TreeView v1.6.6 was used to draw the tree. Using PhyML, an optimized BioNJ distance-based tree was used as a starting tree for likelihood analysis. The JTT model, estimated gamma shape parameter, fixed proportion of invariable sites (0.015 for MAFFT-E and 0.018 for MAFFT-L aligned sequences), and 4 substitution rate categories were applied to calculate the best tree and likelihood score. Bootstrap bipartition with 100 and 500 replicates were used to evaluate support values of tree topology.

Plant materials

To examine the tissue expression patterns of poplar type II MADS-genes, shoot apices, leaf, bark, xylem, and root of 4–5 LD (>16 hours light) grown greenhouse plants of *Populus trichocarpa* Nisqually-1, *Populus tremula* x *alba* clone 717-1B4, and *Populus deltoides* x *trichocarpa* clone 545-4183 were collected, immediately frozen in liquid nitrogen and stored at -80°C until used for RNA purification. Plants of *Populus trichocarpa* Nisqually-1 and *Populus deltoides* x *trichocarpa* clone 545-4183 were established from greenwood stem cuttings that were rooted using Hormodin2 (E.C. Geiger Inc., Harleysville, PA) in Oasis Rootcubes. After 1 to 2 months, rooted cuttings were transferred to 15 cm diameter pots containing a commercial potting mix (Sunshine LC1) and place in a LD greenhouse. For the *Populus tremula* x *alba* clone 717-1B4, *in vitro* propagated rooted shoots were transferred to 15 cm diameter pots containing a commercial potting mix (Sunshine LC1), covered with a clear plastic cup for about 10 days to allow the rooted plants to acclimate in a LD controlled environmental chamber, and transferred to a LD greenhouse after 1 to 2 months. All plants were fertilized with slow released fertilizer. To examine type II MADS-box genes expression in reproductive buds, swollen floral buds of *Populus tremula* x *alba* clone 717-1B4 plants grown outside in natural environments were harvested on March 14, 2007.

For bud dormancy experiments, stem cuttings from greenhouse grown stock plants of *Populus trichocarpa* Nisqually-1 and *Populus deltoides* x *trichocarpa* clone 545-4183 were rooted using Hormodin2 in Oasis Rootcubes. After approximately 1 to 2 months, rooted cuttings were transferred to 15 cm diameter pots containing a commercial potting mix (Sunshine LC1). Plants were then placed in controlled environment chambers (Conviron) under long-days (LD) (16 hours light, 8 hours dark) at 18°C and 50–75% relative humidity for approximately 1 month prior to use for experiments. For the *Populus tremula* x *alba* clone 717-1B4, *in vitro* propagated rooted shoots were transferred to 15 cm diameter pots containing a commercial potting mix (Sunshine LC1), covered with a clear plastic cup for about 10 days to allow the rooted plants to acclimate in a LD controlled environmental

chamber. Vegetative bud development, dormancy and chilling induced dormancy release were performed in controlled environment chambers with SD photoperiods (8 hours light and 16 hours dark at 18°C and 50–75% RH) for 8 weeks followed by additional 10 weeks of SD photoperiods with the day temperature reduced to 10°C and the night temperature reduced to 4°C. Shoot apices or terminal buds from 6–8 plants were harvested right before the treatment started and after 3, 6, 8, 12, 14, and 18 weeks of treatment. After the 18 week experimental treatment, the rest of plants were moved to a LD growth chamber and regrowing shoot apices were harvested 7 weeks later. All harvested samples were immediately frozen in liquid nitrogen and brought back to laboratory and stored at -80°C until used for RNA purification. The labels of SD# and SDC# represent the treatment in each sampling point in the experiment. SD# means # weeks of SD treatment. SDC# means SD# with chilling hours. Only night time at 4°C was counted as chilling temperature. All the chilling treatment started at 8 weeks of SD, so chilling hours is equal to ((# - 8) weeks) x (7 days/week) x (16 hours/ day).

RNA extraction and reverse transcription

Total RNA was purified using the Qiagen RNeasy Plant Mini Kit (QIAGEN 2001) with a slight modification. Terminal buds from 6–8 plants per genotype in each harvest were ground to fine powder in liquid nitrogen using a baked RNase-free mortal and pestle. The frozen fine powder was added to 1–2 mL isothocyanate (GITC)-containing lysis buffer RLT containing 0.01 volume β-mercaptoethanol (β-ME). Samples were vortexed 1 min followed by a 15 min incubation on ice in RLT buffer in which 0.4 volume of 5M potassium acetate (pH6.5) was added. Samples were centrifuged for 10 min at 12,000 rpm and the supernatant was transferred to a fresh tube. A half volume of 100% ice cold ethanol was added to the supernatant which was pipet mixed, then transferred to the RNeasy spin column, with the remaining binding, washing and elution steps performed following the manufacturer's protocols. Total RNA was eluted twice with 50–100 µL RNase free water. Contaminating DNA was removed from the samples by incubating RNA samples with 100–200 units of RQ1 RNase free DNase for 30 min at 37°C. After incubation, RNA samples were extracted with

equal volume of chloroform twice and RNA was precipitated overnight at -20°C using 0.1 volume of 3M sodium acetate (pH 5.2) and 2 volume of 100% ethanol. RNA was collected by centrifuging 30 min at 14000 rpm at 4°C. The RNA pellets were washed twice with ice cold 70% ethanol, dried and resuspended in sterile water. RNA concentrations were determined by measuring absorption at 260 nm with Bio-Tek miniplate UV spectrum (BioTek Instruments, Inc., Winooski, VT, USA). A 0.4 µg/µL diluted RNA sample was prepared from each one for further analysis. RNA samples were stored at -80°C for further analysis.

cDNA synthesis was performed with approximately 1 µg of RNA (2.4 µL of 0.4µg/µL RNA) using the Promega ImProm-II™ reverse transcription system (Promega 2005) following the manufacturer's procedures. These 1st strand cDNAs were stored at -20°C until used for further analysis.

Gene-specific PCR primer design, cloning and sequence analysis

Due to high sequence similarity for genes within each functional class, BioEdit 7.0.5.3 was used to display aligned coding sequences from the same functional class and to highlight different nucleotides or regions. PrimerSelect version 5.51 (Lasergene, DNASTAR, Inc.) was used to design gene specific primer combinations (Table 1-1B). At least one 3' end of forward or reverse primers was located inside the mismatch region or right on that nucleotide in order to get a specific primer combination for each gene, regardless of possible low primer evaluation score. To test the primers, a pooled sample of 1st strand cDNA of shoot apices of experimental treatments and all tissues from *Populus trichocarpa* Nisqually-1 were used to test PCR conditions including annealing temperature (54–64°C) and cycle number of each primer combination to use in PCR analysis. Once a PCR product was observed on an agarose DNA gel, the product was cloned using the Promega pGEM-T Easy vector system and JM109 high efficiency competent cells. Four to thirty-six white colonies were picked from each LB/amipicillin/IPTG/X-gal plate and grown in 5 mL LB supplemented with 100 µg/µL ampicillin. Plasmid DNA was purified using the Promega Wizard Plus SV Minipreps DNA purification system following the manufacturer's procedures. Plasmid DNA was digested with *EcoRI* and the insert size was verified by DNA gel electrophoresis. Based on

this analysis, 2–10 plasmids for each transcript were selected for DNA sequencing. DNA sequences were aligned with respective gene models for the predicted transcripts from JGI to confirm intron-exon structure and the specificity of primer combination. The intron-exon structure was modified when the cDNA sequence showed discrepancies from the predicted transcript in JGI. The modified transcripts were labeled with M in phylogenetic analysis. For example, PtSVP2_LGV_M represents a transcript from PtSVP2_LGV gene with modified intron-exon structure. Alternatively spliced variants were named as as1, as2, etc. For example, PtSVP3as1_LGVII and PtSVP3as2_LGVII represented 2 alternatively spliced variants from the *PtSVP3* gene.

PCR analysis

Each PCR reaction contained 0.8 µL of 1st strand cDNA, 1 µL of 10x Ex Taq™ buffer (Mg^{2+} free), 0.8 µL of 2.5 mM dNTP mixture, 0.56 µL of 25 mM $MgCl_2$, 0.05 µL of TaKaRa Ex Taq™ (5 units/µL), 5.99 µL sterilized distilled H_2O, and 0.4 µL of 5 µM of each primer. PCR reactions with a polyubiquitin (*PtUBQL*) primer combination were used as an internal control. The 10 µL PCR product was mixed with 2.5 µL of 5x Blue Juice DNA loading buffer and run through a 1.2% DNA agarose gel using 1x TAE running buffer. PCR conditions were as follows: 2 min at 94°C, then the specified cycle number of 94°C for 30 sec, the specified annealing temperature for 30 sec, 72°C for 1 min, and then 72°C for 3 min and held at 4°C.

Chilling requirement and dormancy measurement

The chilling temperatures required to overcome bud dormancy can be experimentally quantified by measuring the time required for at least 50% of plants resume growth in permissive conditions after exposure to a given amount of chilling temperature. Plants were treated with SD photoperiods for 8 weeks at 18°C followed by an additional 10 weeks of SD at 10°C (day) and 4°C (night). After 3, 6, 8, 12, 14, 18 weeks of the treatment, 4–5 plants were defoliated and moved from experimental SD and temperature conditions to either a LD controlled environment chamber at 18°C or a LD greenhouse that ranged from 18–36°C. At regular intervals (3 times per week) plants were observed for apical or axillary bud break.

Chapter 3: Characterization of a poplar *FLC-like 2* gene (*PtFLC2*) regulating bud development, dormancy release, and leaf senescence in *Populus alba* x *tremula*

Abstract

Bud dormancy is an adaptive trait allowing survival during unfavorable environmental conditions. It is regulated by environmental cues including photoperiod and cold. A growing body of evidence suggests that analogous or equivalent regulatory circuits may exist between vegetative bud development and flowering. In *Arabidopsis*, the *FLC* gene encodes a type II MADS-box transcription factor that acts as a repressor of flowering. Because of the potential similarities between flowering and vegetative bud dormancy, *FLC*-like homologs to *Arabidopsis FLC* were identified in the poplar genome and the potential role of one of the poplar *FLC*- like genes, *PtFLC2*, was investigated.

Six poplar *FLC-like* genes, *PtFLC1–PtFLC6*, have been identified in the poplar genome. Transcripts for all of the *PtFLC* genes were detected in various poplar tissues (shoot apex, leaf, bark, xylem, and root) except for *PtFLC1* in spring floral buds and *PtFLC4* in the bark and xylem of LD grown plants. *PtFLC2* was expressed in shoot apices of LD grown poplars and during the early stages of short day (SD) initiated bud development. During continued SD exposure in combination with LT, transcript levels of *PtFLC2* decline. The decline of *PtFLC2* expression appears to be associated with cold-mediated dormancy release which is reminiscent of the down-regulation of *FLC* during vernalization in *Arabidopsis*. In contrast to *PtFLC2*, *PtFLC4* transcript abundance increases during dormancy similar to what is observed for *MAF5* during vernalization in *Arabidopsis*. A number of alternatively spliced *PtFLC2* transcripts (*PtFLC2as1–9*) were also detected and the expressions of some of the splice variants were associated with the later stages of bud dormancy. Constitutive overexpression of *PtFLC2* splicing variant 1 (*PtFLC2as1*) in poplar altered the response to photoperiod and cold, resulting in delayed growth cessation and bud formation, reduced

depth of bud dormancy, reduced amount of chilling required to overcome dormancy, and delayed leaf senescence and abscission. The altered dormancy responses could be overcome by prolonged exposure to SD and/or low temperatures. Ectopic expression of *PtFLC2* in late-flowering *Arabidopsis* FRI-Sf2 accelerated flowering while no effect on flowering time was observed in the early-flowering ecotype LER.

Introduction

The growth cycle of temperate trees is characterized by active shoot growth during spring and summer, followed by a quiescent or dormant vegetative phase (Figure 1-1). The length of growth period between spring bud break and fall bud set impacts tree productivity and wood quality. Dormancy is a survival strategy that enables plants to survive unfavorable environmental conditions and also synchronizes shoot growth and influences plant architecture. Phytohormones including gibberellic acid (GA), abscisic acid (ABA), and ethylene and environmental cues such as day length, light quality, temperature, and nutrients regulate the cycle of bud dormancy. Dormancy is defined as "the temporary suspension of visible growth of any plant structure containing a meristem" (Lang 1987). Dormancy has been classified into three types: endodormancy, paradormancy, and ecodormancy (Lang 1987). Endodormancy involves growth control mediated by an endogenous signal within the affected dormant structure. Typically endodormancy is overcome by extended periods of exposure to chilling temperature. Vegetative bud dormancy is an example of endodormancy. Bud development and formation is not a rapid process and can take several weeks from the point of active summer growth to quiescent winter dormancy. This has been suggested as a strategy that allows the plant to prepare for dormancy before the threat of frost (Perry 1971).

The apical bud is one of the major organs involved in vegetative dormancy in trees. The apical bud is produced by the shoot apical meristem and consists of an unextended shoot and leaves and stipules encased by two or more pairs of protective bud scales (Rohde et al. 2002). The activity of the bud and/or shoot apex determines the timing and extent of annual growth and dormancy. Primordia initiated after the critical daylength change their

morphogenetic fate to form bud scales and stipules (Rohde et al. 2002). Bud formation and dormancy in many tree species is induced by shortening day lengths in the fall (Rohde et al. 2000). Bud formation is prerequisite to bud dormancy and coincides with cessation of active vegetative growth and leaf abscission (Rohde et al. 2002). Shoot elongation and growth cessation in woody plants is modulated by multiple light signals, including irradiance, photoperiod, spectral composition, and the ratio of red:far-red (R:FR) wavelengths, in many deciduous trees (Olsen et al. 1997; Olsen et al. 2002), but is primarily a response to daylength.

Temperature and precipitation are the two predominant factors which shape vegetation and plant distribution on the earth. Since plants can not move to avoid harsh environments, they have evolved responses to suboptimal temperatures to optimize their probability of survival and reproduction. Two mechanisms that perennial plants have evolved to survive winters are dormancy and freezing tolerance. To maximize the growing period and avoid possible frost damage, temperate zone perennial plants have evolved a mechanism to measure the length of winter by somehow measuring the exposure to low temperatures. The amount of low temperature exposure required to overcome dormancy by quantified as a chilling hour or chilling unit (hours at certain chilling temperature) (Egea et al. 2003). One approach to quantify the chilling requirement of a plant is to experimentally measure when at least 50% of the plants resume growth in permissive conditions after exposure to a given amount of chilling temperature (0–7 ^{o}C) (Rohde et al. 2000). The range and optimum chilling temperature is species specific (Cesaraccio et al. 2004; Rohde et al. 2000). It has been proposed that exposure to cold temperatures is required for the removal of 1,3-β-D-glucan from plasmodesmata to restore cell-cell communication and symplasmic organization than somehow mediates the transition of the shoot apical meristem from dormancy to an active state (Rinne et al. 2001).

Poplar bud development and dormancy can be defined by five developmental stages - bud initiation and morphogenesis, maturation, dormancy induction, endodormancy, and chilling accumulation (Table 2-3). Bud initiation occurs during the first 3 weeks of SD and

is characterized by initiation and development of bud scales and suppressed embryonic leaf development. Bud maturation follows bud initiation and development and requires continued exposure to SD beyond the initial 3 week period. During bud maturation the organs of the bud grow, storage reserves accumulate, and the bud begins to desiccate. Bud initiation and maturation precede dormancy and the shoot will resume growth in a permissive environment. Bud dormancy is induced with SD exposure beyond 6 weeks and maximum endodormancy results from further exposure to SD and low temperatures. Endodormant buds require exposure to a defined period of chilling temperatures in order to resume growth.

Both vegetative and floral buds arise from the shoot apical meristem. Both vegetative bud development and dormancy and floral bud development are regulated by photoperiod and hormones (Horvath et al. 2003). *Arabidopsis* flowering is regulated by four major pathways: GA, photoperiod, autonomous, and vernalization (Figure 1-3) (Boss et al. 2004; Dennis et al. 2007; He et al. 2005; Henderson et al. 2004; Sablowski 2007). The autonomous pathway promotes flowering through the epigenetic downregulation of *FLC* (Simpson 2004). Vernalization promotes flowering by repressing *FRI* (*FRIGIDA*)-mediated *FLC* expression after exposure to a prolonged period of cold. *FLC* is a point of integration of vernalization and autonomous pathways and is upregulated by *FRI* and *PAF1* complexes. Flowering in juvenile poplar can be induced by overexpression *PtFT1* (Bohlenius et al. 2006) and *PtFT2* (Hsu et al. 2006) and is associated with induction of *PtAP1* expression. This is similar to early flowering in *Arabidopsis* resulting from overexpression of *FT*. This suggests that both poplar and *Arabidopsis* use homologous *CO/FT* regulons to regulate photoperiod processes. This similarity and the observation that floral and vegetative buds share equivalent structures raises the possibility that analogous regulatory factors to flower initiation and development may govern vegetative bud development and dormancy.

MADS-box genes encode a family of transcription factors involved in the regulation of diverse developmental processes in flowering plants including reproductive (flower, seed, fruit) and vegetative (root, leaf) development, differentiation and organ identity. For example, MADS-box flowering time genes such as *FLC, AGL24*, *SVP* and *SOC1* repress or

promote the floral transition mediated by internal (plant age) or environmental factors (day-length, cold) (Hartmann et al. 2000; Hepworth et al. 2002; Michaels et al. 2003a; Sheldon et al. 2002; Sheldon et al. 2000). Furthermore, the floral-quartet-model describes how different combination of class A, B, C, D and E proteins, which are encoded by MADS-box genes, form various tetramers that are involved in the regulation of floral organ identification (sepals, petals, stamens, carpels, ovules) (Figure 1-2A) (Honma et al. 2001; Theissen et al. 2001). In plants, the type II MADS-box proteins are MIKC-type proteins which contain 4 major domains, a highly conserved MADS-box domain of 55–60 amino acids that includes a DNA-binding domain and dimerization motif, a less-well-conserved intervening (I) domain of about 30 amino acids which influences DNA binding specificity and dimerization, a well-conserved keratin-like coiled-coil (K) domain (~70 amino acids) which contains a protein-protein interaction region, and a variable length C-terminal (C) which is involved in transcriptional activation, posttranslational modification, or protein-protein interaction (Alvarez-Buylla et al. 2000b; Hileman et al. 2006; Kaufmann et al. 2005; Nam et al. 2003; Vandenbussche et al. 2003). Several key factors involved in vernalization, flower induction, and floral organ identification are members of type II MADS-box genes; thus it is possible that type II MADS-box genes may also play a role in bud dormancy.

FLC, a type II MADS-box gene, is a floral repressor which quantitatively represses flowering by repressing the flowering time integrators *SOC1*, *FT* and *FD*. A 600–800 kDa multimeric protein complex with more than one FLC peptide binds directly to a region at CArG boxes within the first intron of *FT* and the promoter of *SOC1* and *FD* to repress the expression of *FT*, *SOC1* and *FD* (Helliwell et al. 2006; Searle et al. 2006). The flowering time integrators *SOC1* and *FT* activate the floral meristem identity genes *AP1, AP2, FUL, CAL*, and *LFY* resulting in the activation of the other floral organ identity genes *AP3, PI*, and *AG* (Sablowski 2007). Analyses of *Arabidopsis* FRI-Sf2 (Col) genotype indicates that *FLC* expression is predominately in shoot and root apices and vasculature, and it is also detected in leaves and stems (Michaels et al. 1999; Michaels et al. 2000; Sheldon et al. 1999). Overexpression of *FLC* is sufficient to delay flowering even without an active *FRI*

allele (Michaels et al. 1999). Early flowering *Arabidopsis* accessions have either a naturally occurring non-functional *fri* allele or a weak or non-functional *FLC* allele, whereas the winter-annual (i.e. late flowering) accessions have dominant alleles of *FRI* and *FLC* (Caicedo et al. 2004; Ratcliffe et al. 2001; Shindo et al. 2005; Werner et al. 2005).

There are five homologs of *FLC* in *Arabidopsis; MAF1–4 (MADS AFFECTING FLOWERING1–4*) are flower repressors, while *MAF5* is upregulated during vernalization (Ratcliffe et al. 2003; Ratcliffe et al. 2001). Overexpression of any of the *MAF* homologs delays flowering (Ratcliffe et al. 2003; Ratcliffe et al. 2001; Scortecci et al. 2001). Altered *MAF1* expression through the use of overexpression or *maf1* mutant alleles modifies flowering time comparable to *FLC* overexpression or mutants and does not affect *FLC* expression levels (Ratcliffe et al. 2001; Scortecci et al. 2001). Overexpression of *MAF2* produces a range of flowering times from early to late with the late phenotype consistent between T1 and T2 generations. The 35S::*MAF2* late flowering phenotype becomes insensitive to vernalization similar to overexpression of *FLC* or *MAF1* and does not affect *FLC* expression levels, but rather represses *SOC1* expression (Ratcliffe et al. 2003). This suggests that *MAF1* and *MAF2* act downstream of *FLC* or in different pathways. *FLC* and *MAF1–MAF5* expression is also characterized by the production of alternatively spliced transcripts (Caicedo et al. 2004; Lempe et al. 2005; Ratcliffe et al. 2003; Ratcliffe et al. 2001; Scortecci et al. 2001; Werner et al. 2005). One *FLC* splice variant with MADS-dormain and C-terminal was detected only after 15 days of vernalization (Caicedo et al. 2004). No functional consequence has yet to be ascribed to splice variation among *Arabidopsis FLC*-like genes.

Little is known about the regulatory factors that govern bud dormancy in poplar including what genes are involved in the regulation of bud initiation, development, dormancy, and the release from dormancy. It is possible that analogous regulatory pathways may control flowering and bud dormancy. To test this idea, 6 *PtFLC* genes were identified in the poplar genome. One, *PtFLC2,* was cloned from *Populus deltoides* x *trichocarpa* 545-4183.

This study examines the role of *PtFLC2* in bud development and cold-mediated dormancy release.

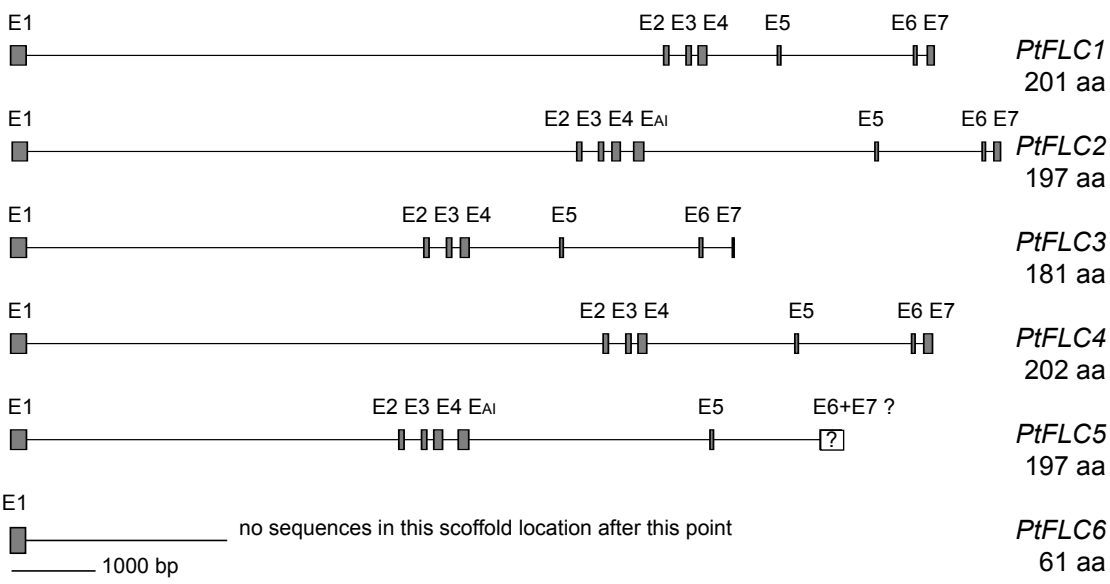
Results

Identification and structure of *PtFLC* genes in the poplar genome

Six putative *PtFLC* gene sequences were identified in the JGI poplar database and were designated *PtFLC1-PtFLC6*. The 6 putative *PtFLC* genes were located on 4 different linkage groups and scaffolds. The linkage group or scaffold location of each gene is presented in Table 1-1B. Based on the predicted sequences or upon the cDNA sequences generated from cloned cDNAs using gene specific primers, the gene structure and intron and exon locations for *PtFLC1-PtFLC5* are shown in Figure 3-1A. All 5 of these *PtFLC* genes show a similar structure and conservation of exons and introns. Typical of many type II MADS-box genes the first exon on all five genes is followed by a large intron that ranges from 7399 bp in *PtFLC1* to 4263 bp in *PtFLC5*. *PtFLC6* is located on scaffold 4342. Scaffold 4342 only has 4142 nucleotides in the JGI poplar database and 2598 of these nucleotides belong to *PtFLC6* gene. Therefore due to a lack of sequences, the *PtFLC6* gene is likely truncated in the database. Based on the predicted transcript sequences and the experimental cDNA sequences, *PtFLC1-PtFLC5* are predicted to encode for proteins comparable in size to the *Arabidopsis* FLC (Figure3-1A). ClustalX alignment of predicted poplar FLC proteins with *Arabidopsis* reveals a high degree of similarity or identity between the poplar FLC proteins with each other and with *Arabidopsis* FLC (Figure 3-1B). The greatest identity was observed with the MADS domain while the least identity was found in the C-terminal domain (Figures 3-1B and 3-1C).

Comparison of the transcript sequences between *PtFLC1* and *PtFLC3* revealed only 8 bp differences while *PtFLC2* only varied by 13 bp from *PtFLC5* without considering the 8 bp difference within E_{AI}. Both *PtFLC1* and *PtFLC2* are located at linkage group III (LG_III), and *PtFLC3* and *PtFLC5* are located at scaffold 690. DNA sequence alignment by ClustalX and BioEdit between 32000 nts of LG_III (15163206-15195206) containing

A



Size (bp) / E/I / Gene	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	Intron 3	Exon 4	Intron 4	Exon AI[a]	Intron 4A[a]	Exon 5	Intron 5	Exon 6	Intron 6	Exon 7
AtFLC	185	3493	58	178	62	90	100	78			42	194	42	992	102
PtFLC1	185	7399	79	185	65	84	109	799			42	1544	42	111	84
PtFLC2	185	6571	79	183	62	87	100	163	125	2747	42	1246	42	102	84
PtFLC3	185	4560	79	188	65	84	109	1039			42	1549	42	347	24
PtFLC4	185	6628	79	170	62	76	100	1706			42	1300	42	107	99
PtFLC5	185	4263	79	184	62	83	100	163	125	2759	42	1226[b]	42[b]	102[b]	84[b]
PtFLC6	185	There are 4142 nucleotides in scaffold 4342. 2598 nts of scaffold 4342 belong to exon 1 (185 nts) and intron 1 (2413 nts) of *PtFLC6*. The comparison between *PtFLC2* and *PtFLC6* gene sequences: (1) The exon 1 and 5'UTR (76 nts) of *PtFLC2* are identical to the ones in *PtFLC6*. (2) There is 77 nts difference out of 2413 nts in intron 1 except 2 gaps (227 and 264 nts).														

Location on scafold 690	Location in *PtFLC3* and *PtFLC5*	Difference in the alignment of LG_III (*PtFLC1*+*PtFLC2*) and scaffold 690 (*PtFLC3*+*PtFLC5*)
1-1026	before 5'UTR of *PtFLC3*	continuous difference
5156-5865	intron 1 of *PtFLC3*	710 bp in *PtFLC3* matchs small pieces within 3488 bp of *PtFLC1*
7253-7500	intron 4 of *PtFLC3*	248 bp gap in *PtFLC1*
10529-11746	between *PtFLC3* and *PtFLC5*	1218 bp gap in LG_III
11947-12526	between PtFLC3 and PtFLC5	580 bp gap in LG_III
15098-15244	intron 1 of *PtFLC5*	147 bp gap in *PtFLC2*
17262-17263	intron 1 of *PtFLC5*	2443 bp gap in *PtFLC5*
23375-23811	after intron 5 of *PtFLC5*	unknown sequence in *PtFLC5,* nucleotide sequences are identical between LG_III and scaffold 690 after *PtFLC2* and *PtFLC5*

Figure 3-1. Poplar *FLC-like* (*PtFLC*) genes.

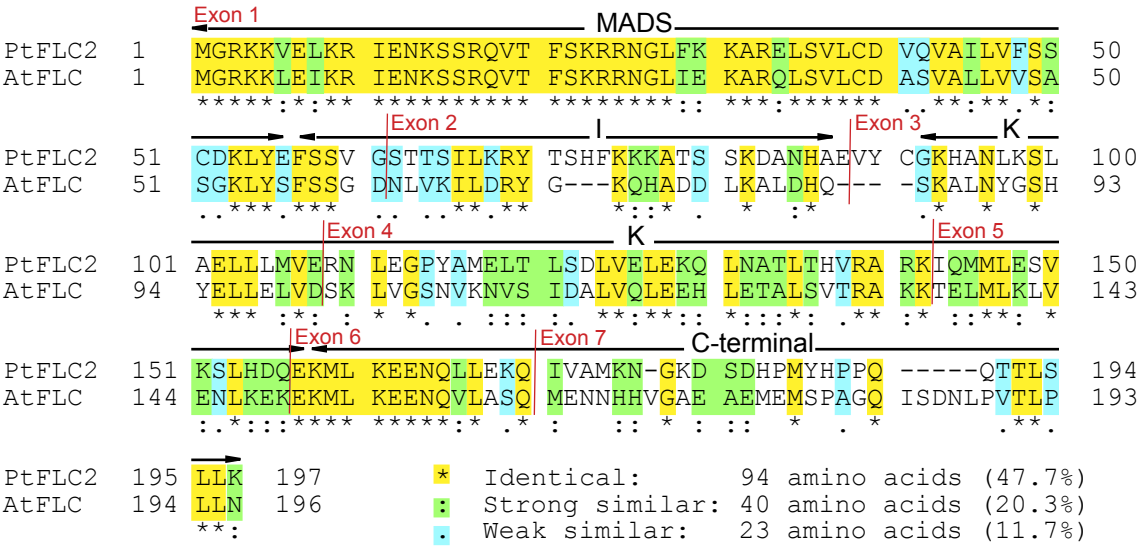
(A) Structure of 6 poplar *FLC-like* (*PtFLC*) genes. Top figures are the exon-intron structures and the positions and sizes of exons and introns. Middle table are the size in base pairs of exons and introns for *Arabidopsis FLC* and 6 *PtFLC* genes. Bottom table is the difference between alignment of LG_III (15163206-15195206) (*PtFLC1*+*PtFLC2*) and entire scaffold 690 (*PtFLC3*+*PtFLC5*). Exons are represented as gray-filled boxes, introns are shown as solid lines, and unsure exons in PtFLC5 are open box with question mark inside.

(B) Protein and (C) CDS alignment between poplar *FLC1-6* and *Arabidopsis FLC*.

Note: a: exon AI might be an alternative internal exon within intron 4 in *PtFLC2* and *PtFLC5* and contains in an early stop codon in *PtFLC2* and *PtFLC5*. Intron 4 consists of intron 4, exon AI, and intron 4A in the predicted functional full-length cDNA of *PtFLC2* and *PtFLC5*.

b: The sequence from end of intron 5 is missing on JGI poplar database. Then there is 1226 bp left in intron 5, but none after that. The current size in exon 6 and 7 and intron 7 is based on PtFLC2 gene structure due to similarity.

B (a) Alignment of PtFLC2 and *Arabidopsis* FLC proteins



(b) Alignment of 6 poplar FLC-like proteins and *Arabidopsis* FLC protein

Figure 3-1. Poplar *FLC-like* (*PtFLC*) genes (continued)

(C) CDS alignment of 6 *PtFLC* genes and *Arabidopsis FLC*

Figure 3-1. Poplar *FLC-like* (*PtFLC*) genes (continued)

PtFLC1 and *PtFLC2* and entire scaffold 690 (26372 nts) containing *PtFLC3* and *PtFLC5* was performed and all differences including indels and gaps were counted and tabulated (Figure 3-1A). Prior to removing any gaps, 67.157% of the nts aligned over a distance of 33,042 bp were identical. After removing large gaps greater than 1 kb from the alignment, the identity between the two sequences increased to 91.10% over 23,244 bp. If three smaller gaps (580, 248 and 147 bp) were removed, the identity increased to 95.092%.

Using gene specific primers, the expression of *PtFLC1-PtFLC5* was assayed in six different tissues (shoot apex, leaf, bark, xylem, root, and floral bud) pooled from 4 LD grown plants and in shoot apices pooled from 8 plants during SD induced vegetative bud dormancy (Figure 3-2). The PCR products were cloned and sequenced and the sequence data was used to verify predicted transcripts from the JGI database as well as to identify differences in the JGI annotation compared to the sequence data. As shown in Figure 3-2, RT-PCR analysis suggested that splicing variants were produced from *PtFLC1, PtFLC2*, and *PtFLC4*. Besides the predicted transcript, one additional splicing variant was found for *PtFLC1* and *PtFLC4* (Figure 2-2 and Table 2-2). For *PtFLC1* the splice variant involves skipping of exon 7 while the splice variant for *PtFLC4* consists of retention of intron 6. At least 13 splice variants were detected and verified for *PtFLC2* (Figure 2-2 and 4-1) and are described in more detail in the following chapter. Some splicing variants of both *PtFLC2* and *PtFLC5* also appear to have an alternative internal exon that would result in an early stop codon resulting in a truncated protein lacking a complete K-domain and C-terminal domain.

PtFLC2 is down-regulated and *PtFLC4* is up-regulated during vegetative bud dormancy

To examine the expression of *PtFLC* genes, the transcript abundance of *PtFLC1-PtFLC4* was analyzed in six various tissues and in the shoot apex or apical bud during vegetative bud development and dormancy for three poplar genotypes (*Populus trichocarpa* Nisqually-1, *Populus deltoides* x *trichocarpa* clone 545-4183, and *Populus alba* x *tremula* clone 717-1B4) using RT-PCR. *PtFLC2* and *PtFLC3* transcripts were detected in floral bud and shoot apices, leaves, bark, xylem and roots of LD grown plants for all three genotypes

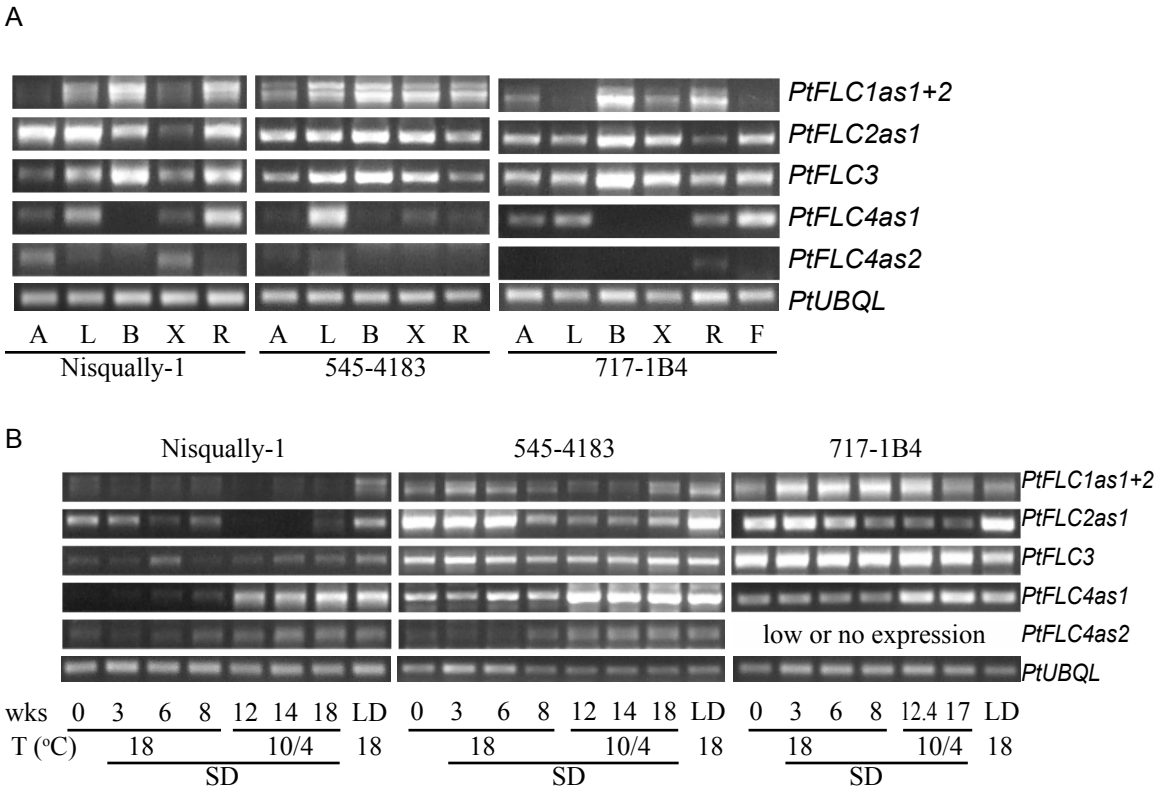


Figure 3-2. Expression of *PtFLC1–PtFLC4* in vegetative and reproductive tissues as well as shoot apices during bud dormancy.
(A) Expression of *PtFLC1, PtFLC2, PtFLC3*, and *PtFLC4* in different tissues of LD grown Nisqually-1, 545-4183, and 717-1B4 poplars. 717-1B4 floral buds were harvested from plants grown outdoors in March 2007.
(B) Expression of *PtFLC1, PtFLC2, PtFLC3*, and *PtFLC4* in shoot apex of Nisqually-1, 545-4183, and 717-1B4 poplars during bud development and dormancy.
(C) Expression of *PtFLC2* in 545-4183 poplar by RT-PCR with *PtFLC2* and PtFLC2asoe primers. PtFLC2asoe primers were used to amplify the full-length *PtFLC2* cDNA used to make 35S::*PtFLC2* constructs for *Agrobacterium* transformation. Unmarked bands are alternatively spliced variants.
(D) Expression of *PtFLC2* in 717-1B4 and Nisqually-1 using RT-PCR and primers specific to *PtFLC2*.
(E) Expression of *PtFLC2* in different tissues of Nisqually-1, 717-1B4, and 545-4183 poplars using RT-PCR with the *PtFLC2* primers.
For all figures, RNA from various tissues was used for RT-PCR using primers specific to each gene. A = shoot apex, L = leaf, B = bark, X = xylem, R = root, F = floral bud. PtUBQL is used as internal control.

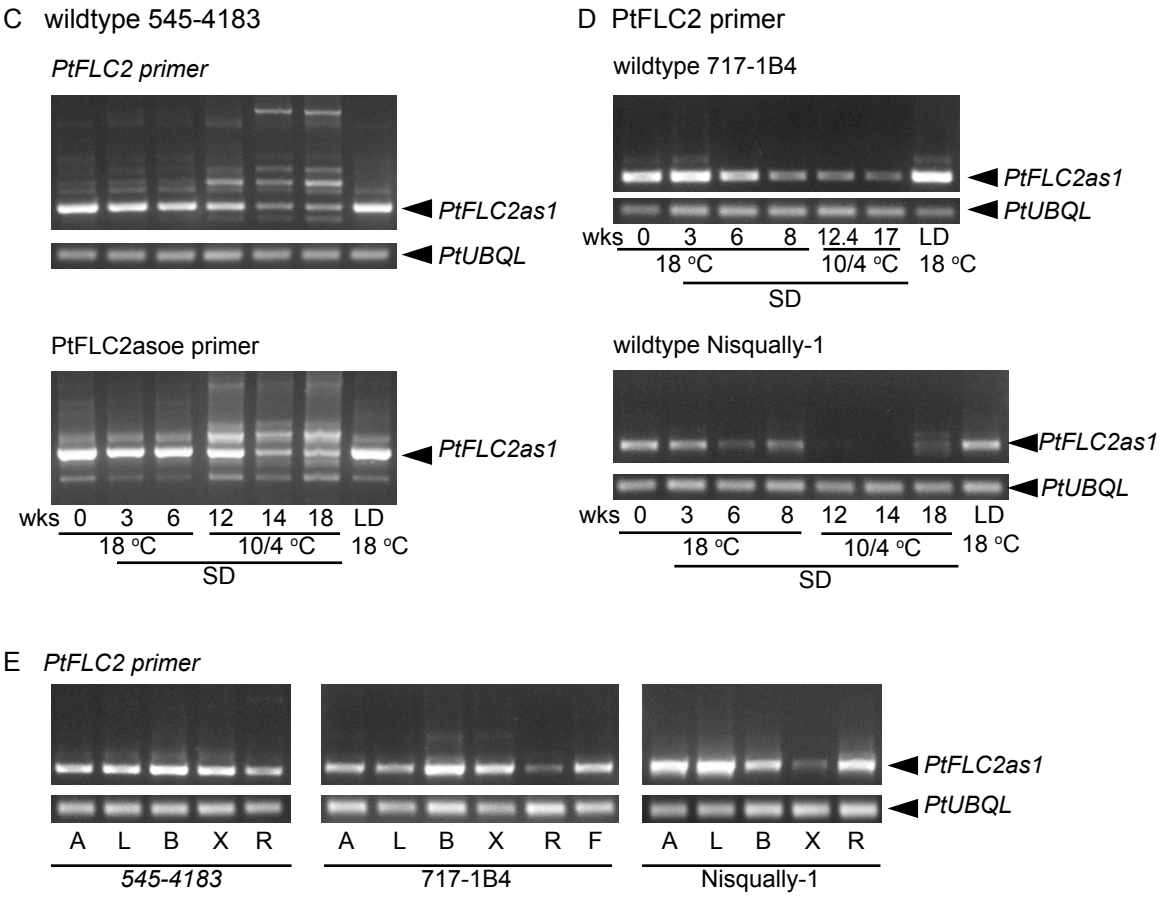


Figure 3-2. Expression of *PtFLC1–PtFLC4* in vegetative and reproductive tissues as well as shoot apices during bud dormancy (continue)

Figure 3-2A). *PtFLC1* transcript was also detected in all tissues except for flower buds. *PtFLC4* was also detected in all tissues except bark.

The expression of *PtFLC1-PtFLC4* was also examined during vegetative bud development and dormancy (Figure 3-2BB). Both *PtFLC1* and *PtFLC3* transcript abundance changed little with SD or SD plus LT treatment. In contrast, *PtFLC2* transcript levels declined in the apical buds of all 3 genotypes after 3–6 weeks of SD treatment and continued SD treatment at reduced temperatures (Figure 3-2B and 3-2C). *PtFLC4* is expressed opposite of *PtFLC2* and transcript abundance increases after 8 weeks of SD treatment followed by additional SD treatment in combination with low temperatures (Figure 3-2B). The expression of *PtFLC4* in some ways mimics that of *MAF5* in *Arabidopsis* during vernalization (Ratcliffe et al. 2003). Surprisingly, *PtFLC2* alternatively spliced transcript variants that were detected during the SD in combination with LT treatment were only detected in the *Populus deltoides* x *trichocarpa* clone 545-4183 genotype and not or low in the remaining two genotypes (Figure 3-2C). In addition, no splice variants were detected in shoot apices, leave, bark, xylem, roots of LD grown plants for all 3 genotypes of in floral buds of *Populus alba* x *tremula* clone 717-1B4.

Characterization of transgenic poplars transformed with *PtFLC2* overexpression and RNAi chimeric genes

In order to study the potential role of *PtFLC2* in vegetative bud development and dormancy, chimeric genes were contstructed that either overexpress *PtFLC2as1* from the 35S promoter (35S::*PtFLC2as1*) or produce double stranded RNA for RNAi (PtFLCR) knockdown studies (vector construction is detailed in the materials and methods section). These chimeric genes were then used in *Agrobacterium* mediated transformation of poplar genoytpe *Populus alba* x *tremula* clone 717-1B4. Transformed cells were selected based on resistance to glufosinate ammonium and resistant callus and regenerated shoot were produced. A total of 4 independently transformed lines for the 35S::*PtFLC2as1* and 5 independently transformed lines for the PtFLCR construct were regenerated. The transcript abundance for *PtFLC2* was then assayed in transgenic and control plants treated with

LD, SD and SD combined with LT using primers specific for *PtFLC2*. Figure 3-3 shows representative results from 4 independent transgenic lines for each construct. Increased transcript abundance was detected for all four transgenic lines transformed with 35S:: *PtFLC2as1*. All of the tested PtFLCR lines showed little if any reduced transcript level. In addition, the PtFLCR lines consistently contained at least two PCR products of greater size than expected. The nature of these larger amplified products is not clear.

Growth of wildtype and *PtFLC2* transgenic poplars

To determine if overexpression of *PtFLC2as1* or *PtFLC2* RNAi influenced the growth of plants, 3 sets of experiments were performed in which growth of transgenic and wildtype control poplars was monitored when treated with either LD or SD. Two of the experiments were carried out using similar controlled environment chambers (Conviron) while a separate experiment used a different controlled environment chamber (EGC) and greenhouse. It is important to note these differences because both the light intensity and spectral quality (Figure 3-4), as well as some temperature parameters differed between the two types of controlled environment chambers. Sylleptic branching was observed in all of the lines 35S::*PtFLC2as1* transgenic plants grown in LD in the Conviron chambers. In contrast, little if any sylleptic branching was observed in either PtFLCR transgenic or wildtype control plants under the same conditions (Figure 3-5). Unexpectedly this phenotype was not observed in plants grown either in LD greenhouse or the EGC chambers. The reason for this difference is unclear; however as shown in Figure 3-5, both light intensity and spectral quality differ in the greenhouse, EGC, and Conviron chambers. This light difference may be the basis for the different branching phenotypes observed.

Growth cessation during SD treatment was compared between transgenic and wildtype control poplars by determining the number of leaves, plant height, leaf increment, and plant height increment at weekly intervals. The data presented in Figure 3-6 represent the means of 40 plants which were10 replicate plants per transgenic or control genotype averaged over 4 independent transgenic lines. During the first 2-3 weeks of SD treatment transgenic poplars grew similar to wildtype controls plants. Beyond 3 weeks of SD

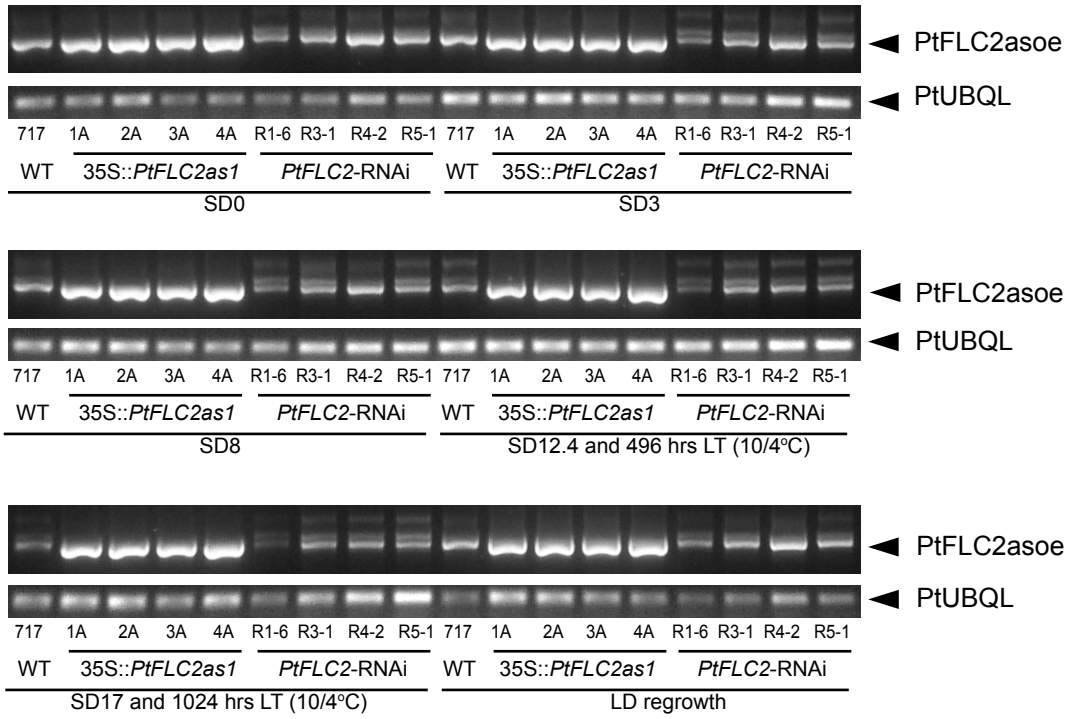


Figure 3-3. *PtFLC2* expression during bud development and dormancy in wild-type and poplars transformed with *PtFLC2* chimeric genes.
AS1-1A, AS1-2A, AS1-3A, and AS1-4A are independent 35S::*PtFLC2as1* transgenic poplar lines. PtFLCR, R1-6, R3-1, R4-2, and R5-1are independent PtFLC2-RNAi transgenic poplar lines. SD0, 3, 8, 12.4, and 17 are poplar RNAs harvested at 0, 3, 8, 12.4, and 17 weeks of SD. Plants with SD12.4 and SD17 are SD combined with 496 and 1024 hours LT (10/4 ºC day/ night). The PtFLC2asoe primers were used for RT-PCR from total RNA isolated from shoot apices or apical buds at the indicated time intervals. *PtUBQL* primer combination was used as internal control.

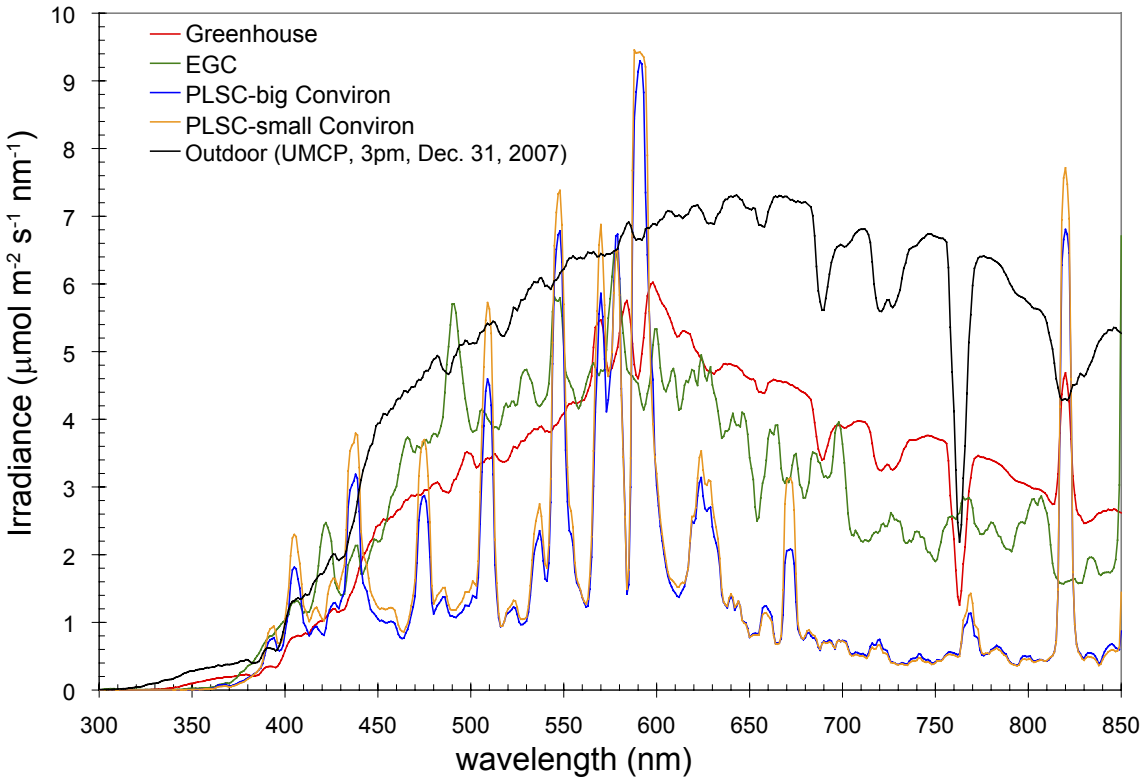


Figure 3-4. The comparison of the irradiances in all experiments.
The irradiance (photon flux density) (µmol m^{-2} s^{-1} nm^{-1}) of outdoor, PLSC Conviron chambers, EGC, and greenhouse were measured with Ocean Optic QE65000. The irradiances of outdoor and greenhouse were measured around 3 pm Dec. 31, 2007 at the Greenhouse complex of University of Maryland, College Park.

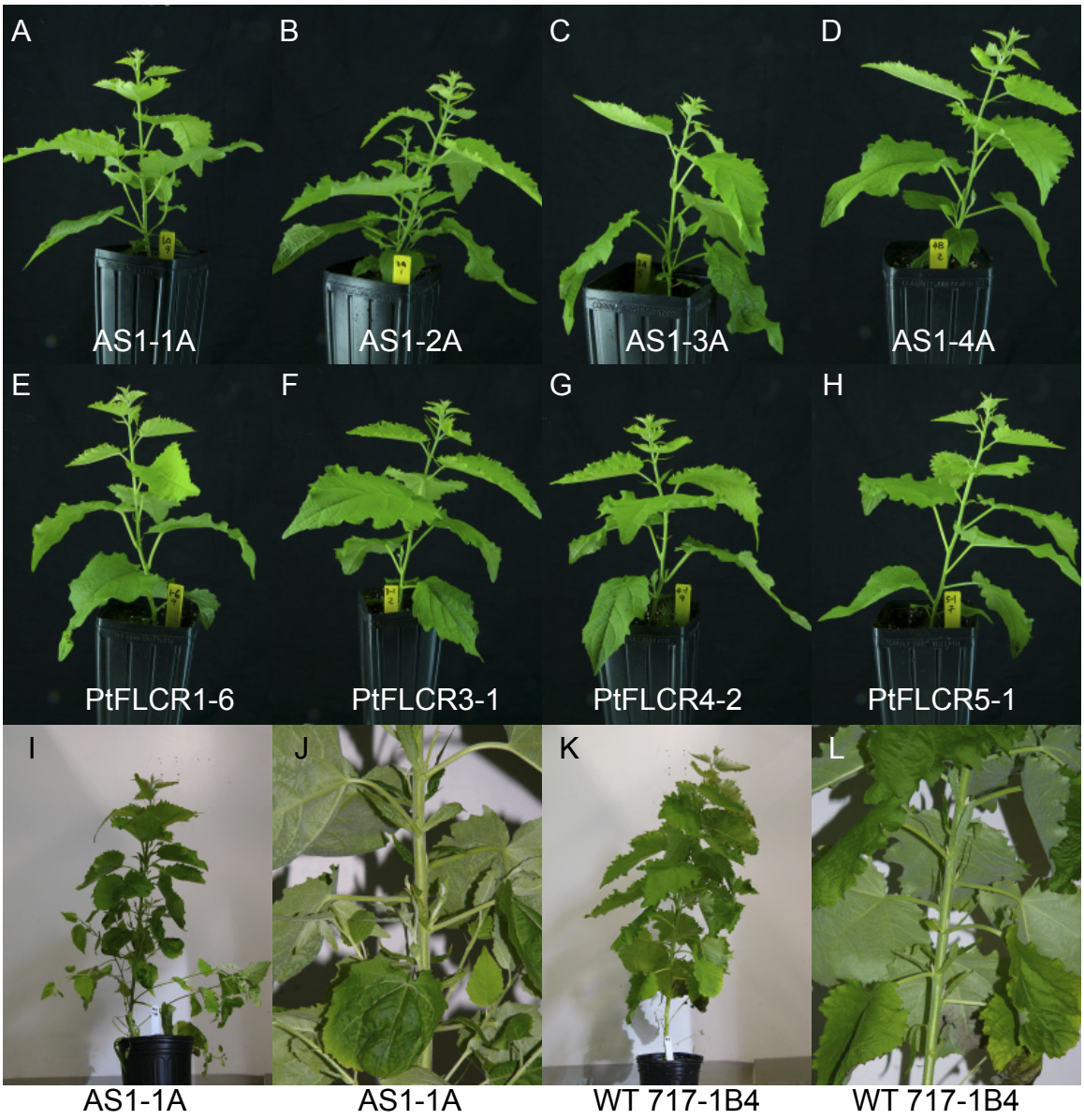


Figure 3-5. Sylleptic branching of transgenic *Populus alba x tremula* clone 717-1B4.
(A-H) Poplars were grown under 1 month of LD in Conviron chamber.
(I-J) Poplars were grown under 2 months of LD in Conviron chamber. Photos J and L are closeups of photo I and K.
For all figures panels labeled 717-1B4 are wildtype plants, AS1-X are 35S::*PtFLC2as1*, and PtFLCRX-X are *PtFLC2*-RNAi lines.

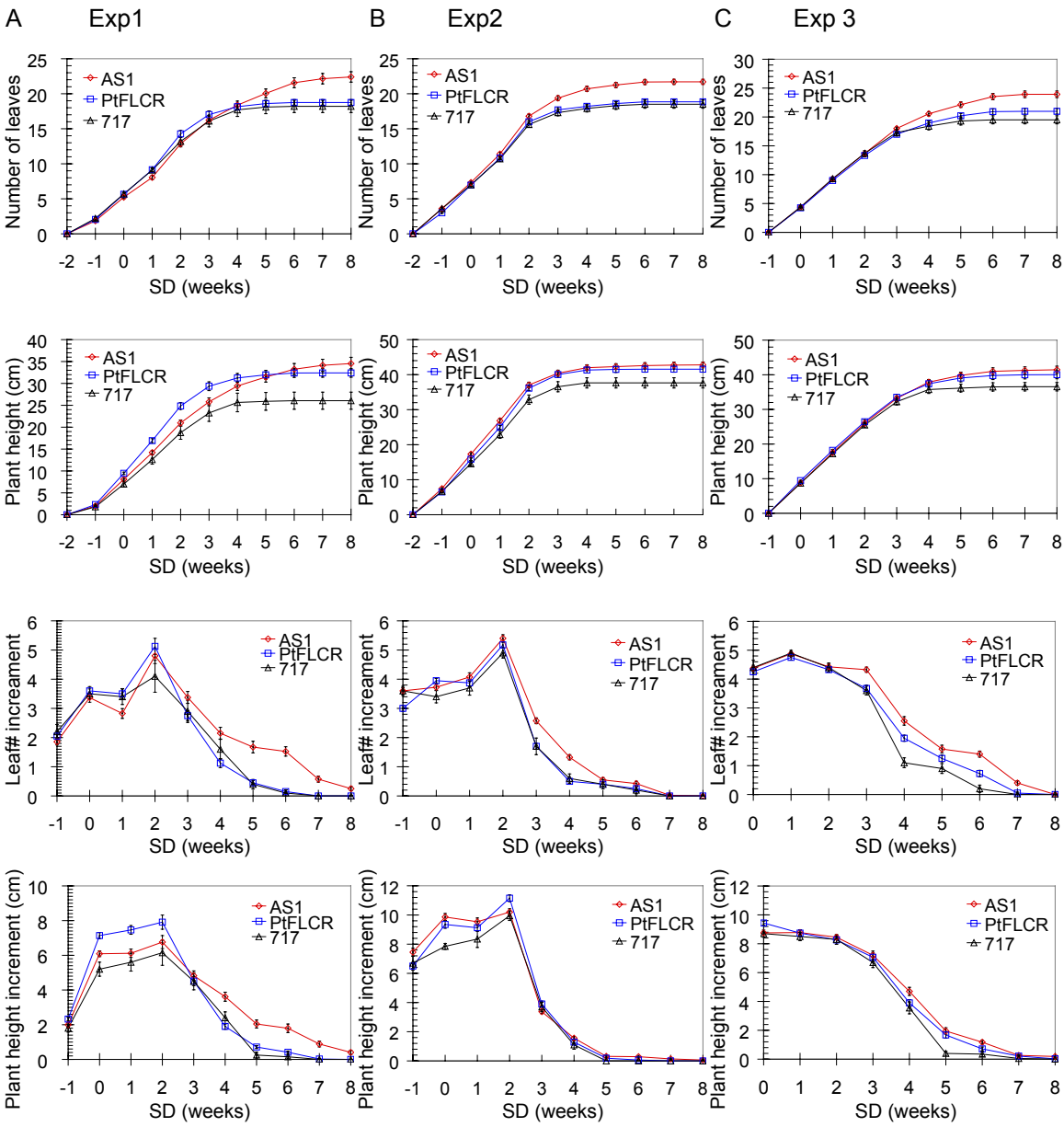


Figure 3-6. Growth cessation of wildtype and transgenic poplars in SD.
(A) Column A is experiment 1 carried in Conviron chamber in 2006–2007.
(B) Column B is experiment 2 carried in EGC chamber in 2007.
(C) Column C is experiment 3 carried in Conviron chamber in 2007–2008.
Overexpressing *PtFLC2as1* poplars (AS1) includes 10 plants from each AS1-1A, AS1-2A, AS1-3A, and AS1-4A transgenic line. *PtFLC2*-RNAi (PtFLCR) poplars includes 10 plants from each PtFLCR1-6, PtFLCR3-1, PtFLCR4-2, and PtFLCR5-1 transgenic line. Values given in A–C are means+/-StdError of 40 plants with same construct from 4 different lines. The minus (-) sign in x-axis means the number of week before switching from LD to SD. Leaf# and plant height were equal to (measured value - 1st measurement) of that particular line. The 1st measurements were measured at 2, 2, and 1 weeks before switching to SD in experiment 1, 2, and 3 respectively.

treatment a significant delay in growth cessation was observed for 35S::*PtFLC2as1* plants. By comparing the number of weeks required for leaf increment and height increment to approach zero, it was observed that 35S::*PtFLC2as1* plants required 3 additional weeks to cease growth. The data for growth cessation for individual lines is consistent within the same construct (data not shown). Two-way ANOVA with main effects of weeks of SD, constructs (35S::*PtFLC2as1*, 35S::*PtFLC2R*, and wildtype) (or individual lines), and their interaction showed that leaf number increment and plant height are significantly different ($P<0.001$) in all 3 experiments with the exception that construct effects were not significant in experiment 2 and the interaction with plant height in experiment 3 was not significant (Appendix A-2). Significant differences due to constructs in leaf increment and plant height were observed after 4 weeks of SD treatment except for experiment 2, where the significance was only observed at 3 or 4 weeks of SD treatment.

PtFLC2as1 overexpression impinges on bud formation, leaf senescence, leaf abscission, and chilling requirements

The vegetative apical bud initiation was delayed during the first 3 weeks of SD in 35S::*PtFLC2as1* poplars compared to PtFLCR and control poplars (Figures 3-7 and 3-8). A small newly initiated apical bud was readily observed in wildtype 717-1B4 and PtFLCR poplars but was not detected in 35S::*PtFLC2as1* poplars. Histological analysis confirmed the initiation of bud scales and stipules in wildtype 717-1B4 and PtFLCR poplars but not in 35S::*PtFLC2as1* poplars (Figure 3-8). After 6 and 8 weeks of SD treatment vegetative apical buds were present in all transgenic lines and wildtype poplars; however it was obvious that bud development was delayed in 35S::*PtFLC2as1* poplars as evidenced by reduced bud size (Figures 3-8). SD treatment beyond 8 weeks resulted in little, if any, change in the morphology and size of buds for wildtype and PtFLCR plants. Continued SD treatment beyond 8 weeks did result in continued development of bud from 35S::*PtFLC2as1* poplars and after 12.4 weeks of SD with or without LT, the apical buds of these transgenic plants were comparable to wildtype plants.

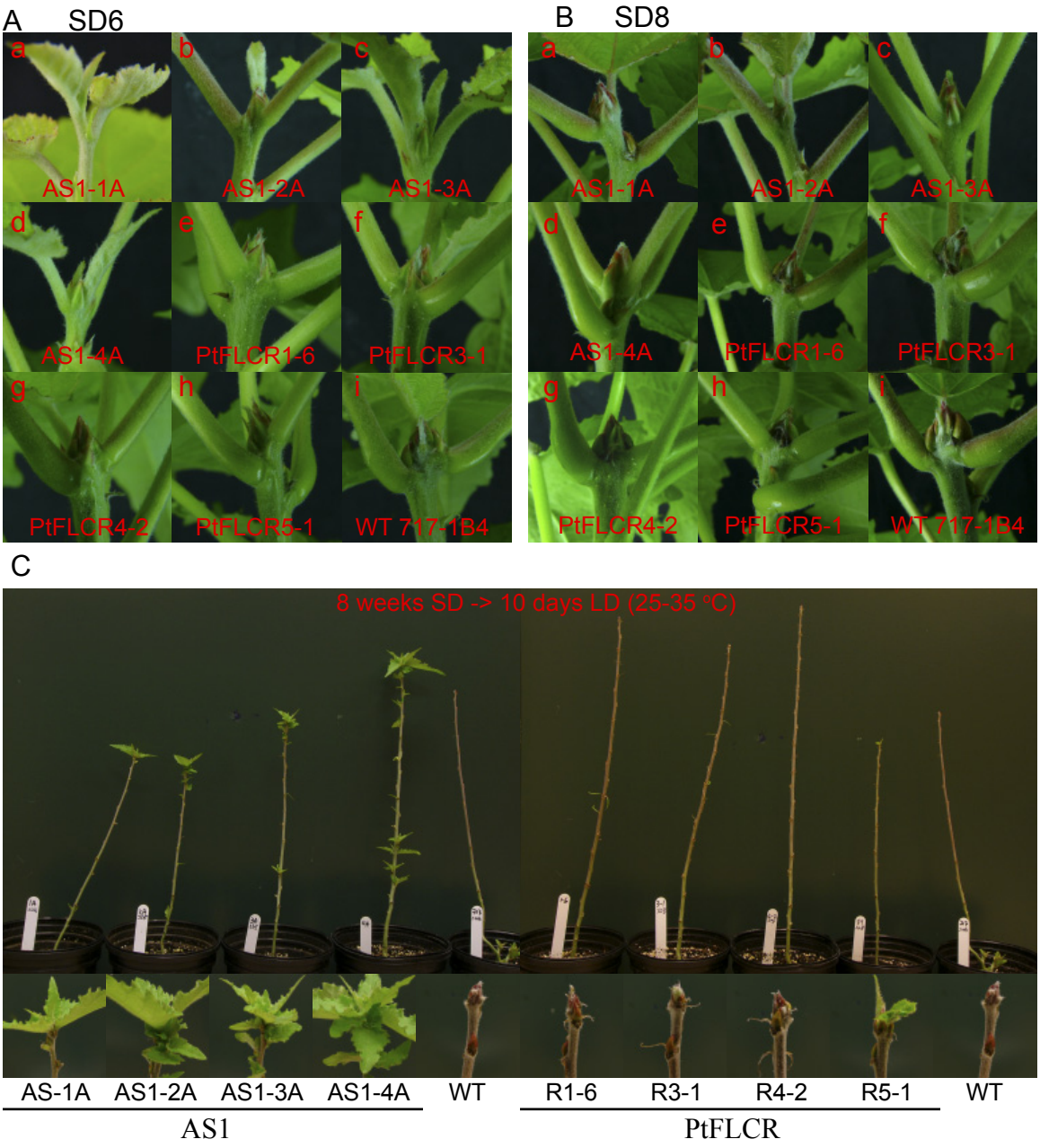


Figure 3-7 Delayed SD bud formation and dormancy in transgenic poplars overexpressing *PtFLC2as1*.

(A and B) Apical buds of AS1 (panels a, b, c, d) and PtFLCR (panels e, f, g, h) transgenic poplars and wildtype 717-1B4 poplar (panel i) treated for 6 (A) or 8 (B) weeks with SD. (C) Bud break and shoot growth 10 days after switching plants to LD after 8 weeks of SD treatment. For all figures AS1-1A, AS1-2A, AS1-3A, and AS1-4A are independent 35S:: *PtFLC2as1* transgenic poplars, R1-6, R3-1, R4-2, R5-1 are independent lines of *PtFLC2*-RNAi transgenic poplars and WT is wildtype control plants.

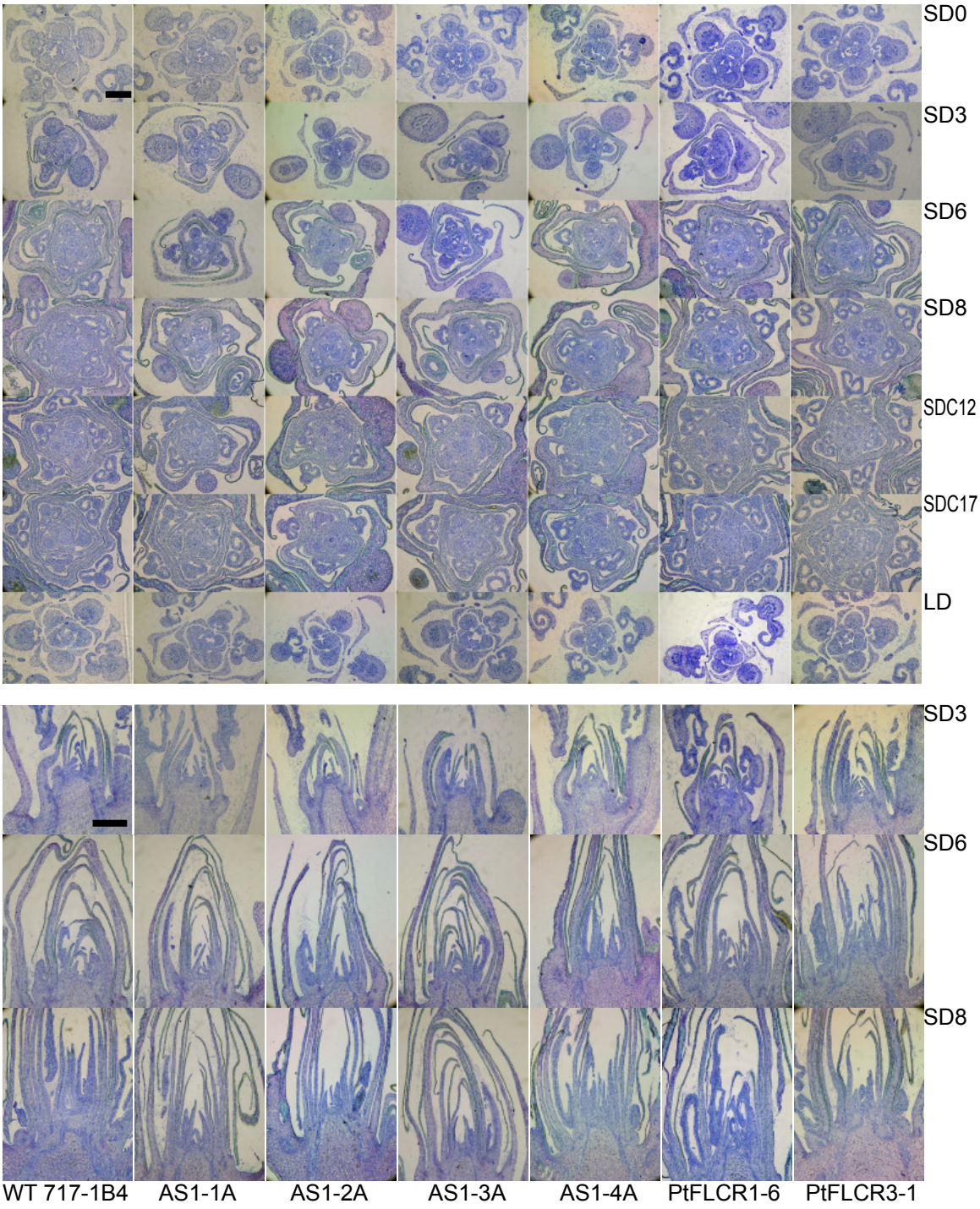


Figure 3-8. Morphology of apical buds in *PtFLC2* transgenic poplar.
Cross and longitudinal sections of apical buds of wildtype, AS1 (35S::*PtFLC2as1*) and PtFLCR (*PtFLC2*-RNAi) transgenic poplars during SD bud development and dormancy. All bars = 0.5 mm.

ANOVA revealed that the gene construct (i.e genotype) and chilling hours had a significant effect ($P<0.001$) on the number of days to bud burst and that these two variables have a significant interaction (Appendix A-2). Apical bud break in 35S::*PtFLC2as1* lines treated with 8 wks of SD occurred after less than 10 days when transferred to LD permissive conditions. In contrast wildtype control poplars required a longer time period in LD permissive conditions and the average time period ranged from 11.8 (exp 1) to 26.2 (exp 3) days depending upon the experimental replication. The difference in the time required for apical bud break between 35S::*PtFLC2as1* transgenic and wildtype control poplars increased substantially when plants were first treated with 8 weeks of SD followed by 2.3 weeks of SD combined with LT (10/4 ^{o}C day/night) (i.e. 256 chilling hrs). After this combination of SD and LT, 35S::*PtFLC2as1* transgenic poplar lines required an average of 7.8 (exp1, AS1-1A) to 26 (exp 3, AS1-2A) days for bud break in LD conditions for experiments 1 and 3 while wildtype control plants required an average of 139.3 (exp 1) to >152 (exp 3) days of LD exposure in order for bud break to occur in the same experiments (1 and 3). The difference between 35S::*PtFLC2as1* transgenic lines and wildtype poplars was also observed in experiment 2 but not to the extent observed in experiments 1 and 3. The difference observed between experiments 1 and 2 is most likely related to the elevated temperatures that occurred in the greenhouse during LD conditions. Endodormancy was induced in 35S::*PtFLC2as1* with further SD treatment combined with LT (10/4 ^{o}C day/night) when this treatment was extended for an additional 4.4 weeks (i.e. 496 chilling hrs) beyond the initial 8 week SD treatment. After this extended SD and LT treatment bud break averaged 52.0 to 135.0 days of LD for *PtFLC2as1* overexpressing poplars in experiment 1 and 3 and is similar to the time required for bud break in wildtype poplars (averaged 106.5–121.8 days). Continued SD and LT treatment resulted in a decline in the number of days required for bud break to occur for both 35S::*PtFLC2as1* and wildtype poplars. To determine the influence of extended SD and SD with LT on endodormancy induction, transgenic poplars and wildtype plants were treated for 10.3 or 12.4 weeks of SD with or without LT during the SD treatment beyond 8 weeks of the initial SD treatment (i.e 256 or 496 chilling hours). Extended SD treatment (10.3 or

12.4) induced endodormancy in wildtype poplars and these plants required more than 126 or 150 days for bud burst to occur in LD conditions and opposed to 35S::*PtFLC2as1* transgenic poplars which averaged 6.2–8.6 or 9.0–47.0 days in LD for bud burst to occur (Figure 3-9B). However, when this extended SD period also included a LT treatment, the35S::*PtFLC2as1* transgenic plants required average of 51.6–134.6 days in LD before bud burst occurred. These results suggest that overexpression of *PtFLC2as1* delays the onset of endodormancy by SD which can be compensated by LT, but the chilling requirement for overcoming endodormancy is not affected. Three-way ANOVA shows that the effect of constructs, chilling hours, and the interaction of constructs and chilling temperature are significant (P<0.001). The response of PtFLCR poplars did not differ from wildtype control plants (Appendix A-2).

In addition to the effect on bud endodormancy, leaf senescence and abscission was also delayed or reduced in 35S::*PtFLC2as1* transgenic plants (Figure 3-10). Leaves remained green under SD (at least up to 12.4 weeks of SD) and warm environment and started senescence and abscission between 256–752 chilling hrs treatment. Leaves of wildtype and PtFLCR poplars abscised completely before 752 chilling hours while at least 1/3–2/3 of leaves still remained intact on 35S::*PtFLC2as1* poplars (Figure 3-10B). Leaf chlorophyll a, b, c and carotenoids contents were measured in the various transgenic lines as well as wildtype poplars. Both chlorophyll a and total chlorophyll contents were greater in 35S::*PtFLC2as1* transgenic plants that were treated with SD combined with 752 hrs LT compared to wildtype control or PtFLCR plants irrespective of leaf position (Figure 3-10A). Carotenoids content did not differ among the various genotypes (Figure 3-10A). The delayed senescence phenotype was consistently observed in three different experiments. A more detailed analysis of this response shows that delayed loss of chlorophylls a, b was consistently observed between different 35S::*PtFLC2as1* transgenic lines when treated with SD combined with LT treatment, and occurred irrespective of leaf position (data not shown). The two-way ANOVA (construct (or genotype) x LPI) indicates that leaf chlorophyll a and b content was significantly (P<0.001) affected by genotypes (i.e. transgenic and wildtype)

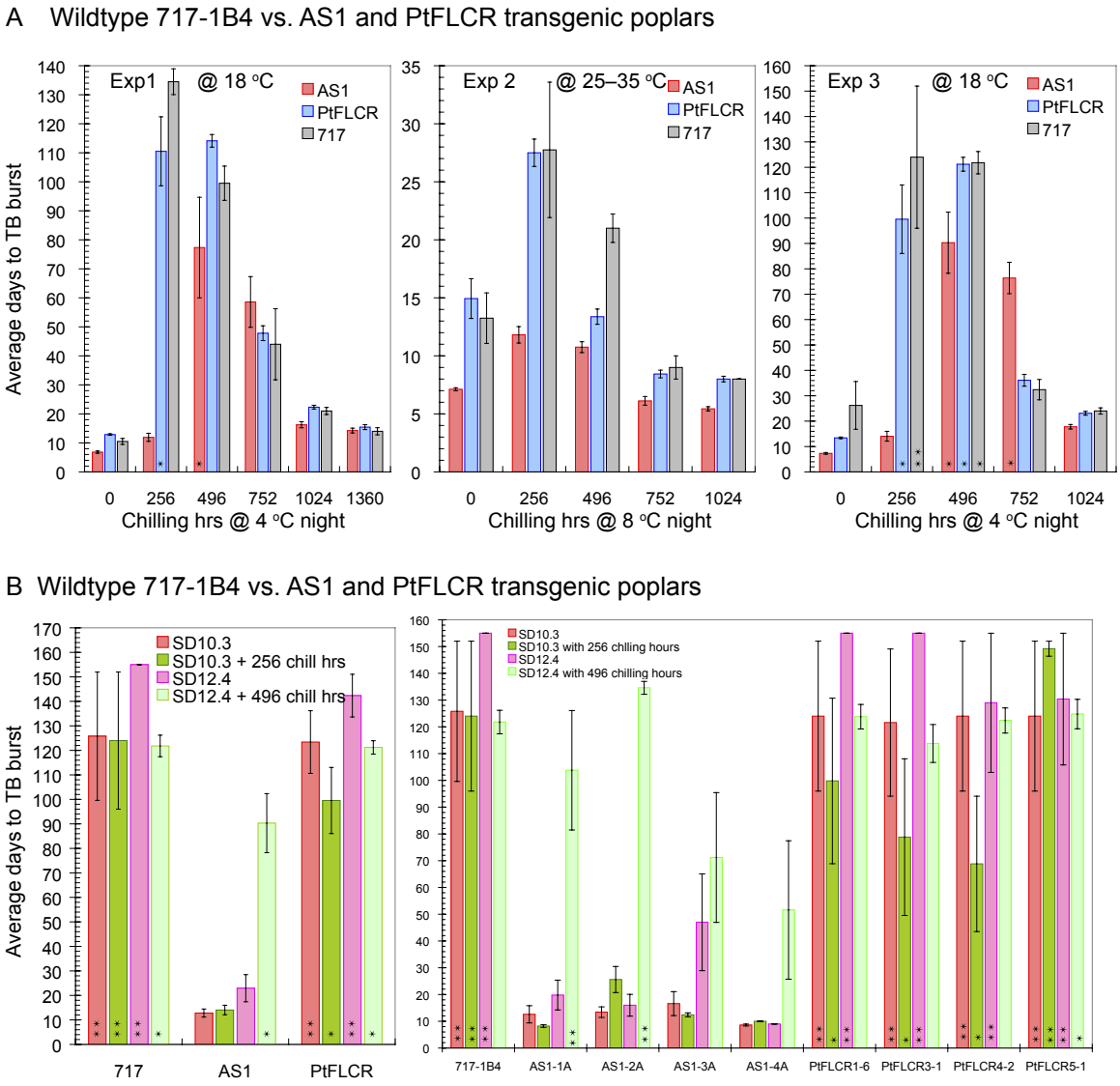


Figure 3-9. Bud dormancy of transgenic poplars overexpressing *PtFLC2as1*.
(A) Dormancy of terminal bud (TB) of wildtype, 35S::*PtFLC2as1* (AS1) and *PtFLC2*-RNAi (PtFLCR) transgenic poplars in different chilling and regrowth temperatures.
(B) Dormancy of terminal bud (TB) of wildtype, AS1 and PtFLCR transgenic poplars with and without chilling.
· and ⁚ mean 5–40% and 60–100% buds were still in dormant when the experiment was terminated. The number of days when experiment was terminated was recorded and used to calculate mean and Std. Error to TB burst. Values in average days to TB burst are mean +/- Std. Error.

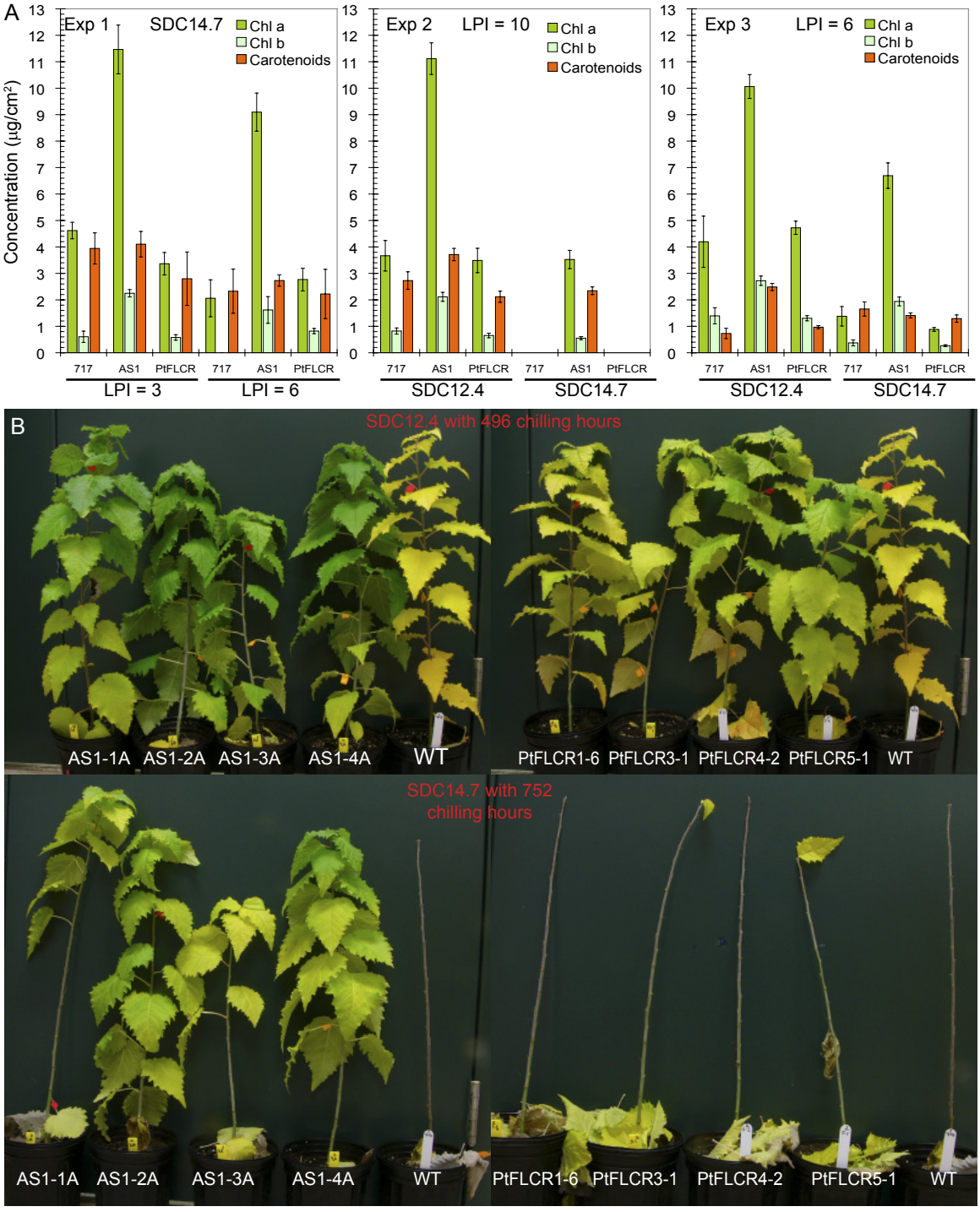


Figure 3-10. Chlorophyll content of leaves from transgenic poplars overexpressing *PtFLC2as1*.
(A) Chlorophyll and carotenoid concentration of wildtype, AS1 (35S::*PtFLC2as1*) and PtFLCR (*PtFLC2*-RNAi) transgenic poplars from 3 repeated experiments during 2006–2008.
(B) Leaf senescence and abscission in wildtype, AS1 (35S::*PtFLC2as1*) and PtFLCR (*PtFLC2*-RNAi) transgenic poplars after SDC12.4 and SDC14.7 treatments
Irradiance and chilling temperature are different between Conviron (10/4 °C day/night) (experiment 1 and 3) and EGC chambers (12/8 °C day/night) (experiment 2) due to equipment capacity. LPI = leaf position index. SDC12.4 = 12.4 weeks of SD combined with 496 chill hrs. SDC14.7 = 14.7 weeks of SD combined with 752 chill hrs. There is no leaves on any plant at SDC17. Values given are means +/- StdError.

and chlorophyll a and carotenoids content were significantly (P=0.004 and P=0.057) affected by leaf position (LPI) (Appendix A-2). In the two-way ANOVA (construct (or genotype) x chilling hours), leaf chlorophyll a, b, c, and carotenoids were significantly (P<0.001) affected by genotype (transgenic genotypes and wildtype), chilling hours, and their interaction. Differences among lines within same construct are not significant. PtFLCR and wildtype control plants did not appear to differ in leaf senescence and abscission responses to SD and LT treatment. These results are consistent with altered *PtFLC2* impinging on the photoperiod and low temperature response of poplar.

Expression of type II MADS-box genes, *PtFT1, PtFRI*, and *PtVIN3* during dormancy

Because of the central role of *FLC* in regulating flowering in *Arabidopsis,* it was determined if expression of poplar homologs to *Arabidopsis* genes related to floral organ identity (A, B, C, D, and E classes), flowering time (F and FLC classes), vegetative development (G class), upstream *FLC* regulators (*FRI, VIN3*) and downstream *FLC* targets (*FT, AP1,* and *SOC1*) were altered in *PtFLC2* transgenic and wildtype control poplars. Little if any difference was observed by RT-PCR of putative homologs to floral organ identity genes (Figure 3-11A). The only exception is for the putative B class gene *PtBs2* which was expressed in the combined SD plus LT treatments in 35S::*PtFLC2as1* transgenic poplars but was not detected in either the wildtype control or PtFLCR plants under the same conditions. Similar to floral identity gene, little if any change in expression was observed between transgenic and wildtype plants for genes related to flower timing (Figure 3-11B) with the exception of *PtF2* which appeared to be reduced in 35S::*PtFLC2as1* poplars after 3 weeks of SD treatment. For other flowering associated genes that were assayed, no obvious expression differences were observed between transgenic and wildtype poplars (Figure 3-11C and 3-11E). It was noted however that the expression of the poplar homolog to *VIN3*, *PtVIN3*, appeared to modulated by temperature and the transcript abundance declined in apical buds upon exposure to LT in the transgenic lines as well as *Populus trichocarpa* Nisqually-1, *Populus deltoides* x *trichocarpa* clone 545-4183, and *Populus alba* x *tremula* clone 717-1B4

A

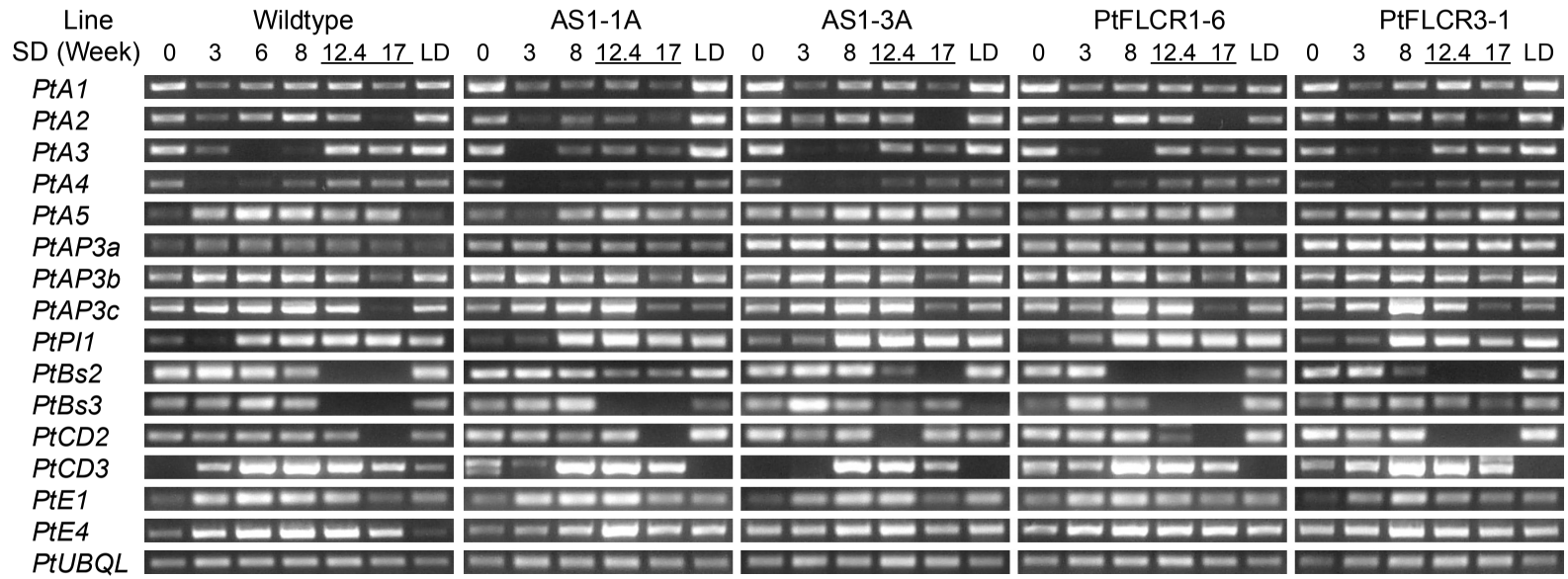


Figure 3-11. Gene expression in *PtFLC2* transgenic poplars.
(A) Expression of floral organs related genes during dormancy in *PtFLC2* transgenic poplars. RNA from shoot apices or buds was used for RT-PCR analysis using primers to detect *PtA, PtAP3, PtPI, PtBs, PtCD*, and *PtE* class genes.
(B) Expression of flowering time related genes during dormancy in *PtFLC2* transgenic poplars. RNA from shoot apices or buds was used for RT-PCR analysis using primers to detect *PtF, PtFLC*, and *PtSVP* class genes.
(C) Expression of other genes during dormancy in *PtFLC2* transgenic poplars. RNA from shoot apices or buds was used for RT-PCR analysis using primers to detect *PtG, PtFRI, PtFT1,* and *PtVIN3* genes.
(D) Expression of other genes during dormancy and in different tissues and clones (Nisqually , 545-4183, 717-1B4). RNA from shoot apices or buds was used for RT-PCR analysis using primers to detect *PtFT1, PtFT2, PtFRI*, and *PtVIN3* genes
(E) Expression of other genes during dormancy in *PtFLC2* transgenic poplars. RNA from shoot apices or buds was used for RT-PCR analysis using primers to detect *PtFT1, PtFRI*, and *PtVIN3* genes.
Weeks of SD with underscore are SD combined with LT beyond initial 8 weeks of SD. Clear and consistent expression patterns of expression for *PtPI2, PtBs1, PtBs4, PtCD1, PtCD4, PtE2, PtE3, PtE5,* and *PtFT2* could not be obtained.

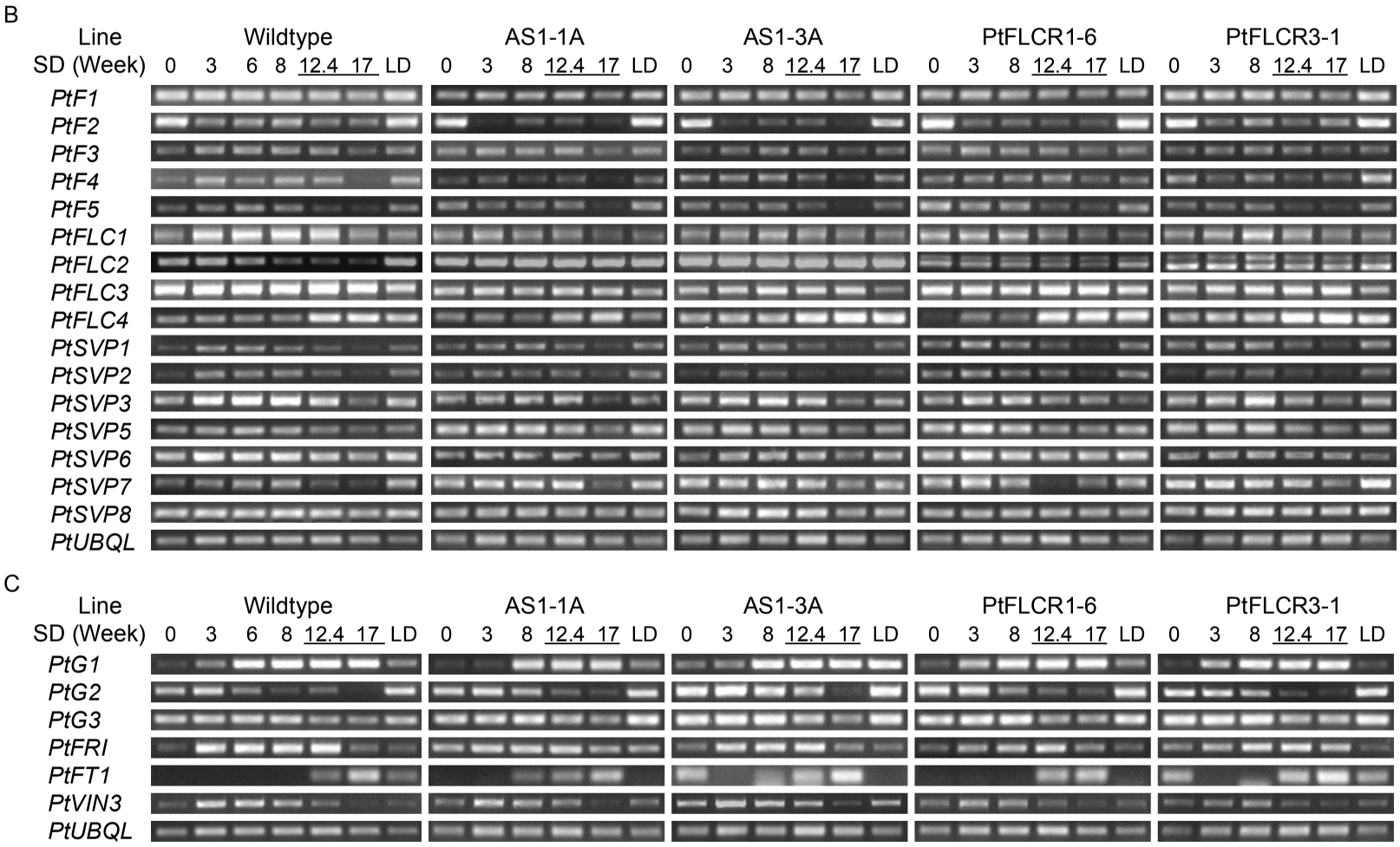


Figure 3-11. Gene expression in *PtFLC2* transgenic poplars (continued)

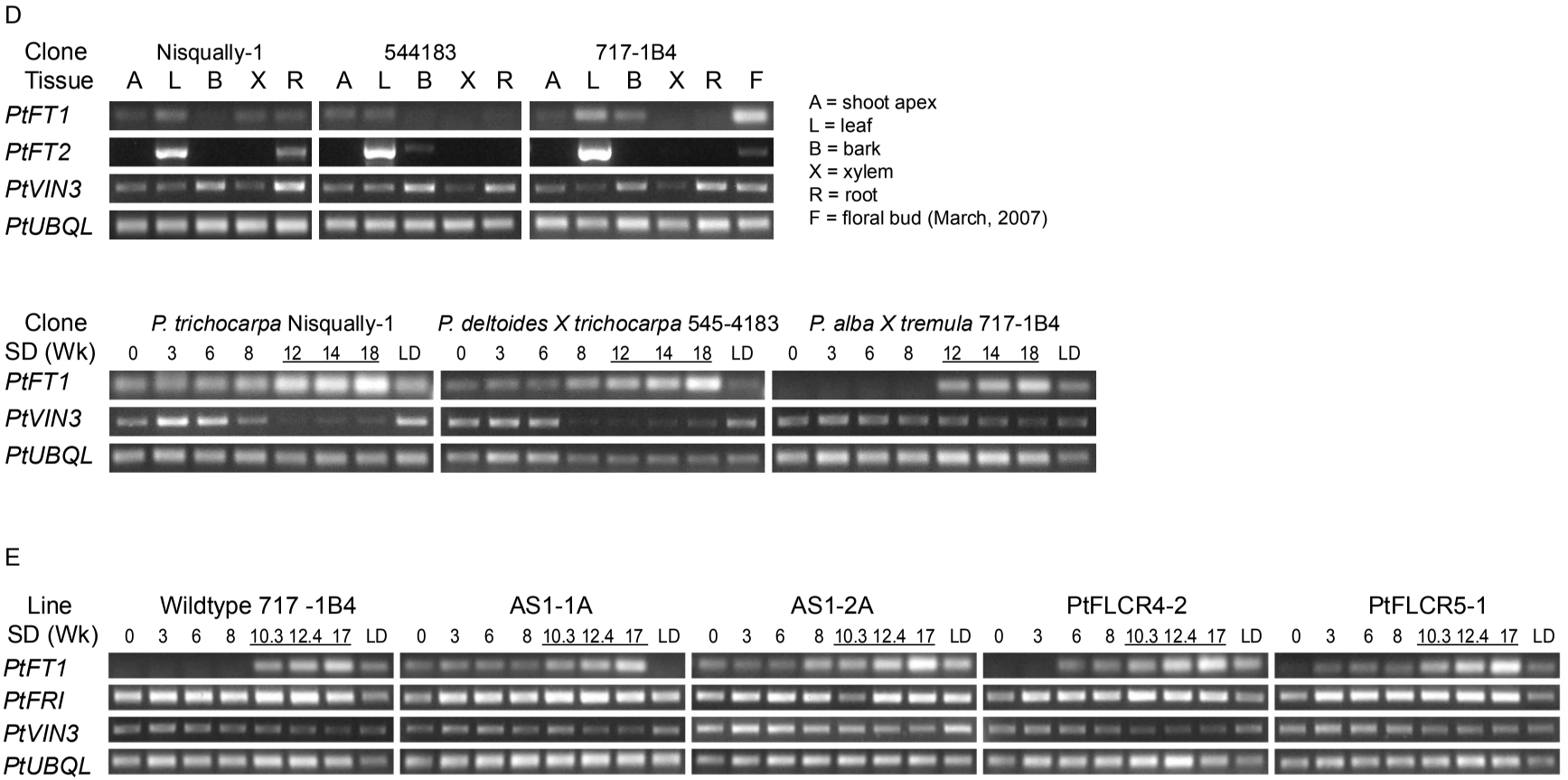


Figure 3-11. Gene expression in *PtFLC2* transgenic poplars (continued)

(Figure 3-11D). Whether reduced expression of *PtVIN3* plays a role in bud dormancy is not known but the possibility of *PtVIN3* being somehow involved in bud dormancy is intriguing.

PtFLC2 induces early flowering in *Arabidopsis*

To determine if FLC function is conserved between poplar and *Arabidopsis*, early and late flowering *Arabidopsis* ecotypes LER and FRI-Sf2 were transformed with 35S::*PtFLC2as1* and 35S::*PtFLC2R* constructs. No significant differences were observed in flowering times in the T1 generation of the LER background when comparing 12 wildtype, 12 *PtFLC2as1* overexpression lines, and 47 *PtFLC2* RNAi lines under LD at 20°C. Unexpectedly, plants of the T1 generation of the FRI-Sf2 background transformed with 35S::*PtFLC2as1* (18 independent lines) flowered significantly earlier than wildtype (6 lines) or plants transformed with *PtFLC2* RNAi (29 lines) (Figure 3-12B). Some of the overexpression lines flowered in half the time required for wildtype *Arabidopsis* FRI-Sf2.

Segregation analysis of 5 early flowering 35S::*PtFLC2as1* transformed lines (AS1-2, AS1-3, AS1-6, AS1-7, and AS1-8) used 48 seedlings per line that were randomly picked 5 days after germination. The plants were grown in LD 20 °C and the number of leaves and days to bolt were recorded every other day. Hierarchical, K-means, and two-step cluster analyses have grouped 48 plants into 2 clusters (early vs. late flowering) for each line using either number of days or number of leaves as the variable (Figure 3-12C). Binomial and Chi-square tests of a 3:1 segregation ratio indicate a single locus for early vs. late flowering (Figure 3-12D). Analysis of gene expression by RT-PCR confirmed the expression of *PtFLC2as1* in the early flowering plants (Figure 3-12D). In addition, RT-PCR analysis shows that *AtFLC* was expressed in the early flowering lines and the expression of *PtFLC2* did not result in co-suppression of *AtFLC*. Examination of the expression of the targets of *AtFLC*, *SOC1, FT,* and the downstream regulatory gene *AP1* revealed that their expression was similar in the early flowering and wildtype lines. Therefore it is not clear at this time how overexpression of *PtFLC2as1* results in early flowering. This question will require further study with later sampling points (30 and 40 days after germination) to examine RNA

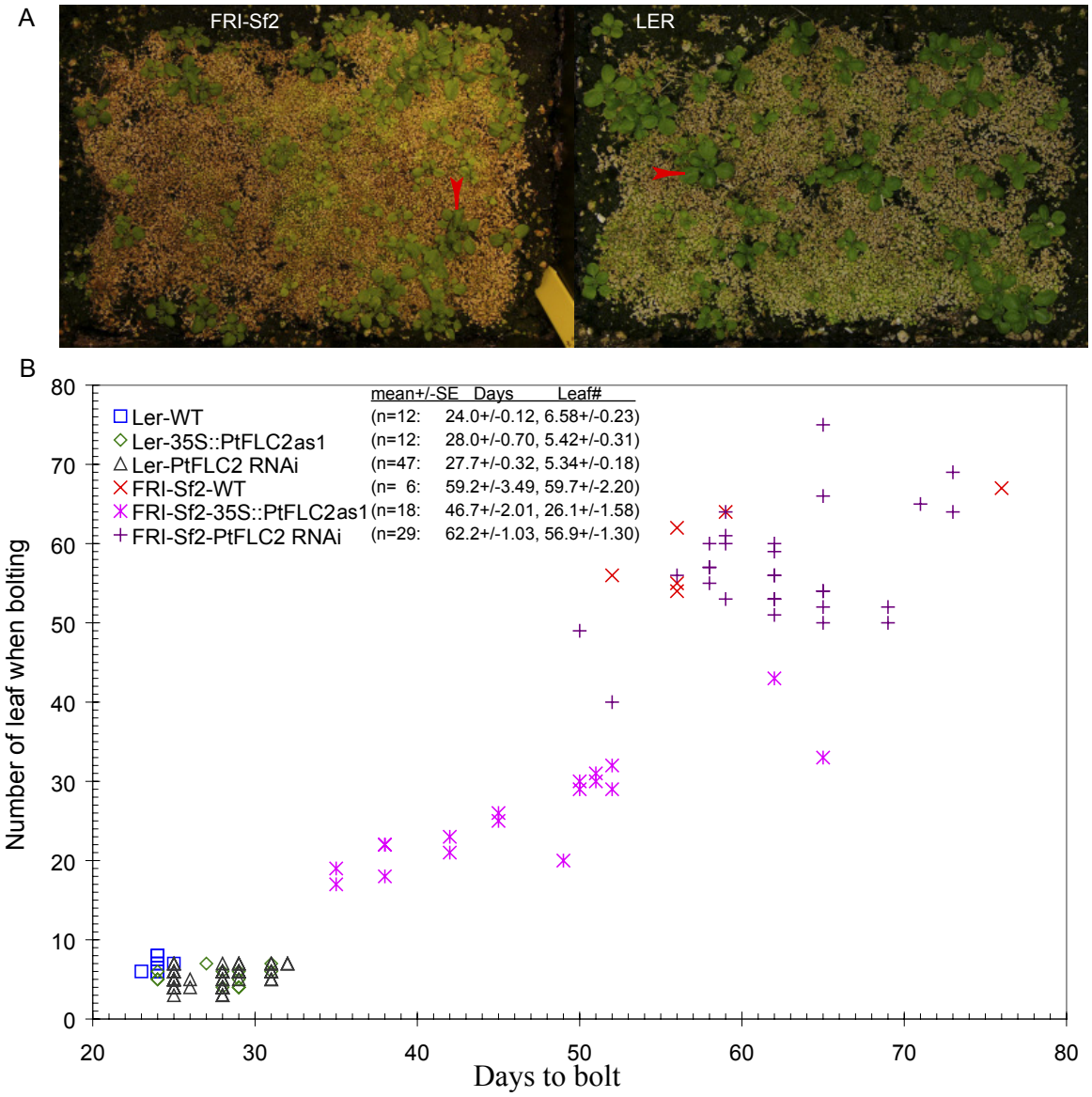
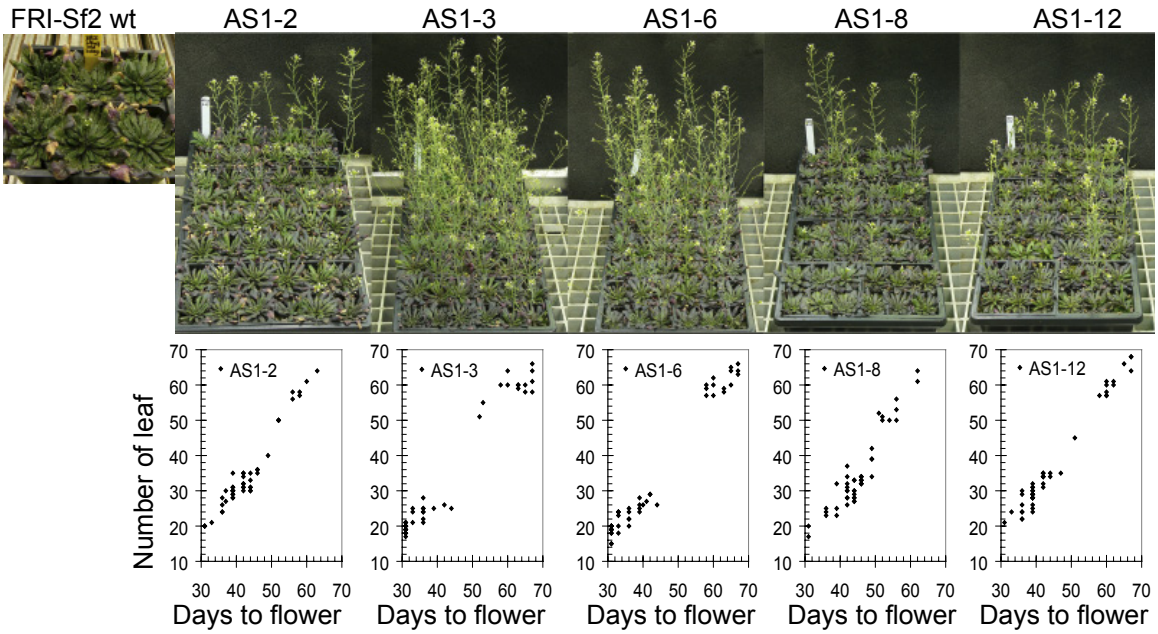


Figure 3-12. Early flowering in *Arabidopsis* late flowering ecotype FRI-Sf2 overexpressing *PtFLC2as1*.
(A) Basta to selection of 35S::*PtFLC2as1* transformed *Arabidopsis (*FRI-Sf2 and LER background*)*. Arrowhead points to the transformed seedling.
(B) Flowering time (leaf# and days to bolt) of T1 generation and wildtype Ler and FRI-Sf2.
(C and D) Analysis of flowering time in 35S::*PtFLC2as1 Arabidopsis* (FRI-Sf2) T2 generation and wildtype *Arabidopsis* (FRI-Sf2) after 51 days LD growth. The flowering time of all nonflowering plants in have no statistic difference to wildtype *Arabidopsis* FRI-Sf2.
(D) RT-PCR analysis of the accumulation of *AP1, FLC, SOC1*, and *FT* transcripts in transgenic and wildytpe *Arabidopsis* leaves harvested from 17-day-old LD grown plants. *AtUBQ10* is internal control.

C



D

Construct	35S::*PtFLC2as1*								WT	
Line	2	3	6	7	8	10	12	18	FRI-Sf2	
T1 generation										
Leaf# to bolt	26	19	17	43	18	32	25	31	59.7	
Days to bolt	45	35	35	62	38	52	45	51	59.0	
Gene expression of T2 generation and WT at 17 days LD										PCR cycle
PtFLCoeas										28
AtAP1										37
AtFLC										26
AtSOC1										28
AtFT										34
AtUBQ10										24
n	39	34	34	N/A	39	N/A	35	N/A	11	
Leaf# to bolt	29.9	21.3	22.0	N/A	30.3	N/A	29.1	N/A	60.2	
Std Error	0.7	0.5	0.6	N/A	0.8	N/A	0.7	N/A	1.4	
Days to bolt	40.0	33.0	34.6	N/A	42.8	N/A	39.1	N/A	56.5	
Std Error	4.3	0.6	0.7	N/A	0.7	N/A	0.5	N/A	2.5	
H0: proportion of the number of plant with early flowering = 0.75										
Plant# (early flowering)	39	34	34	N/A	39	N/A	35	N/A		
Plant# (late flowering)	9	12	12	N/A	9	N/A	13	N/A		
Binomial	0.205	0.301	0.301	N/A	0.205	N/A	0.423	N/A		
Chi-square	0.317	0.505	0.444	N/A	0.317	N/A	0.739	N/A		

Figure 3-12. Early flowering in *Arabidopsis* late flowering ecotype FRI-Sf2 overexpressing *PtFLC2as1* (continued)

around bolting time or other downstream genes such as *AP3, PI*, or *AG* in case PtFLC2as1 targets them instead of *FT, SOC1* and *AP1*.

Discussion

Overexpression *PtFLC2as1* alters photoperiod response and delays growth cessation and bud development

Shoot growth consists of both cell proliferation and organogenesis in the meristem and cell elongation in subapical tissues. The initial physiological and developmental events in dormancy are the cessation of shoot elongation and the formation of a vegetative apical bud. Shoot growth cessation and bud development in many deciduous trees is a response to daylength (Rohde et al. 2000). The critical daylength (or photoperiod) is the longest photoperiod that induces growth cessation and is inherited as a quantitative character (Rohde et al. 2000). The production of new leaves (leaf number or increment) and shoot elongation (plant height or increment) were used as shoot growth parameters to evaluate any differences in growth cessation between wildtype and *PtFLC2* transgenic poplars. The results of this study revealed that overexpression of poplar *PtFLC2as1* delayed the onset of growth cessation in transgenic poplars. In addition, the development of the apical bud was monitored through a histological study of bud development which showed that bud development was also delayed in *PtFLC2as1* overexpressing poplars. Since shoot growth cessation and bud development does occur in the *PtFLC2as1* overexpressing poplars, albeit at a slower rate, it is possible that photoperiod perception still occurs in these plants yet the response is somehow delayed. The nature or basis for these responses is not known.

It appears that photoperiod sensitivity or the response to photoperiod may be altered in some manner, but not eliminated in 35S::*PtFLC2as1* poplars. Further studies that focus on photoperiod perception and response could provide clues as to how altered *PtFLC2as1* expression impinges on this response. Since photoperiod responses are known to be phytochrome mediated, determining the effect of *PtFLC2as1* overexpression on other aspects of photoperiod responses, such as the shade avoidance response, might provide insight.

It was observed that axillary bud growth was enhanced in poplars that overexpressing *PtFLC2as1*. Furthermore, this was observed only in the controlled environmental chambers with lower irradiance (Figure 3-4). It is intriguing to speculate that this increase in axillary shoot growth may represent an altered shade avoidance response. Further studies might also focus on the R/FR and end of FR responses of these plants.

Morphological comparison between vegetative bud (scales, stipules, embryonic leaves) (Rohde et al. 2002) and floral organs (sepals, petals, stamens, carpels) (Krizek et al. 2005) suggest analogous morphological structures between the poplar vegetative apical bud and *Arabidopsis* floral bud. If the poplar vegetative bud is viewed as an *agamous* flower (i.e. no expression of C class genes) or mutant flower with leaf-like oranges (i.e. no expression of E class genes) of *Arabidopsis* (Figure 1-2), the transition from active growth to a vegetative apical bud is similar to the transition from active vegetative growth to flowering in *Arabidopsis*. An *agamous* mutant flower consists of sepals in the first whorl, petals in the second and third whorls and reiterations of this pattern in interior whorls (Krizek et al. 2005). A mutant flower with leaf-like organs is a quadruple mutant (*sep1 sep2 sep3 sep4*) that lacks class E activity which results in loss of floral determinacy (Ditta et al. 2004). In RT-PCR analysis, expression of 5 *PtA*, 3 *PtAP3*, *PtPI1,* 2 *PtCD*, and 2 *PtE* genes were detected in shoot apices whereas apical bud expression of *PtCD2, PtCD3*, and *PtE4* genes was reduced by comparison (Figure 3-11A). The poplar genome appears to have 4 *PtCD* and 5 *PtE* genes. and the low expression of these genes in shoot apices (or apical buds) suggests that the poplar apical bud may represent an *agamous* leaf-like organ.

Overexpression of *PtFLC2as1* alters bud dormancy

Once bud dormancy has been induced, exposure to a certain period of chilling temperature is required to release buds from dormancy (Rinne et al. 2001; Rohde et al. 2000). Under the experimental conditions used in this study, bud endodormancy was induced in *Populus alba* x *tremula* clone 717-1B4 after 8 weeks of SD treatment and warm temperatures. This differed from timing reported for this same genotype by Ruttink et al (2007) in which only 6 weeks of SD were required. The difference between the current study and the report

by Ruttink et al. (2007) is likely to be a consequence of the experimental conditions used in the two studies. In the current study the photon flux density in the controlled environment chambers ranged from 300–800 μmol m^{-2} s^{-1} while the Ruttink et al. (2007) study averaged 100 μmol m^{-2} s^{-1}. It is likely that the 3x-8x increase in irradiance resulted in more vigorous plants that required a longer SD exposure to induce bud dormancy. Photoperiod induced apical bud dormancy was significantly reduced in poplars overexpressing *PtFLC2as1* after 8 weeks of SD treatment. This difference became even more apparent when SD treatments were extended by 2 to 4 weeks resulting in an 80% reduction in the number of days in LD that were required for the apical bud of *PtFLC2as1* overexpressing poplars to resume growth. Interestingly, bud endodormancy could be induced to a level comparable to wildtype plants if exposure to cold temperature accompanied SD photoperiod. Furthermore, the 35S:: *PtFLC2as1* plants required about 2.5 additional weeks of SD combined with LT, to induce a comparable level of bud endodormancy. Once *PtFLC2as1* overexpressing plants become endodormant, they required further exposure to LT to overcome bud dormancy. Surprisingly, the *PtFLC2as1* overexpressing plants required a similar amount of chilling temperature to overcome dormancy compared to wildtype plants indicating that chilling requirement for release from endodormancy was unchanged by overexpression of *PtFLC2as1*. This suggests that PtFLC2 may somehow be involved in promoting vegetative growth. In addition, this also suggests that the establishment of bud dormancy is regulated by two pathways, photoperiod and cold, and *PtFLC*2 may play a role in integrating these two environmental signals.

Every autumn, deciduous trees undergo leaf senescence and proceed with leaf abscission by forming an abscission layer in the petiole after phloem transport stops to allow the senescent leaf to separate from the plant (Keskitalo et al. 2005). The most obvious event associated with autumn senescence is the leaf color changes from green to yellow, red, or orange mediated by chlorophyll degradation and anthocyanin accumulation (Hoch et al. 2001). It has been suggested that photoperiod is the major environmental signal that induces fall leaf senescence in trees (Keskitalo et al. 2005). However, in poplar visual signs

of leaf senescence fail to develop when exposed only to SD but rather SD appears to be a prerequisite for LT mediated leaf senescence. This observation was confirmed by analysis of leaf chlorophyll contents which showed that levels did not decline in wildtype until exposure to SD and LT. Even with extended exposure to SD, the chlorophyll content of wildtype leaves did not change appreciably. Only upon exposure to chilling temperatures did the chlorophyll content of wildtype plants decline which confirmed the observation that leaf senescence is regulated by both photoperiod and chilling temperature. Autumn senescence has been separated into four phases. Phase 1 is a pre-senescent stage with stable chlorophyll levels and during phase 2 is a stage with chlorophyll concentrations decrease to about 10% of that of phase 1 (Keskitalo et al. 2005). In *Populus alba* x *tremula* clone 717-1B4, it appears that photoperiod initiates the autumn senescence process of phase 1 while subsequent chilling temperature regulates subsequent phases including chlorophyll degradation and leaf abscission.

In contrast to wildtype plants, leaf senescence was delayed in *PtFLC2as1* overexpressing poplars. Leaf contents of both chlorophyll a and b were consistently greater in these poplars irrespective of leaf development (LPI) (Figure 3-10). In some ways this result is surprising since chilling requirement for bud endodormancy does not appear to be appreciably different in these plants compared to wildtype plants. Thus the temperature response for dormancy release appears to be maintained while the temperature response for leaf senescence appears different. This could suggest that these two physiological responses somehow sense temperature differently. However, this difference in response may be explained by an altered photoperiod response. Since SD photoperiod is prerequisite to leaf senescence, then an alteration in SD response could impact subsequent processes. Since other photoperiod responses including growth cessation, apical bud development, and bud dormancy are altered in *PtFLC2as1* overexpressing poplars, it is just as likely that reduced photoperiod sensitivity somehow inhibits the LT phase of leaf senescence.

PtFLC2 may not regulate the expression of poplar homologs of *SOC1, AP1*, and *FT*

genes

FLC represses the expression of *Arabidopsis* flowering time integrators genes (*SOC1* and *FT*) as has been demonstrated by 35S::*FLC* and *FRI flc-3* null mutant *Arabidopsis* (Hepworth et al. 2002; Michaels et al. 2001; Michaels et al. 2005; Searle et al. 2006). Overexpression of *FD* or *FT* in *Arabidopsis* upregulates the expression of *AP1* and *FUL* (Abe et al. 2005; Wigge et al. 2005). The lack of significant up- or down-regulation of type II MADS-box genes among wildtype and all *PtFLC2* transgenic poplars (Figure 3-11) might be due to the sensitivity of DNA detection, not enough sampling points to detect the change when the effect is only at a certain developmental point, or the failure select the correct downstream genes regulated by *PtFLC2*. Down-regulation of *PtVIN3* during dormancy suggests that it does not repress *PtFLC2* expression as *the Arabidopsis* homolog *VIN3* does with *FLC* in *Arabidopsis*. Thus from the information generated from this study, it is not clear what the targets of *PtFLC2* are or how they may impinge on bud development and dormancy. *Arabidopsis* FLC binds to a region with CArG boxes within the first intron of *FT* and promoter of the *SOC1* and *FD* to repress the expression of *FT*, *SOC1* and *FD* (Helliwell et al. 2006; Searle et al. 2006). Studies that identify the targets of *PtFLC2* may prove valuable in understanding the altered response of *PtFLC2* overexpression.

PtFLC2 induces early flowering in late flowering *Arabidopsis* (FRI-Sf2)

Overexpression of *FLC* in *Arabidopsis* is sufficient to delay flowering even in genotypes without an active *FRI* allele (Michaels et al. 1999). The extreme late flowering of *Arabidopsis* FRI-Sf2 (Col) is eliminated in the presence of the *flc-3* null mutation (Michaels et al. 1999). If the function of *FLC* as a floral repressor is conserved across species, then overexpression of *PtFLC2* in *Arabidopsis* would be expected to act as a floral repressor resulting in delayed flowering. Thus overexpression of *PtFLC2* in early-flowering *Arabidopsis* LER will generate late flowering transgenic plants. Two *Arabidopsis* wildtypes with late (FRI-Sf2 in Col) and early (LER) flowering phenotypes were selected to test this hypothesis. Unexpectedly, overexpression of *PtFLC2as1* in *Arabidopsis* resulted in early flowering on late flowering genotypes while it had no effect on early flowering genotypes.

The effect of overexpressing *PtFLC2as1* was not as great as that reported for *flc-3* (Michaels et al. 1999), yet reduced flowering time approximately 50%.

Two possible explanations could account for the early flowering phenotype - PtFLC2as1 may serve as a dominant negative repressor which reduces FLC repression on flowering time by competing with FLC, or PtFLC2as1 may by itself induce flowering. In the first case in acting as a dominant negative repression, PtFLC2as1 may compete with FLC to bind on the DNA binding site of downstream genes but fail to act as a repressor of these genes. PtFLC2as1 could also compete with FLC in the formation of a protein complex. Since FLC is part of a 600–800 kDa multimeric protein complex that acts to repress the expression of *FT*, *SOC1* and *FD,* by binding directly to a region with CArG boxes within the first intron of *FT* and promoter of the *SOC1* and *FD* (Helliwell et al. 2006; Searle et al. 2006), the inclusion of PtFLC2as1 in this complex may render it nonfunctional. The likelihood of PtFLC2as1 acting as a non-functional repressor of downstream genes seems remote. It is more likely that PtFLC2as1 is included in a multimeric protein complex resulting in a nonfunctional complex.

The possibility that PtFLC2as1 can induce flowering can not be eliminated from the current study. In fact similar phenotypes of delayed growth cessation, leaf senescence, and dormancy are observed in transgenic poplar overexpressing *BpMADS4*, a birch homolog of *FRUITFULL* (Hoenicka et al. 2008). *BpMADS4* is also a homolog of *PtA1* and *PtA*5 with 83% bootstrap support in a phylogenetic NJ tree using MAFFT aligned protein and cDNA sequences (data not shown). Phylogenetic analysis also suggests that genes in both the *FLC* and *A* classes may have evolved from a common ancestor gene (Figure 2-3A). The similar phenotypes between 35S::*BpMADS4* and 35S::*PtFLC2* poplars combined with the likelihood that all homologs of FLC found are in eudicots (Reeves et al. 2007) could mean the FLC class evolved from the A class after the divergence of monocots and eudicots and the PtFLC2 still retains A class gene function while evolving the neofunction of FLC. If *PtFLC2* is able to mimic the function of *Arabidopsis AP1/CAL/FUL*, then overexpression *PtFLC2* in *Arabidopsis* FRI-Sf2 would be expected to promote early flowering which is similar to

overexpression of *AP1* (Mandel et al. 1995) or homologs of *AP1* in *Arabidopsis* (Gocal et al. 2001; Kotoda et al. 2002).

These findings suggest that both *PtFLC2* and *FLC* may function differently *in vivo* with regard to the regulation of flowering time in *Arabidopsis* due to some sequence divergence. Therefore it is possible that PtFLC2 either competes with the endogenous functional FLC protein to form a multimeric protein complex which is unable to repress downstream gene expression and/or the PtFLC2 protein itself serves as a flowering meristem identity protein similar to AP1/CAL/FUL, which activates the expression of other floral organ identity genes and converts the vegetative meristem to a floral fate.

In this study, constitutive expression of *PtFLC2as1* had little if any effect on the transcript abundance of *Arabidopsis FLC* expression suggesting that cosuppression is likely not to be the cause of the early flowering phenotype. In addition, little change in the expression of *SOC1* or *FT* was observed in the early flowering transgenic *Arabidopsis*. The up-regulation of *AP1* and *SOC1* during transition from vegetative to flowering in *Arabidopsis* takes place in the shoot apical meristem after bolting with slight delay in *AP1* activation relative to *FT* activation (Abe et al. 2005; Wigge et al. 2005). Since this study sampled leaf tissue, it is possible that the effect on gene expression of *AP1* and *SOC1* will not be significant.

PtFLC2 is an essential component of bud dormancy

In *Arabidopsis* flowering pathways, *FLC* is the integration point of multiple pathways including the vernalization, autonomous pathway, and epigenetic regulation, and *FLC* represses the flowering transition independent of the pathways regulated by light signals (photoperiod and light quality) and gibberellins (Figure 1-3). Photoperiod has no effect on the levels of *FLC* expression in wildtype and the *vil1* (*vin3-like 1*) *Arabidopsis* mutant (Sung et al. 2006). *MAF1* (or *FLM*), a flower repressor, is expressed mainly in root and shoot apices and young leaves (Scortecci et al. 2001). Mutant *maf1* lines flower early under both LD and SD and *MAF1* overexpression delayed flowering under both LD and SD which suggests that plants still retain a photoperiod response (Scortecci et al. 2001). VIL1 regulates flowering

time by attenuating *MAF1* expression in noninductive photoperiods (i.e. SD for *Arabidopsis*) and modifying chromatin structure of *FLC* and *MAF1* to a silent state with VIN3 together in the vernalization pathway (Sung et al. 2006).

Six identified *PtFLC* genes are homologs of six *Arabidopsis FLC* class genes. They form a single clade joined together as 2 monophyletic groups in the phylogenetic analysis of poplar MADS-box genes (Figure 2-1A, 2-3). This suggests that gene duplication and divergence events of *FLC* and *PtFLC* class genes happened after speciation between *Arabidopsis* and poplar. Each *FLC* and *PtFLC* class genes might evolve their own neo-function and/or inherit the original or sub-functions of their ancestor in a different way between poplar and *Arabidopsis*. Both *FLC* and *MAF1* are regulated by vernalization. *FLC* is not regulated by photoperiod, but *MAF1* is down-regulated by SD. This study indicates that overexpression *PtFLC2as1* interferes with both photoperiod and cold regulatory pathways. It is possible that *PtFLC2as1* inherited or evolved both regulatory mechanisms (photoperiod and chilling) to fine tune the regulation of dormancy and senescence.

I propose that *PtFLC2as1* promotes active vegetative growth directly or by inhibiting the repression of active vegetative growth and can be down-regulated by prolonged SD and cold by 2 different pathways. This leads to enhance axillary branching under both LD and SD, delays SD induced transition from active growth to growth cessation and bud formation, delays SD and chilling-induced endodormancy, leaf senescence and abscission under SD combined with cold.

Materials and Methods

Plant materials and growth conditions

Plant propagation and growth conditions for *Populus trichocarpa* Nisqually-1, *Populus deltoides* x *trichocarpa* 545-4183 and *Populus tremula* x *alba* 717-1B4 are as described in chapter 2. Light sources used in all controlled environment chambers were a combination of incandescent and high intensity discharge (HID) bulbs. Photosynthetically active radiation (PAR) at 50 cm above the surface of pots ranged from 310–470 μmol m^{-2}

s^{-1} in the Conviron environmental chambers, 400–800 µmol m^{-2} s^{-1} in the EGC controlled environmental chambers, and 400–800 µmol m^{-2} s^{-1} in the University of Maryland Research Greenhouse. Irradiances of each growth environment were measured with Ocean Optic QE65000.

Vegetative bud development, dormancy and chilling induced dormancy release was performed in controlled environment chambers with SD photoperiods (8 hours light and 16 hours dark at 18°C and 50–75% RH) for 8 weeks followed by an additional 9 weeks of SD photoperiods with the day temperature reduced to 10°C and the night temperature reduced to 4°C. Shoot apices or terminal buds from 4 plants were harvested from LD grown plants before the treatment started and after 3, 6, 8, 12.4, 14.7, and 17 weeks of treatment. SD treatments up to 8 weeks SD were at 18°C while after 8 weeks of SD the temperature was reduced to 10°C day and 4°C night. After the 17 week experimental treatment, the remaining plants were moved to a LD growth chamber and growing shoot apices were harvested 5 weeks later. All harvested samples were immediately frozen in liquid nitrogen and stored at -80°C until used for RNA purification. The shoot apices (or apical buds) of 2–4 plants were harvested at 0, 3, 6, 8, (10.3), 12.4, and 17 weeks of treatment, and 3–5 weeks after LD regrowth and fixed in FAA (formalin - acetic acid - alcohol) for histological analysis. As with the previous treatments, the SD treatments beyond 8 weeks were combined with 10°C day and 4°C night temperatures.

Isolation of *PtFLC2* and RT-PCR analysis

Total RNA isolation and cDNA synthesis was performed as described in chapter 2. The poplar *FLC-like* homologs (*PtFLC1–PtFLC6*) were identified in chapter 2 via phylogenetic analysis of poplar MADS-box genes from JGI poplar database.

In RT-PCR, a 478 bp *PtFLC2as1* fragment was amplified with *PtFLC2* specific primers (forward primer, 5'-TCTCTAGCGTTGGCAGTACAACC- and reverse primer 5'-TGCCATTATAGCCGCCTCCAG-3'). The forward primer spanned intron 1 to avoid genomic DNA contamination. The length of this fragment is 61 bp longer than the predicted gene model in the JGI poplar database because one more exon (62 bp) was added and 1 bp

is removed from JGI predicted gene based on cloned sequencing results (Table 2-1). This PCR product was cloned using the Promega pGEM-T Easy vector system and sequenced more than 30 times using total RNA from three different poplar clones (*Populus deltoides* x *trichocarpa* clone 545-4183, *Populus alba* x *tremula* clone 717-1B4 and *Populus trichocarpa* clone Nisqually-1) to verify the DNA sequence and intron-exon borders.

The specificity of all gene specific primers of poplar type II MADS-box genes was verified by cloning the PCR product with Promega pGEM-T Easy vector cloning system and sequencing the cloned products as described in chapter 2. DNA sequences of *PtVIN3a* (fgenesh1_pg.C_LG_XVIII000799), *PtVIN3b* (estExt_fgenesh1_pg_v1.C_1210022) and *PtFRI* (eugene3.00150894) were retrieved from the JGI poplar database after searching gene model hits with Smith-Waterman criteria by locus ID of *Arabidopsis VIN3* (At5g57380) and *FRI* (At4g00650). Gene specific primers for *PtVIN3a* (forward 5'-CGCAGGTCTATCTCCAGCCACAG-3', reverse 5'-TCGCCATTCCCCACTTCTCTTC-3') amplified a 682 bp product while the *PtFRI* primers (forward 5'-CCTTCCATGGCCATTACTCTCA-3' reverse 5'-ACGGCGTTTTTGCTTTTTCTGT-3') amplified a 349 bp product. Both the *PtVIN3a* and *PtFRI* products were cloned and sequenced to verify amplification. *PtVIN3*b primer combination yielded a PCR product smaller than 300 bp which is far shorter than the expected 1009 bp. *PtVIN3a* primer combination gave a 682 bp PCR product which is 15 bp smaller than the size based on the predicted transcript in the JGI gene model. Cloned sequencing results showed that it is more likely to derive from *PtVIN3b* instead of *PtVIn3a*. Gene specific primers for *PtFT1* (fgenesh1_pm.C_LG_VIII000284, forward 5'-CAGAACTTCAACACCAGAGA-3' reverse 5'-TCCTACCACCAGAGCCACT-3' as reverse primer) produced a 103 bp product and *PtFT2* (eugene3.14090001, forward 5'-ATGCCTAGGGATAGAGAACCTCTTAG-3' reverse 5'-GATACCTACAATAATCATAGTTTCCC-3' as reverse primer) resulted in a 700 bp product. Primers for *PtFT1* and *PtFT2* were based on published reports (Bohlenius et al. 2006; Hsu et al. 2006). All PCR analyses followed the same procedure described in chapter 2.

PtFLC2 overexpression and RNAi constructs

Several alternatively spliced *PtFLC2* variants have been identified (detailed in chapter 2). The largest *PtFLC2* transcript (*PtFLC2as1*) was selected for use in transgenic studies. Based on *PtFLC2* cDNA sequences, the primers used to generate the 35S::*PtFLC2as1* and *PtFLC2* RNAi (35S::*PtFLC2R*) constructs were identified using PrimerSelect (DNASTAR Lasergene). The 647 bp full-length *PtFLC2as1* CDS was amplified with forward primer 5'-CACCATGGGCCGTAAGAAAGTGGAG-3' and the reverse primer 5'-TGCCATTATAGCCGCCTCCAG-3' located in the 3'UTR. For use in constructing an RNAi chimeric gene, a 379 bp *PtFLC2* fragment consisting of partial K-domain and complete C-terminal was amplified using the forward primer 5'-CACCTGGTGAGCATGCGAACTTGAAAT-3' and the same reverse primer used above to obtain a full length transcript. The "CACC" nucleotides in the beginning of both forward primers were added to the original sequences to enable directional cloning into the pENTR plasmid using the Directional TOPO Cloning kit (Invitrogen). RNA from shoot apices of *Populus deltoides* x *trichocarpa* clone 545-4183 was used for RT-PCR.

PCR products (2 μL PCR product) were mixed with 0.5 μL pENTR™ D-TOPO entry vector (15–20 ng/μL linearized plasmid DNA) following the instructions of the Invitrogen pENTR™ Directional TOPO Cloning Kit. OneShot Top10 chemically competent *E. coli* cells were transformed following the manufacturer's procedures. Plasmid DNA from transformed colonies was isolated using the Wizard Plus SV Minipreps DNA purification system (Promega) and analyzed by restriction enzyme analysis to confirm DNA cloning. After restriction analysis, selected plasmid DNA samples were verified by DNA sequencing

Invitrogen GATEWAY™ technology was used to facilitate vector construction. Approximately 200–300 ng of pENTR entry clone and 300 ng destination vector (i.e. GATEWAY T-DNA binary vector) were mixed with toposiomerase I and LR Clonase enzyme mix following the manufacturers directions (Invitrogen). Plasmid DNA was used to transform Top10 chemically competent *E. coli* cells. Plasmid DNA was isolated from transformed colonies and analyzed by restriction enzyme digestion.

Both PCR products were subcloned into the pENTR D-TOPO entry vector and then recombined into the GATEWAY™ T-DNA binary vector pB7WG2 and pB7GWIWG2(II) (Karimi et al. 2002) mediated by LR Clonase™ enzyme mix. The pB7WG2 T-DNA binary vector is designed to express sequences from the 35S promoter and key components consisting of RB (right border), p35S (cauliflower mosaic virus (CaMV) 35S promoter), attR1 (site-specific recombination site), CmR-ccdB (chloramphenicol resistance marker-ccdb positive selection gene), attR2, T35S (35S terminator), *nos* (nopaline synthase) promoter, Bar (bialaphos acetyltransferase gene), *nos* terminator, LB (left border), and Sm/SpR (spectinomycin-Streptomycin resistant gene). The pB7GWIWG2(II) T-DNA binary vector is designed to produce double stranded RNA and key components consist of RB, p35S, attR1, ccdB, attR2, intron, CmR, intron, attR2, ccdB, attR1, T35S, *nos* promoter, Bar, *nos* terminator, LB, and Sm/SpR. The resulting construct 35S::*PtFLC2as1* produced in the binary vector pB7WG2 contains an expression cassette consisting of the p35S::poplar cDNA::T35S and the plant selectable marker *bar* (bialaphos acetyltransferase) which confers resistance to glufosinate ammonium. The resulting construct *PtFLC2*-RNAi (35S::*PtFLC2R*) produced in the binary vector pB7GWIWG2(II) contains an expression cassette consisting of the p35S::cDNA sense–*Arabidopsis* intron–cDNA antisense::T35S and the plant selectable marker *bar*.

T-DNA binary vectors were used to transform *Agrobacterium tumefaciens* strain C59/pMP90 by the freeze-thaw method. 1 μg plasmid DNA was added to a tube with just thawed 0.1 mL C58/pMP90 *Agrobacterium* competent cells and mixed gently. The cells were frozen in liquid nitrogen for a few seconds and then thawed in a 37°C waterbath for 5 minutes. 1 mL LB medium in room temperature was added and the cells were incubated for 4 hours at 28°C shaker (150 rpm). The cells were spun down, re-suspended to 200 μL LB, spread onto 2 LB plates with suitable antibiotics, and incubated overnight at room temperature (20–25°C). The antibiotics used for *Agrobacterium* C58/pMP90 cells with 35S::*PtFLC2as1* construct are 20 μg/mL gentomycin and 50 μg/mL spectinomycin. The antibiotics used for *Agrobacterium* C58/pMP90 cells with 35S::*PtFLC2R* construct are 20 μg/mL gentomycin,

50 μg/mL spectinomycin, and 50 μg/mL chloramphenicol. Transformed colonies appeared in 2 days. Plasmid DNA was isolated from picked colonies and analyzed with multiple combinations of restriction enzymes.

Production of transgenic poplars

Populus alba x *tremula* clone 717-1B4 was used to generate transgenic poplars and was *in vitro* propagated on hormone-free 0.5X Linsmair-Skoog's (LS) medium. The protocol for *Agrobacterium tumefaciens* transformation has been well established for *Populus alba* x *tremula* clone 717-1B4 (Han et al. 2000; Leple et al. 1992; Zhu et al. 2001). Longitudinally cut internodal stem explants were preconditioned for 48 hours on M1 media (34.73 g/L LS, 2 g/L gelrite, 10 μM 1-naphthylacetic acid (NAA), and 5 μM N6-(2-isopenthy) adenine(2iP)) at room temperature in the dark. A single transformed *Agrobacterium* colony for each construct was picked and cultured in 5 mL LB 28°C overnight and 0.1 mL *Agrobacterium* of the overnight culture was added to 250 mL LB medium with proper antibiotics and incubated on a 28°C shaker overnight. Preconditioned nodal explants were inoculated with *Agrobacterium* at a cell concentration of about 5 x 10^8 cfu/mL, (OD660 = 0.3–0.4) at room temperature for 16 hours with gentle shaking (10–20 rpm). Explants were then co-cultivated by blotting the explants on sterile paper towels and incubating on M1 media at room temperature in the dark for 48 hours. Explants were then de-contaminated by washing 4 times in 30 mL sterile water with 0.2% Tween-20 and 2 times in 30 mL water with antibiotics (250 mg/L cefotaxime, 500 mg/L carbenicillin and 5 mg/L tetracycline) with vigorous vortexing. Explants were blotted on sterile paper towels after each wash and transferred to M2 callus induction media (34.73 g/L LS, 2 g/L gelrite, 10 μM NAA, 5 μM 2iP, 250 mg/L cefotaxime, 500 mg/L carbenicillin and 5 mg/L tetracycline). Stem explants were incubated at room temperature in the dark for 2 weeks, then transferred to new M2 plates containing 5mg/L Basta. After additional 2 weeks of culture, explants were transferred to shoot induction media M3B (34.73 g/L LS, 2 g/L gelrite, 0.1 μM TDZ (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea,), 250 mg/L cefotaxime, 500 mg/L carbenicillin, and 5 mg/L Basta) and cultured in the light. Regenerated shoots were excised with a piece of callus and transferred to hormone-free media for root induction (34.73

g/L LS, 1 g/L gelrite, 3 g/L agar, and 5 mg/L Basta). All regenerated and rooted shoots resistant to Basta were propagated monthly and maintained *in vitro* in hormone-free 0.5X Linsmair-Skoog's (LS) medium with 5mg/L Basta. Transformed plants were verified by RT-PCR analysis (Figure 3-3).

Production of transgenic *Arabidopsis*

The floral dip method for *Agrobacterium*-mediated transformation for *Arabidopsis thaliana* was used to generate transgenic *Arabidopsis* (Clough et al. 1998). The early flowering ecotype LER and late flowering ecotype FRI-SF2 (Col) (TAIR stock# CS6209) were transformed with both 35S::*PtFLC2as1* and 35S::*PtFLC2R*. Transformed 2–5 day old seedlings were selected for Basta resistance by spraying a diluted non-selective herbicide solution (1 mL 5.79% Basta Concentrate, 0.5 mL Silwet L-77, and 1 L water) evenly onto all seedlings daily for 7–10 days.

The days and number of leaves to bolt were recorded 3 timers per week for transformed T1 plants. Some early flowering transgenic lines were selected for segregation and RNA analysis.

Leaves of 10 plants were harvested after the light was on 6–8 hours for 17 days after germination. Qiagen RNeasy Plant Mini Kit was used to isolate total RNA. Transformed plants were verified by RT-PCR analysis (Figure 3-12). PCR primers for *Arabidopsis* genes (*AP1, FLC, SOC1, FT,* and *UBQ10*) were based on published reports (Abe et al. 2005; Michaels et al. 2003a). PCR was performed at 62°C annealing temperature for all primer combinations and cycle numbers were 37, 26, 28, 34, and 24 respectively.

The segregation analysis used 5 early flowering 35S::*PtFLC2as1* transformed T2 lines in the FRI-Sf2 background. Forty-eight seedlings per line without Basta selection were randomly picked and transferred to individual pots 5 days after germination. Numbers of leaves and days to bolt were recorded every other day. SPSS 13.0 was used for all statistic analyses. Hierarchical, K-means, and two-step cluster analyses with Schwarz's Bayesian criterion, Ward's method, and squared Euclidean distance interval were used to group 48 plants into 2 clusters (early vs. late flowering) for each line using either number of days

or number of leaves to bolt as variables. Nonparametric binomial and Chi-square tests were used to test the null hypothesis, H0: segregation is 3:1. Equality and homogeneity of variances were checked by Levene's test. Multiple comparisons of means among lines within the same cluster with equal variance were carried out by one-way ANOVA. Post Hoc tests were Bonferroni, Tukey HSD, and Scheffe methods.

Measurement of growth cessation and leaf senescence

The plant height and number of leaves of 10 plants from each poplar line were measured weekly starting 2 weeks before SD and continuing until 8 weeks of SD. Plant height, number of leaves, and increments of plant height and number of leaves were used to evaluate growth cessation during SD treatment.

Leaf senescence was evaluated by measuring the leaf content of chlorophyll a, b, c, and carotenoids that were extracted with 80% acetone (Lee et al. 2003; Lichtenthaler 1987; USEPA September, 1997; Vernon 1960). Leaves from 3 plants were harvested for each line after 6, 8, and 12.4 weeks of SD without LT (10°C day and 4°C night) and 10.3, 12,4, and 14.7 weeks of SD combined with 256, 496, and 752 hrs LT beyond first 8 weeks of SD. Three 1.3 cm^2 leaf disks per plant were cut from leaves at LPI (leaf position index) 3, 6 or 10 with a cork borer for pigment extraction. Chlorophylls and carotenoids were extracted from 3 leaf disks without tissue disruption in 2 mL 80% acetone for 1 week at 4°C in darkness. The absorption of 5 wavelengths, 750 nm, 664 nm, 647 nm, 630 nm, and 470 nm, were measured by Perkin-Elmer Lambda Bio 2.3 UV/VIS spectrometer. The absorbance at 750 nm is used to assess turbidity in the sample and was subtracted from all absorbance values at other wavelengths before concentration calculation. Concentrations (mg/L) of each pigment were calculated by the following equations (Lichtenthaler 1987; USEPA September, 1997) and then converted to $\mu g/cm^2$.

Chla = 11.85 (A664) - 1.54 (A647) - 0.08 (A630)

Chlb = 21.03 (A647) - 5.43 (A664) - 2.66 (A630)

Chlc = 24.52 (A630) - 7.60 (A647) - 1.67 (A664)

Total Chl = 4.75 (A664) + 11.9 (A647) + 21.8 (A630)

Carotenoids (xanthophylls+β-carotene) = [1000 (A470) - 1.82 (Chla) - 85.02 (Chlb)] /198

Chilling requirement and dormancy measurement

The chilling temperatures required to overcome bud dormancy can be experimentally quantified by measuring the time required for at least 50% of the plants resume growth in permissive conditions after exposure to a given amount of chilling temperature. Plants were treated with SD photoperiods for 8 weeks at 18°C followed by an additional 10 weeks of SD at 10°C (day) and 4°C (night). After 8, 10.3, 12.4, 14.7, 17 weeks of SD treatment with 0, 256, 496, 752 and 1024 chilling hours, 4–5 plants were defoliated and moved from the experimental SD and temperature condition to either a LD controlled environment chamber at 18°C or a LD greenhouse that ranged from 18–36°C. At regular intervals (3 times per week) plants were observed for apical or axillary bud break.

Histology and light microscopy

All tissues were fixed in FAA, dehydrated and infiltrated with ethanol, xylene and paraffin, and embedded in paraffin (Kelly et al. 1995; Ruzin 1999; Sass 1958). Every step was extended longer to make sure each solution had penetrated through tightly encased bud scales. All freshly dissected shoot apices (or apical buds) from the same line were placed in 10 mL FAA (3.7% formaldehyde, 5% glacial acetic acid, 50% ethanol) and fixed for 3 hours under light vacuum with occasional swirling. Tissues were returned to 1 atm for 10 minutes at the beginning of each hour of the vacuum cycle to let air bubbles encased inside the bud dissolve. All tissues were kept in FAA and stored in 4°C cold room for at least 2 weeks before proceeding with dehydration and infiltration. Tissues were dehydrated in an ethanol series (50%, 60%, 70%, 80%, 95%, and 100% ethanol (8 hours each)) followed by an ethanol/xylene (0%, 0%, 25%, 50%, 75%, 100%, 100%, and 100% xylene (1–3 hours each)) series. Tissues were infiltrated overnight at room temperature with type 1 paraffin (Richard-Allan Scientific, category no. 8336) in 100% xylene. More type 1 paraffin was added over a 6 hours period until the xylene solution was saturated. The xylene solution was replaced with 1/3, 1/2, and 1/2 equal amount of preheated type 1 paraffin every 2 hours and then kept in 58°C incubator overnight. Type 1 paraffin was replaced with preheated type 1 paraffin

twice and then incubated at 58°C overnight. Type 1/type 6 paraffin series (25%, 50, 75%, and 100%) was used over a two-day period to replace type 1 with type 6 paraffin followed by replacing with 100% type 6 paraffin twice to make sure no type 1 paraffin remained. All tissues were embedded in type 6 paraffin. Serial longitudinal and cross sections (~8–10 μm) of each line at each sampling point were sectioned from the blocks using a Leica rotary microtome. Sections were mounted onto Superfrost ProbeOn Plus (Fisher) slides, air dried in room temperature, then air dried on a 40°C slide warmer overnight.

Selected sections were stained with 0.05% toluidine blue O in 0.2 M sodium phosphate (pH ~9.15) for 4 minutes (Sakai 1973). A Nikon BIOPHOT microscope equipped with polarization optics was used to examine the sections. Images of selected stained sections were taken with a digital camera (C5050 Zoom, Olympus America Inc., Center Valley, PA, USA) that was attached to the microscope with a universal digital camera microscope coupler (Edmund Optics, Barrington, NJ, USA).

Statistical analysis

SPSS 13.0 was applied for all statistic analyses. The univariate analysis of variance for two factors (two-way ANOVA) of GLM (general linear model) was used to test (1) the effects of SD, constructs, and the interaction of SD and construct (SD x Construct) on growth cessation, (2) the effects of chilling hours, constructs, and interaction of chilling hours and construct (chilling hours x construct) on leaf senescence, and (3) the effects of chilling hours, constructs, and interaction of chilling hours and construct (chilling hours x construct) on dormancy status. GLM univariate analysis can test both balanced and unbalanced models. Equality and homogeneity of variances were checked by Levene's test. The null hypotheses are SD, chilling hours, lines, and constructs have no effect on growth cessation, leaf senescence and dormancy status. Multiple comparisons of means among lines within same construct and among construct were carried out by one-way ANOVA Post Hoc tests with Tukey HSD, Scheffe, Tamhane's T2 and Games-Howell methods because the assumption of equal variance was sometimes unsatisfied.

Chapter 4: Analysis of the alternatively spliced transcripts of the poplar *FLC-like 2* gene (*PtFLC2*)

Abstract

Six putative poplar *FLC-like* genes (*PtFLC1–PtFLC6*) were identified in the poplar genome. One of these genes *PtFLC2* is expressed in shoot apices of LD grown poplars and transcript abundance declines in vegetative buds during cold temperature exposure. This decline appeared to be associated with cold-mediated dormancy release which is similar to the down-regulation of *FLC* during vernalization in *Arabidopsis*. The *PtFLC2* transcript is highly alternatively spliced and at least 13 splice variants have been identified. The expression of some of specific splice variants is associated with the later stages of bud dormancy.

Sequence analysis of 2 *PtFLC2* splice variants, *PtFLC2as1* and *PtFLC2as2*, predicts that they encode proteins with complete MADS-domain and C-terminal. In addition PtFLC2as1 consists of also I and K domains whereas PtFLC2as2 only has a partial K domain. Poplars transformed with either of these splice variants and expressed from the constitutive 35S promoter showed altered bud development and dormancy. Constitutive ectopic expression of *PtFLC2as1* and *PtFLC2as2* in late-flowering *Arabidopsis* FRI-Sf2 results in early flowering while three different splice variants (*PtFLC2as3, PtFLC2as4,* and *PtFLC2as6a*) tested had no effect on flowering time. These two alternatively spliced transcripts may encode proteins that act as dominant negative repressors by competing with DNA-binding, dimerization, or the formation of protein complexes, thereby reducing FLC repression and initiating early flowering, or they may by themselves induce flowering. These results suggest that *PtFLC2* has diverged significantly from *Arabidopsis FLC*. These results also hint at a possible mechanism for manipulating flowering.

Constitutive overexpression of *PtFLC2as1* and *PtFLC2as2* in poplar altered the response to photoperiod and cold resulting in changes in sylleptic bud growth, growth cessation, bud formation, dormancy, chilling requirement and leaf senescence. These data

suggest that *PtFLC2* plays diverse roles in poplar and may promote active growth in long days, inhibit the short day photoperiod induced growth cessation, and promote dormancy release. Therefore, *PtFLC2* is an important component in the regulatory pathways of short day photoperiod induced bud formation and cold-mediated bud dormancy release in poplar.

Introduction

Most eukaryotic genes contain one or more introns that are spliced out from pre-mRNA by spliceosomes to produce mRNA that is translated into protein. Plants tend to have smaller genes with fewer exons and introns per gene and the introns are typically smaller with a higher AT content compared to human genes (Reddy 2007).

Four types of short conserved sequences within the pre-mRNA are involved in the specificity of splicing mediated by spliceosomes (Figure 1-4). They include the splice donor at the 5' site of the intron consisting of GU dinucleotide flanked by few purines (Pu, i.e. A or G) that is recognized by U1 small nuclear ribonucleoprotein particle (snRNP); the splice acceptor at the 3'site of the intron consisting of AG dinucleotide that is recognized by U2 auxillary factor 35 (U2AF35); the branch site UACUAAC in yeast and vertebrates and CURAY in plant (R is purines (A, G) and Y is pyrimidine (C, U)) located within the intron about 30 nucleotides upstream of the polypyrimidine tract and is recognized by U2 snRNP; the polypyrimidine tract (12–14 pyrimidines (Py, i.e. C or U)) or several U-rich sequences between the 3' splice site and branch site which is recognized by U2AF65 (Hartwell et al. 2004; Reddy 2007; Simpson et al. 2002). Splicing is catalyzed by the spliceosome which consists of 5 small nuclear RNAs (snRNAs) and nearly 300 proteins (Reddy 2007).

Variable splicing patterns produce multiple transcripts from a single gene and can increase protein diversity. Alternative splicing of pre-mRNA is an important post-transcriptional regulatory mechanism that affects mRNA stability and increases the transcriptome and proteome complexity within and between cells and tissues (Reddy 2007; Wang et al. 2006). Six types of alternative splicing (AS) have been observed, including exon skipping, alternative donor site, alternative acceptor, alternative position, intron retention, and

mutually exclusive exons (Black 2003; Hartwell et al. 2004; Reddy 2007; Wang et al. 2006) (Figure 1-4). Most AS events are mutually independent and it has been estimated that 67% of *Arabidopsis* and 60% of rice alternatively spliced genes involve a single AS event while only 10.6% of *Arabidopsis* and 7.4% of rice AS involved multiple events within the same transcript (Wang et al. 2006). Based on EST and cDNA comparisons it has been suggested that 35–60% of human genes are alternatively spliced while microarray data indicates as many as 70% (Reddy 2007; Wang et al. 2006). The majority of alternative splicing in human genes involves exon skipping (58%) while only about 5% involve intron retention (Reddy 2007; Wang et al. 2006). In the Plant Gene and Alternatively Splicing variant Annotator (PGAA) studying of 7 plants (rice, wheat, maize, barley, sorghum, soybean, and *Arabidopsis*), 36.9% genes were estimated to undergo alternatively spliced (Chen et al. 2007). From *Arabidopsis* and rice EST/cDNA sequences, it was shown that at least 21.8% (*Arabidopsis*) and 21.2% (rice) of genes have alternatively spliced events, with the majority involving intron retention (56.1% for *Arabidopsis* and 53.5% for rice) while exon skipping (8.1% for *Arabidopsis* and 13.8% for rice) occurred less often. Almost half (41.7%) of the alternatively spliced genes in *Arabidopsis* were also alternatively spliced in rice (Wang et al. 2006). Thus human genes tend to favor the exon definition model while plants favor the intron definition model in splice site recognition, possibly due to the larger intron size in humans compared to plants (Reddy 2007). 35% of the human, 43% of *Arabidopsis*, and 36% of rice AS events alter the reading frame and possibly produce premature termination codons (PTCs) (Wang et al. 2006). PTCs in human genes have been defined as an in-frame stop codon residing >50 bp upstream of the 3' most exon-exon junction (Lewis et al. 2003). PTCs may be the targets of a mRNA surveillance mechanism, nonsense-mediated mRNA decay (NMD) (Wang et al. 2006).

Several observations suggest that splice variants may have biological roles (Reddy 2007). These observations include: (a) AS is predominant in some gene families but not evenly distributed in most intron-containing genes, (b) many AS events occur in genes that encode multidomain proteins, (c) AS in plants is tissue-specific and also regulated by

developmental stages, stresses and hormones, (d) the position of AS introns is conserved among distantly related plant species, (e) many splicing variants with retained introns are not removed by RNA surveillance mechanisms and are recruited for translation, (f) biological functions (photosynthesis, defense response, flowering, and stress response) of AS variants from several genes have been identified, and (g) various biotic (viral and bacterial pathogens) and abiotic stresses (heat, cold, and heavy metals) affect alternative splicing of pre-mRNA in plants. A number of examples of alternative splicing in higher plants include, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase in spinach and *Arabidopsis* (Werneke et al. 1989), diacylglycerol kinase in tomato (Snedden et al. 2000), N resistance gene in tobacco (Dinesh-Kumar et al. 2000; Reddy 2007), chloroplast ascorbate peroxidase in spinach (Yoshimura et al. 1999), flowering time control gene *FCA* in *Arabidopsis* (Macknight et al. 2002; Quesada et al. 2003), stress and hormone regulated conserved family of splicing regulators - serine/argining-rich SR proteins (Palusa et al. 2007), cold stress upregulated nuclear protein and pre-mRNA splicing factor *STABILIZED1* in *Arabidopsis* (Lee et al. 2006), and temperature-sensitive splicing of mutant *apetala3-1* gene in *Arabidopsis* (Sablowski et al. 1998).

Twenty-seven *Arabidopsis* MADS-box genes (i.e. 25% of all *Arabidopsis* MADS-box genes) in the A, AGL15, ANR1, Bs, CD, FLC, E, F, M_{α}, M_{γ}, and M_{δ} classes appear to have alternatively spliced events based on data in three database (Alternative Splicing in Plants (ASIP) (http://www.plantgdb.org/ASIP/), Alternative Splicing and TRanscription Archives (ASTRA) (http://alterna.cbrc.jp/), and National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez)) and are summarized (Table 1-2). In poplar, splicing variants were detected in 11 genes in the PtA, PtANR1, PtAP3, PtCD, PtFLC, and PtSVP classes of 56 poplar type II MADS-box genes (Table 2-2, Figure 2-2). However it is likely that the estimate of alternative splicing in poplar is underestimated due to the lower number of cDNAs/ESTs used to identify splicing variants compared to the number used for *Arabidopsis*.

FLC, a type II MADS-box gene, is a floral repressor which quantitatively represses flowering by repressing expression of the flowering time integrators genes (*SOC1* and *FT*) and *FD* (Boss et al. 2004; Dennis et al. 2007; He et al. 2005; Henderson et al. 2004; Sablowski 2007) (Figure 1-3). The levels of *FLC* mRNA are regulated positively by *FRI* and negatively regulated by vernalization (Michaels et al. 1999) and the autonomous pathway. Several alternatively spliced variants of *FLC* have been reported (Lempe et al. 2005; Werner et al. 2005) that encode a truncated protein without C-terminal or I + K domains. One splicing variant encoding the MADS-domain (exon 1), C-terminal (exon 7), and partial intron 1 and 6 was detected after 15 days of vernalization but not during normal growth temperatures (Caicedo et al. 2004). There is no functional consequence yet ascribed to splice variation among *Arabidopsis FLC*-like genes. *FLC* homologs have been identified in *Brassica* (Schranz et al. 2002) and sugar beet (Reeves et al. 2007) and *BvFL1*, the sugar beet *FLC* homolog, is downregulated by cold. Furthermore, four alternatively spliced variants of *BvFL1* delay flowering in transgenic *Arabidopsis* null mutant *flc-3* (Reeves et al. 2007).

There are five homologs of *FLC* in *Arabidopsis,* with *MAF1–4 (MADS AFFECTING FLOWERING1–4*) acting as flower repressors while *MAF5* is upregulated during vernalization (Ratcliffe et al. 2003; Ratcliffe et al. 2001). Overexpression of any of the *MAF* genes in *Arabidopsis* delays flowering (Ratcliffe et al. 2003; Ratcliffe et al. 2001; Scortecci et al. 2001). *MAF1–MAF5* all produce alternatively spliced transcripts with three reported for *MAF1/AGL27/FLM* giving predicted peptides of 196, 192, and 173 amino acids, four for *MAF2/AGL31* (196, 145, 145 and 80 amino acids), five for *MAF3/AGL70* (196, 185, 118, 77 and 77 amino acids), five for *MAF4/AGL69* (200, 136, 63, 66, and 69 amino acids), and two for *MAF5/AGl68* (198 and 184 amino acids) (Ratcliffe et al. 2003; Ratcliffe et al. 2001; Scortecci et al. 2001).

Little is known about the functional regulation of transcript splice variants in plant development. Alternative splicing events have been found in all *Arabidopsis FLC* and several poplar *FLC-like* class genes. Their biological significance, if any, is not understood. In this study *PtFLC2* was selected for further study as to the possible significance of

alternative splicing in the regulation of bud initiation, development, dormancy and the release from dormancy. Since a number of *PtFLC2* splice variants have been identified in *Populus deltoides* x *trichocarpa* clone 545-4183, specific variants were selected and expressed in both *Arabidopsis* LER and FRI-Sf2 ecotypes and *Populus alba* x *tremula* clone 717-1B4. This study examines the role of splicing variants of *PtFLC2* in bud development and cold-mediated dormancy release in poplar and the effect of these poplar splice variants on flowering time in *Arabidopsis*.

Results

The *PtFLC2* transcript is alternatively spliced

I identified 13 different alternatively spliced transcripts for *PtFLC2* based on cDNA sequencing (Figure 4-1). *PtFLC2* contains 8 exons designated, E1, E2, E3, E4, E_{AI}, E5, E6, and E7 where E_{AI} represents an alternative internal exon that is not present in the full length transcript but is used in some of the splice variants. Exons 1, 4, 5, 6, and 7 are present in all transcripts that were sequenced. The splicing variants involved exon skipping, alternative donor and acceptor sites, and intron retention. The majority of the splicing variants involved exon skipping or intron retention. The *PtFLC2as1* transcript contains 7 exons (E1, E2, E3, E4, E5, E6, and E7) and lacks E_{AI} while encoding for a 197 amino acid protein that consists of all four domains (MADS-box, I, K, and C-terminal). This PtFLC2as1 protein is 47.7% identical and 32% similar (PAM250) to the *Arabidopsis* FLC protein. *PtFLC2as2* transcripts consist of 5 exons (E1, E4, E5, E6, and E7) and splices out E2, E3 and E_{AI}. *PtFLC2as2* would encode a 154 amino acid protein with complete MADS-box domain and C-terminal, but with a partial K domain and lacking an I domain. Both *PtFLC2as1* and *PtFLC2as2* transcripts have the in-frame stop codon TAG at the same location in exon 7. *PtFLC2as3, as4, as5, as6a, as6b*, and *as7* all include E_{AI} which contains an early TAG stop codon in E_{AI} for *PtFLC2as3* and *PtFLC2as6a*. An earlier TGA stop codon in E4 results from an alternative acceptor site in E3 in *PtFLC2as4* and from E2 skipping combined with the alternative acceptor site in E3 for *PtFLC2as5*. Intron 4 retention produces an early TAA stop codon in

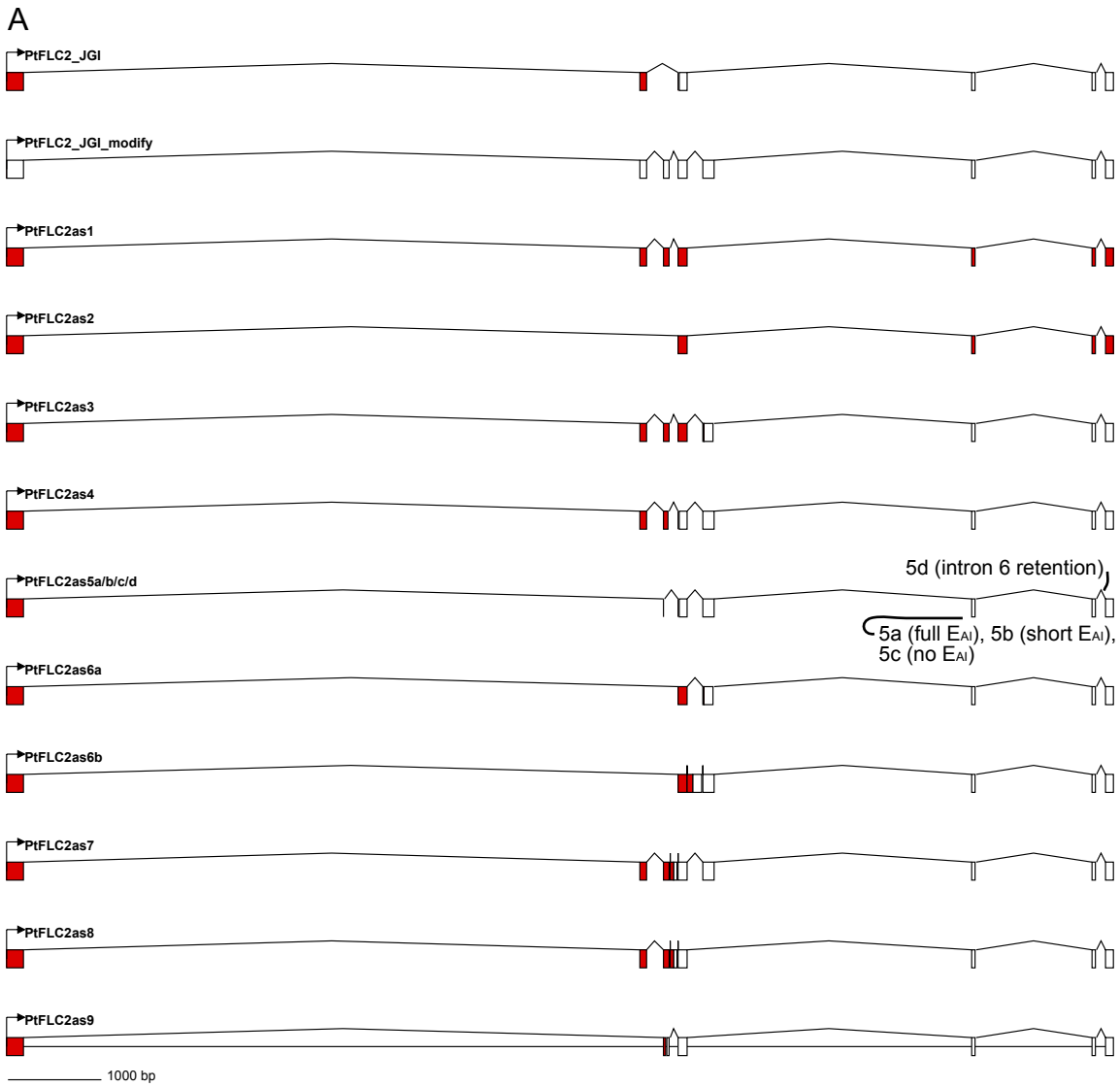


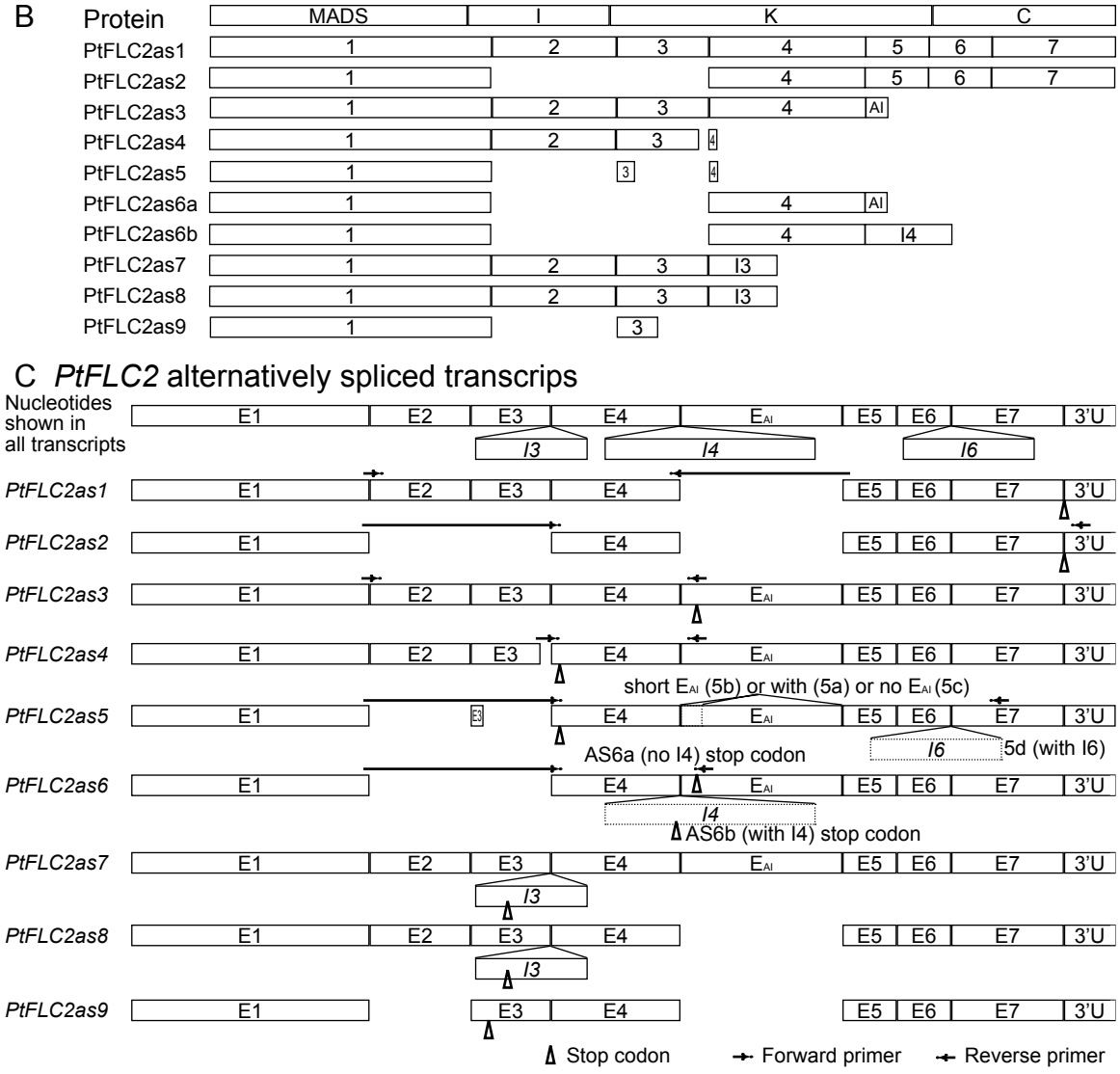
Figure 4-1. Intron-exon structure and translated proteins of *PtFLC2* alternatively spliced variants.

(A) *PtFLC2* genes. Coding exons are represented as red filled boxes, non-coding exons due to early stop codon are open boxes, introns by solid lines, and splicing events are represented by angled lines. PtFLC2_JGI is the gene model predicted in JGI poplar database which has stop codon in the beginning of exon 4 due to lack of exon 3. PtFLC2_JGI_modify is a modified intron-exon border including all 8 exons found in cloned sequencing results. Others are alternatively spliced variants verified by cDNA cloning and DNA sequencing.

(B) Protein and MADS, I, K, and C-terminal domain structure of *PtFLC2* alternatively spliced variants.

(C) Primer design for detecting *PtFLC2* alternatively spliced variants. Arrowhead toward right is forward primer and toward left is reverse primer. *PtFLC2as5* has 4 variants (shorter EAI, with or without EAI, with or without I6). *PtFLC2as6* has 2 variants (with or without I4).

(D) Sequence alignment of *PtFLC2* alternatively spliced variants. There is no full-length sequenced result for *PtFLC2as4, as5*, and *as6*. Those nucleotides with lower case in *PtFLC2as4, as5*, and as*6* are based on the sequenced results of *PtFLC2as1, as2*, and *as3*. RNA source is *Populus deltoides* x *trichocarpa* clone 545-4183 for *PtFLC2as4–as6* and *Populus trichocarpa* Nisqually-1 for *PtFLC2as1–3* and *PtFLC2as7–9*.



Transcript	PrimerID	Primer Sequence	Length	Ta (°C)
PtFLCas1	AS1F	TTCTCTAGCGTTGGCAGTAC	274	62
	AS1R	CCAACATCATTTGTATCTTTCTA		
PtFLCas2	AS2F	CTCTAGCGTTGGCAGGAACCTTG	337	64
	AS2R	GTGCCATTATAGCCGCCTCCAG		
PtFLCas3	AS3F	CTCTAGCGTTGGCAGTACAACCAG	287	64
	AS3R	GGAAGAAGACGCCCTAAACCAA		
PtFLCas4	AS4F	TGCAGAGCTACTACTAATGGAACC	149	62
	AS3R	GGAAGAAGACGCCCTAAACCAA		
PtFLCas5abcd	AS5F	GCAGGTATATTATGGAACCTTGA	378/360/253/480	64
	AS5R	GTGGAGGATGATACAGAGGGTGAT		
PtFLCas6ab	AS6F	TCTCTAGCGTTGGCAGGAACCT	151/314	64
	AS6R	GTATGGAAGAAGACGCCCTAAGC		

Figure 4-1. Intron-exon structure and translated proteins of *PtFLC2* alternatively spliced variants (continued)

D Sequence alignment of *PtFLC2* alternatively spliced variants

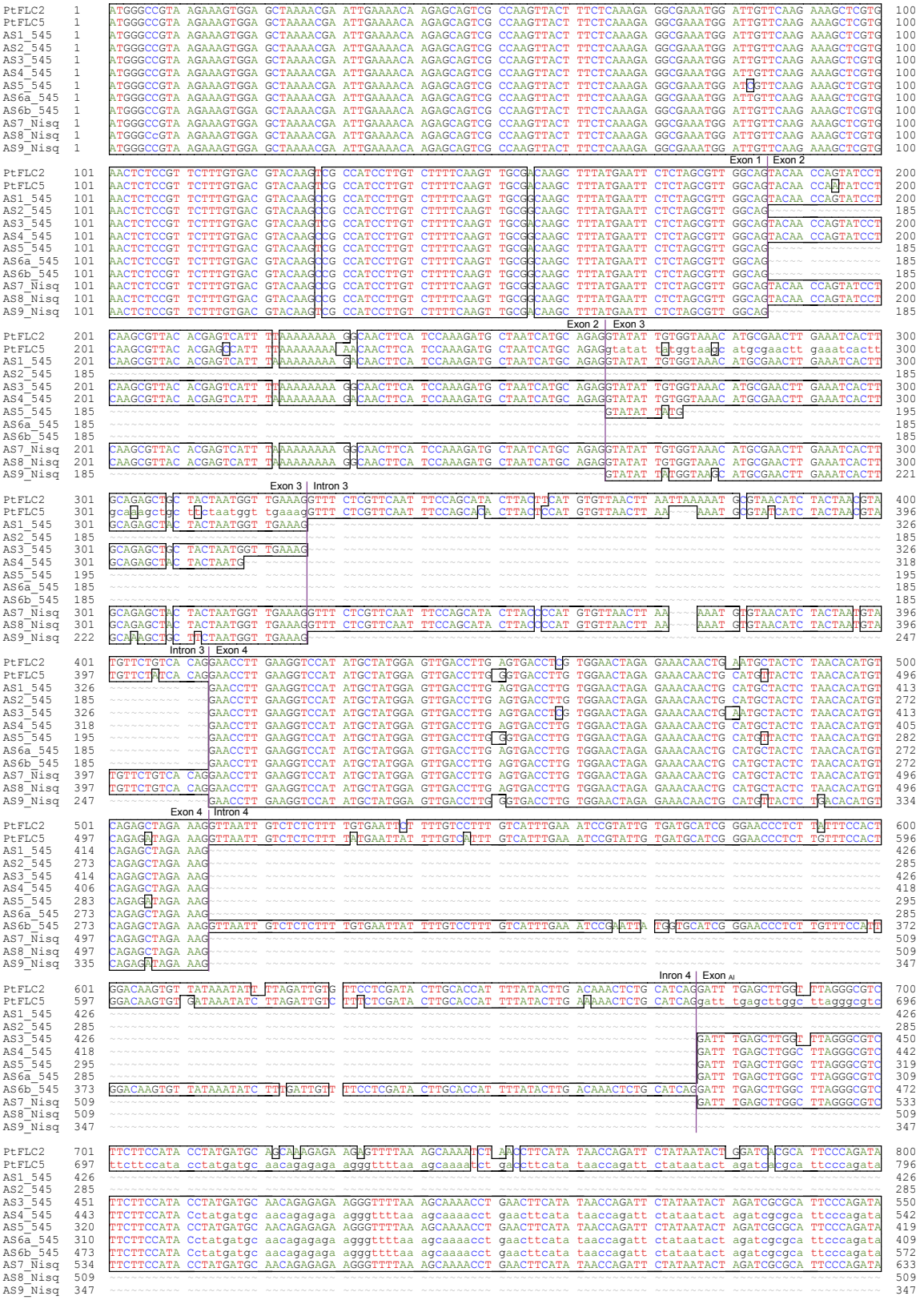


Figure 4-1. Intron-exon structure and translated proteins of *PtFLC2* alternatively spliced variants (continued)

D Sequence alignment of *PtFLC2* alternatively spliced variants (continue)

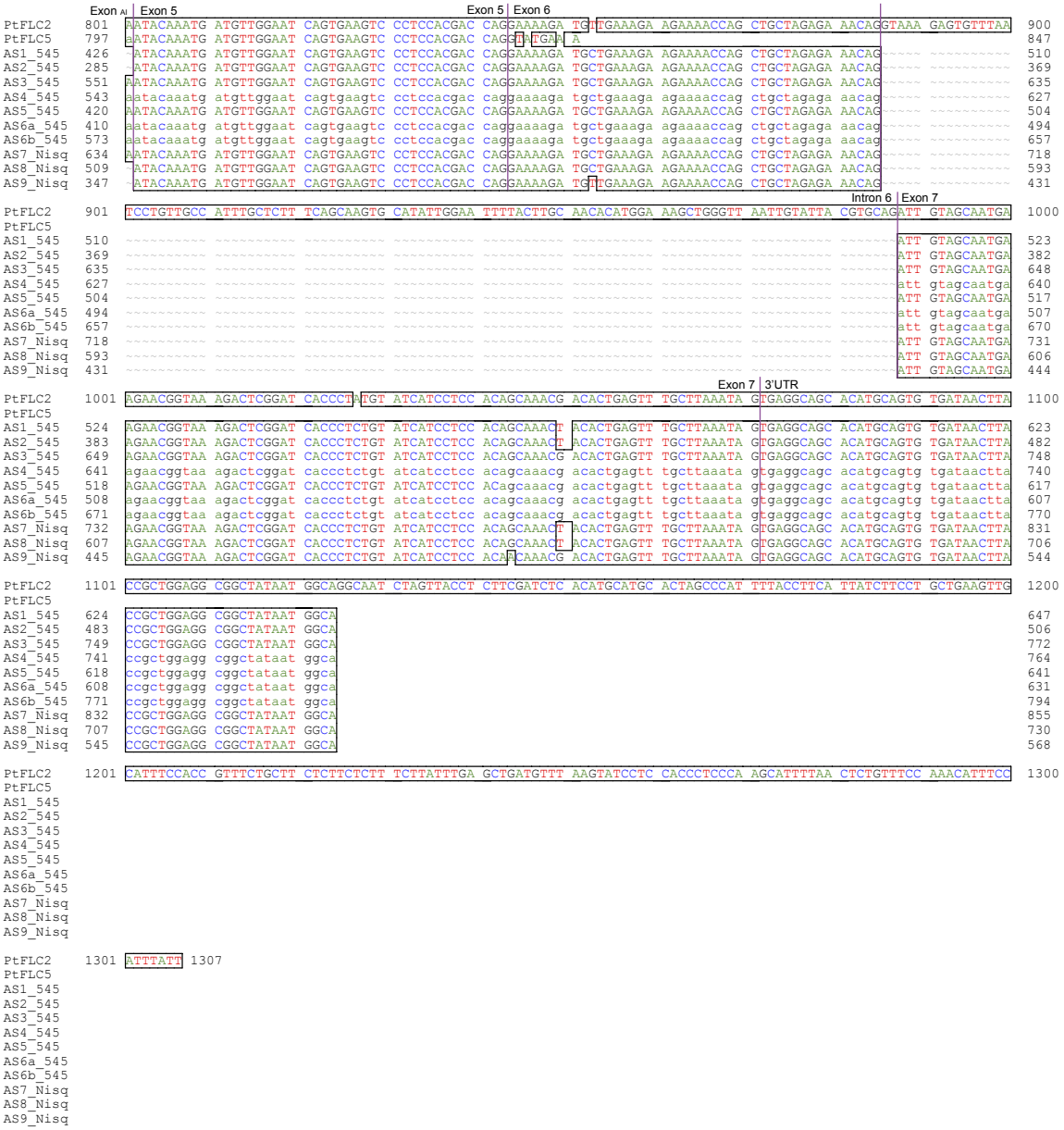


Figure 4-1. Intron-exon structure and translated proteins of *PtFLC2* alternatively spliced variants (continued)

intron 4 for *PtFLC2as6b* while intron 3 retention produces an early TAA stop codon in intron 3 for *PtFLC2as7* and *PtFLC2as8*. Finally, E2 skipping results in early TGA stop codon in E3 for *PtFLC2as9*. The transcript variants *PtFLC2as3–PtFLC2as9* appear to possess premature termination codons (PTCs) or encode a protein with complete MADS-box domains but lacking complete protein-protein interaction domains I and K domains and also lacking the C-terminal (Figure 4-1B).

Expression of *PtFLC2* alternatively spliced variants during bud dormancy

Using RT-PCR with primers designed to either detect *PtFLC2* transcripts (designated *PtFLC2* primer), amplify full-length *PtFLC2* cDNA (designated *PtFLC2asoe* primer), or specific to splice variant *PtFLC2as1* showed that in *Populus deltoides* x *trichocarpa* clone 545-4183 *PtFLC2as1* transcript abundance declines during bud development and dormancy is associated with low temperatures (Figure 4-2A and 4-2B). A similar expression pattern was observed in *Populus alba* x *tremula* clone 717-1B4 and *Populus trichocarpa* Nisqually-1 (Figure 3-2B–E). Several *PtFLC2* alternatively spliced variants (*PtFLC2as2, as3, as4, as5a, as5b, as6a,* and *as6b*) are induced with cold treatment in poplar clone 545-4183 (Figure 4-2A and 4-2B). *Populus deltoides* x *trichocarpa* clone 545-4183 appears to either produce more *PtFLC2* splice variants or express them at a greater level compared to *Populus alba* x *tremula* clone 717-1B4 and *Populus trichocarpa* Nisqually-1 (Figures 4-2C and 4-2D).

Interestingly, level of the *PtFL2Cas2* splice variant was upregulated in transgenic poplar that overexpressing the *PtFLC2as1* transcript (35S::*PtFLC2as1*) (Figure 4-2C). Furthermore, expression of the other *PtFLC2* splice variants detected in the transgenic plants (*PtFLCas3, 4, 6a, 6b)* did not differ from wildtype plants. DNA sequence analysis of the cloned *PtFLCas2* transcript from these transgenic poplars revealed that the splice variant originated from the transgene as opposed to the endogenous *PtFLC2* transcript.

PtFLC2as1 and *PtFLC2as2* induce early flowering in *Arabidopsis* FRI-Sf2

To determine if *PtFLC2* alternatively spliced transcripts affect flowering time in *Arabidopsis*, early and late flowering *Arabidopsis* ecotypes LER and FRI-Sf2 were transformed with 35S::*PtFLC2as1,* 35S::*PtFLC2as2,* 35S::*PtFLC2as3,* 35S::*PtFLC2as4,*

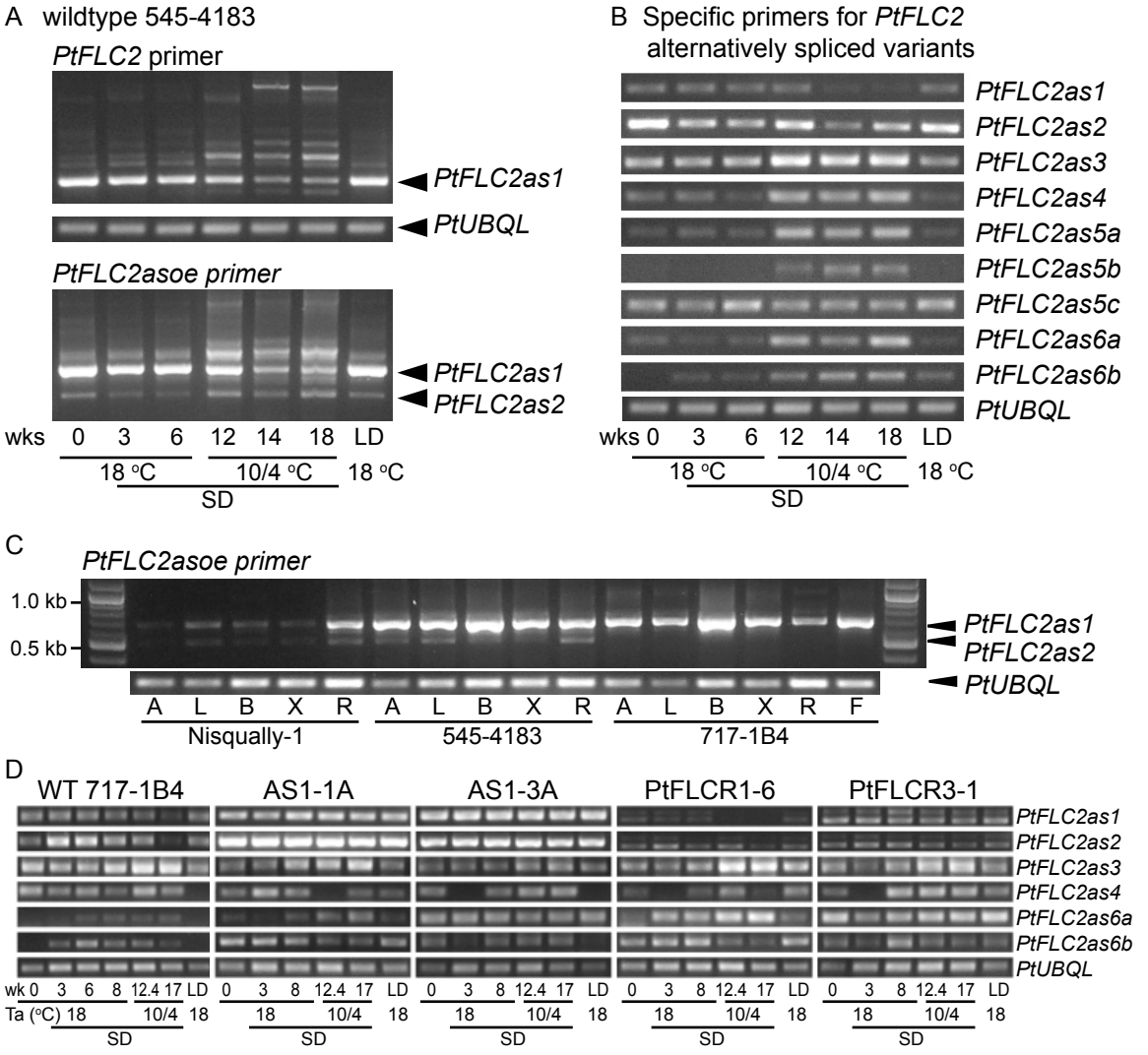


Figure 4-2. Expression of *PtFLC2* alternatively spliced variants in wildtype and *PtFLC2* transgenic poplars.

(A) Expression of *PtFLC2* with *PtFLC2* and *PtFLC2asoe* primers in *Populus deltoides* x *trichocarpa* clone 545-4183.

(B) Expression of *PtFLC2* alternatively spliced variants.

RT-PCR was used to assay *PtFLC2* expression with specific primers to *PtFLC2as1–PtFLC2as6* in *Populus deltoides* x *trichocarpa* clone 545-4183. Locations of primers are shown in Figure 4-1C.

(C) Expression of *PtFLC2* alternatively spliced variants in different tissues of 3 different poplar genotypes. The genotypes are *Populus deltoides* x *trichocarpa* clone 545-4183, *Populus alba x tremula* clone 717-1B4 and *Populus trichocarpa* Nisqually-1. A = shoot apex (LD), L = leaf (LD), B = bark (LD), X = xylem (LD), R = root (LD), and F = floral bud (March 2007, outdoor in UMCP Greenhouse complex).

(D) Expression of *PtFLC2* alternatively spliced variants in *Populus alba* x *tremula* clone 717-1B4 and transgenic poplars. RT-PCR was used to assay *PtFLC2* expression with specific primers to *PtFLCas1,2,3,4,6a,6b*. Locations of primers are shown in Figure 4-1C.

and 35S::*PtFLC2as6a* constructs. No differences were observed in flowering times in the T1 generation of the LER background between 12 wildtype, 12 35S::*PtFLC2as1*, 44 35S::*PtFLC2as2*, 10 35S::*PtFLC2as3*, 43 35S::*PtFLC2as4*, and 36 35S::*PtFLC2as6a* lines under LD 20°C environment (Figure 4-3B).

In the T1 generation of the *Arabidopsis* FRI-Sf2 background, there are 18 independent lines transformed with 35S::*PtFLC2as1*, 24 lines transformed with 35S::*PtFLC2as2*, 24 lines transformed with 35S::*PtFLC2as3*, 24 lines transformed with 35S::*PtFLC2as4*, 24 lines transformed with 35S::*PtFLC2as6a,* and 30 lines transformed with 35S::*PtFLC2R* Unexpectedly, several 35S::*PtFLC2as1,* 35S::*PtFLC2as2*, and 35S::*PtFLC2as6a* lines flowered significantly earlier than wildtype under LD at 20°C (Figure 4-3). Some of the 35S::*PtFLC2as1* and 35S::*PtFLC2as2* lines flowered 20–70% earlier (21.3–30.3 leaves compared to 59.7 leaves and 24.9–38.4 days to bolt compared to 59.2 days) than required for wildtype *Arabidopsis* FRI-Sf2). Few 35S::*PtFLC2as6a* lines flowered ~40% earlier than wildtype. Few 35S::*PtFLC2as3* and 35S::*PtFLC2as4* lines flowered ~30% earlier than wildtype.

Segregation analysis of five early flowering 35S::*PtFLC2as1* transformed lines (AS1-2, AS1-3, AS1-6, AS1-7, and AS1-8), 5 35S::*PtFLC2as2* transformed lines (AS2-2, AS2-7, AS2-14, AS2-15, and AS2-17) and 4 35S::*PtFLC2as6a* transformed lines (AS6a-4, AS6a-5, AS6a-10, and AS6a-16) used 48 seedlings per line that were randomly picked 5 days after seed germination. The plants were grown in LD 20°C and the number of leaves and days to bolt were recorded every other day. Hierarchical, K-means, and two-step cluster analyses grouped 48 plants into 2 clusters (early vs. late flowering) for each 35S::*PtFLC2as1* and 35S::*PtFLC2as2* line using either number of days or number of leaves as the variable (Figure 4-4, Table 4-1). Binomial and Chi-square tests of a 3:1 segregation ratio indicated a single locus for early vs. late flowering in 35S::*PtFLC2as1* and 35S::*PtFLC2as2* lines (Table 4-1). In 4 AS6a lines, only AS6a-5 showed a 3:1 segregation and AS6a-10 was clustered into 3 groups. The average number of T-DNA insertions per line is ~1.5 in *Agrobacteria* transformation of *Arabidopsis* (Alonso et al. 2003; McElver et al. 2001), so it is possible that

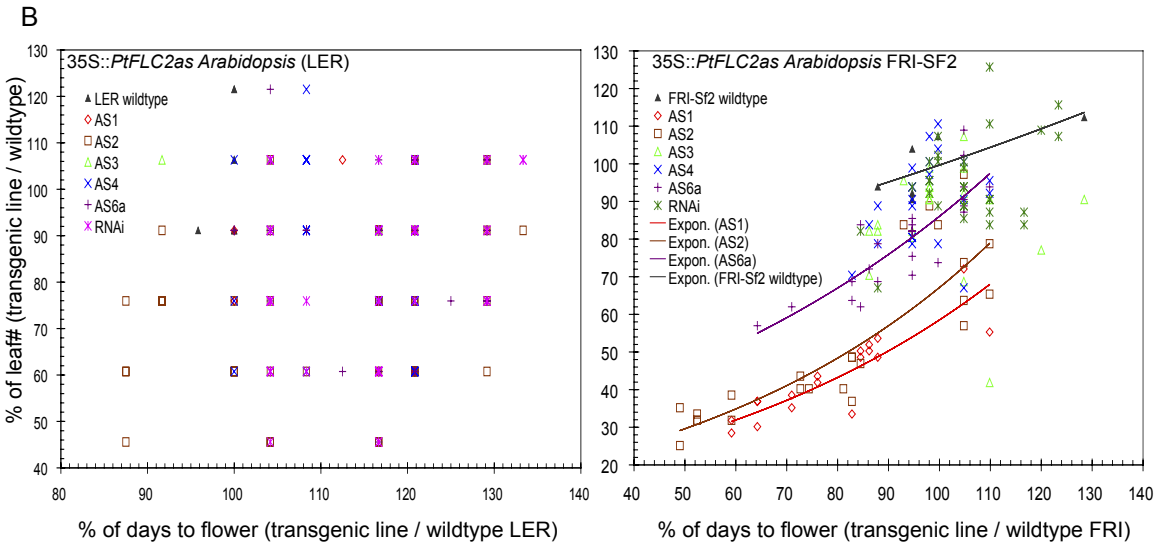


Figure 4-3. Flowering in the *Arabidopsis* late flowering ecotype FRI-Sf2 transformed with *PtFLC2* alternatively spliced variant.
(A) Examples of flowering in 35S::*PtFLC2as1–PtFLC2as6a* (AS1-AS6a) and *PtFLC2*-RNAi transformed *Arabidopsis* (FRI-Sf2) T1 generation along with wildtype *Arabidopsis* (FRI-Sf2) after 59 days LD growth.
(B) Distribution of flowering time of all T1 plants and wildtypes (LER and FRI-Sf2) with leaf# as y-axis and days to flower as x-axis. The mean of wildtype is set as 100% of leaf# and 100% of days to bolting. LER is 24 days and 6.58 leaves. FRI-Sf2 is 59.2 days and 59.7 leaves.

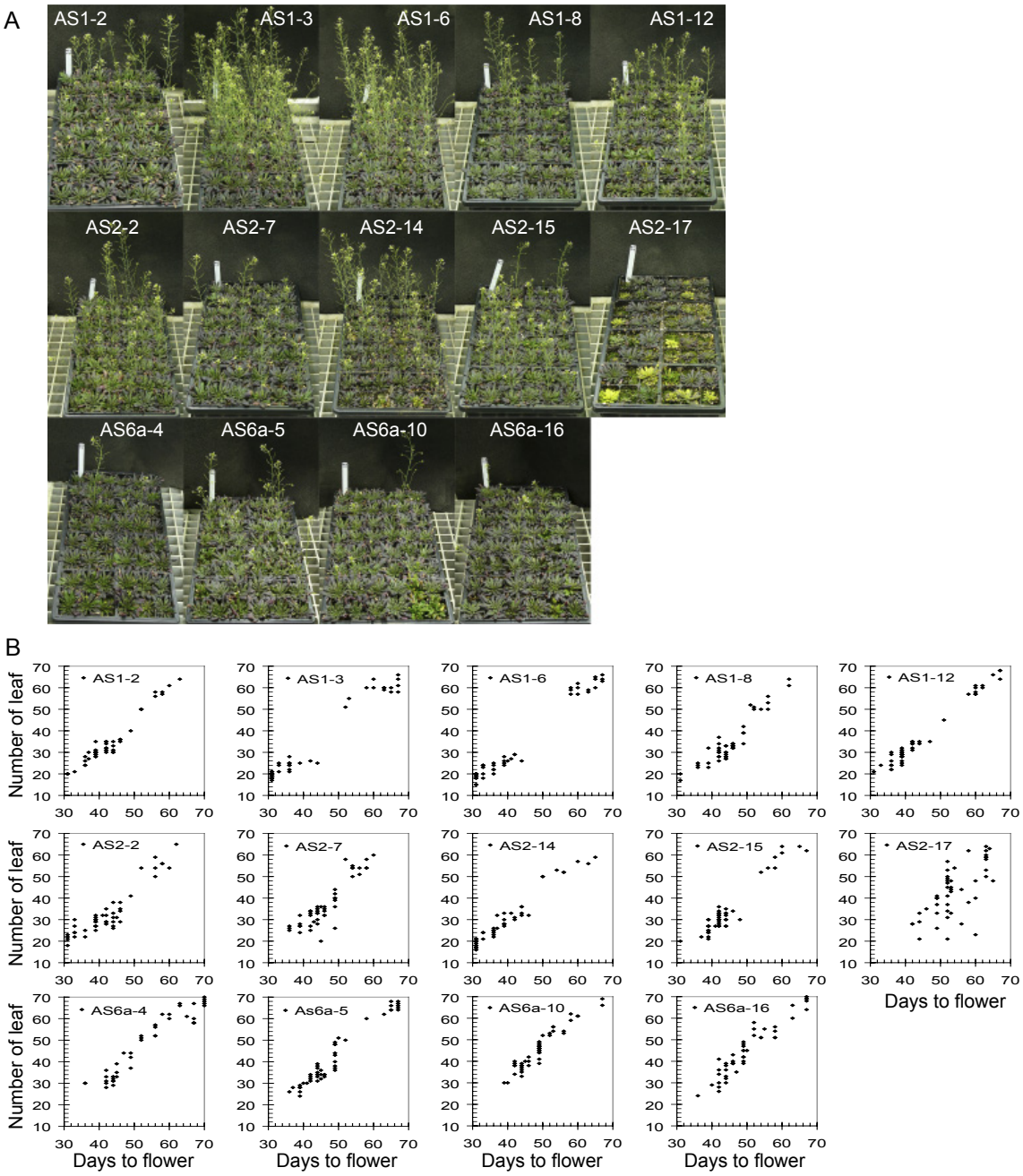


Figure 4-4. Analysis of flowering time in *Arabidopsis* late flowering ecotype FRI-Sf2 transformed with *PtFLC2* alternatively spliced transcripts.
(A) Examples of flowering of five 35S::*PtFLC2as1* (AS1), five 35S::*PtFLC2as2* (AS2) and four 35S::*PtFLC2as6a* (AS6a) transformed *Arabidopsis* (FRI-Sf2) T2 generations after 51 days LD growth.
(B) Distribution of flowering time of 48 T2 plants per line with leaf# as y-axis and days to flower as x-axis.

Table 4-1. Cluster analysis and one-way ANOVA multiple mean comparison of flowering time in *Arabidopsis* (FRI-Sf2) transformed with *PtFLC2* alternative splice variants
(A) Cluster analysis with H0: 3:1 ratio (cluster 1 : cluster 2 = early flowering phenotype : wildtype late flowering phenotype) of five 35S::*PtFLC2as1*, five 35S::*PtFLC2as2* lines, and 4 35S::*PtFLC2as6a* lines.
(B) Multiple comparison of mean among plants transformed with 3 constructs and *Arabidopsis* FRI-SF2. Homogeneity of variance test (Levene statistic) is significant. Conservative Tamhane's T2 and liberal Games-Howell were used because the assumption of equal variance is rejected.

A

Construct	Line	# of plants per cluster		H0: Proportion of Cluster1 = 0.75 Binomial	Chi-square	
		cluster 1	cluster 2	Asymp. Sig.(1-tailed)		Conclusion
PtFLC2as1	2	39	9	0.205	0.317	unable to reject H0
	3	34	14	0.301	0.505	unable to reject H0
	6	34	14	0.301	0.444	unable to reject H0
	8	39	9	0.205	0.317	unable to reject H0
	12	35	13	0.423	0.739	unable to reject H0
PtFLC2as2	2	40	8	0.119	0.182	unable to reject H0
	7	37	11	0.445	0.739	unable to reject H0
	14	40	8	0.119	0.182	unable to reject H0
	15	40	8	0.119	0.182	unable to reject H0
	17	36	12	0.555	1.000	unable to reject H0
PtFLC2as6a	4	20	28	0.008	0.008	reject H0
	5	37	11	0.445	0.739	unable to reject H0
	10	cluster1=19, 2=23, 3=6		N/A	N/A	N/A
	16	30	18	0.037	0.046	reject H0

B

Construct	lines	plant number	mean	Std. Error	Tamhane's T2	Games-Howell
Multiple mean comparison of cluster 1 among 3 constructs and Arabidopsis FRI-SF2 wildtype						
			Leaf# variable			
PtFLC2as1	2, 3, 6, 8, 12	181	26.73	0.429	a	a
PtFLC2as2	2, 7, 14, 15, 17	193	30.40	0.541	b	b
PtFLC2as6a	5, 16	67	36.51	0.845	c	c
wildtype	FRI-SF2	17	60.00	1.144	d	d
			Days variable			
PtFLC2as1	2, 3, 6, 8, 12	181	38.11	0.396	a	a
PtFLC2as2	2, 7, 14, 15, 17	193	42.11	0.470	b	b
PtFLC2as6a	5, 16	67	44.79	0.469	c	c
wildtype	FRI-SF2	17	57.41	1.969	d	d
Multiple mean comparison of cluster 2 among 3 constructs and *Arabidopsis* FRI-SF2 wildtype						
			Leaf# variable			
PtFLC2as1	2, 3, 6, 8, 12	59	58.69	0.668	b	b
PtFLC2as2	2, 7, 14, 15, 17	49	56.06	0.675	a	a
PtFLC2as6a	5, 16	29	61.55	1.240	b	b
wildtype	FRI-SF2	17	60.00	1.144	b	b
			Days variable			
PtFLC2as1	2, 3, 6, 8, 12	59	60.22	0.650	a	ab
PtFLC2as2	2, 7, 14, 15, 17	49	58.62	0.623	a	a
PtFLC2as6a	5, 16	29	61.90	1.036	a	b
wildtype	FRI-SF2	17	57.41	1.969	a	ab

there is no significant effect on flowering time when overexpressing the *PtFLC2as6a* splicing variant in plants and there might be more than one insertion site, which made it difficult to distinguish them into 2 groups in cluster analysis (Figure 4-4B). Multiple comparisons of means among wildtype plants and those plants transformed with 3 different constructs suggests that overexpression of the *PtFLC2* splicing variant *PtFLC2as1*, *PtFLC2as2*, and *PtFLC2as6a* change flowering time significantly (Table 4-1B). The full-length PtFLC2as1 containing complete 4 domains (MADS, I, K, and C) promotes earliest flowering followed by the PtFLC2as2 with complete 2 domains (MADS and C) and partial K, PtFLC2as6a with MADS and partial K domains, and PtFLC2as3 and PtFLC2as4 with MADS, I, and partial K.

Overexpression of *PtFLC2* splicing variants did not affect RNA levels of genes downstream of *FLC* in *Arabidopsis* FRI-Sf2

Analysis of gene expression by RT-PCR confirmed the expression of *PtFLC2as1, PtFLC2as2*, and *PtFLC2as6a* in *Arabidopsis* FRI-Sf2 (Table 4-2). In addition, RT-PCR analysis shows that *AtFLC* was expressed in the early flowering lines and the expression of *PtFLC2* did not result in co-suppression of *AtFLC*. Examination of the expression of the targets of *AtFLC*, *SOC1, FT,* and the downstream regulatory gene *AP1* revealed that their expression was similar in the early flowering and wildtype lines. Therefore it is not clear at this time how overexpression of *PtFLC2as1* results in early flowering. This question will require further studies with additional sampling points (30 and 40 days after germination) and examination of the expression of other flower related genes such as *AP3, PI*, or *AG* in case PtFLC2as1 acts as an A class gene and activates their expression.

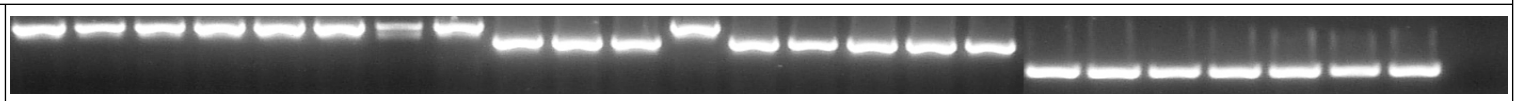
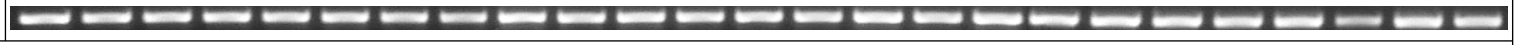
Growth of wildtype and *PtFLC2* splicing variants transgenic poplars

To determine if overexpression of *PtFLC2* alternatively spliced transcripts influenced the growth of plants, experiments were performed in which growth of transgenic and wildtype control poplars was monitored when treated with either LD or SD. Growth cessation during SD treatment was compared between transgenic and wildtype control poplars by determining the number of leaves, plant height, mean leaf increment and plant height increment at weekly intervals (Figure 4-5). The data presented in Figure 4-5 represent

Table 4-2. Ectopic expression of *PtFLC2* alternatively spliced transcripts induced early flowering under long days in transgenic *Arabidopsis* (FRI-Sf2).

RNA samples were collected from leaves of 17 days LD grown plants. *AtUBQ10* is used as internal control.

Means and standard errors of T2 generation in 35S::*PtFLC2as1* and 35S::*PtFLC2as3 Arabidopsis* are based on the values of plants in cluster 1. Means and standard errors of T2 generation in 35S::*PtFLC2as6a Arabidopsis* were calculated from plants with <50 days to bolt due to unable to cluster to 2 groups.

35S::*PtFLC2as1*, 35S::*PtFLC2as2* and 35S::*PtFLC2as6a* transformed *Arabidopsis* late flowering ecotype FRI-Sf2																									
Construct	35S::*PtFLC2as1*								35S::*PtFLC2as2*									35S::*PtFLC2as6a*							
Line	2	3	6	7	8	10	12	18	1	2	7	9	14	15	17	19	21	4	5	7	10	16	17	21	WT
T1 generation																									
Leaf# to bolt	26	19	17	43	18	32	25	31	28	21	19	29	15	26	24	58	47	34	37	43	41	38	54	56	59.7
Days to bolt	45	35	35	62	38	52	45	51	50	29	31	49	29	43	43	62	65	38	42	51	49	49	62	65	59
Gene expression of T2 generation on 17th day after germination																									
PtFLCoeas																									
AtAP1																									
AtFLC																									
AtSOC1																									
AtFT																									
AtUBQ10																									
T2 generation																									
Leaf# to bolt	29.9	21.3	22.0	N/A	30.3	N/A	29.1	N/A	N/A	28.8	32.0	N/A	24.9	28.9	38.4	N/A	N/A	34.0	34.6	N/A	41.1	N/A	37.8	N/A	60.2
Std.Error	0.7	0.5	0.6	N/A	0.8	N/A	0.7	N/A	N/A	0.8	0.9	N/A	0.9	0.6	1.6	N/A	N/A	1.1	1.1	N/A	0.9	N/A	1.2	N/A	1.4
Days to bolt	40.0	33.0	34.6	N/A	42.9	N/A	39.1	N/A	N/A	39.6	43.6	N/A	35.9	41.4	51.1	N/A	N/A	43.8	44.0	N/A	46.0	N/A	45.3	N/A	56.5
Std. Error	4.3	0.6	0.7	N/A	0.7	N/A	0.5	N/A	N/A	0.8	0.6	N/A	0.8	0.4	0.7	N/A	N/A	0.8	0.7	N/A	0.5	N/A	0.6	N/A	2.5
Number of plant	39	34	34	N/A	39	N/A	35	N/A	N/A	40	37	N/A	40	40	36	N/A	N/A	20	35	N/A	34	N/A	30	N/A	11

the means of 40 plants for 35S::*PtFLC2as1* and 35S::*PtFLC2R* (Figure 4-5A, C, E, G) and the means of 10 plants for wildtype, 35S::*PtFLC2as2*, 35S::*PtFLC2as3*, 35S::*PtFLC2as4*, and 35S::*PtFLC2as6a* plants (Figure 4-5B, D, F, H). By comparing the number of weeks required for leaf increment and height increment to approach zero, it is observed that 35S::*PtFLC2as1* plants required 3 additional weeks to cease growth compared to wildtype while 35S::*PtFLC2as2*, 35S::*PtFLC2as3*, 35S::*PtFLC2as4*, and 35S::*PtFLC2as6a* plants ceased growth 0–1 week earlier than wildtype.

Sylleptic branching was observed in all 35S::*PtFLC2as1* transgenic lines (Figure 3-6) and three out of five 35S::*PtFLC2as2* transgenic lines (AS2-2B, AS2-4, and AS2-5) (Figure 4-6A) grown in LD. In contrast, little if any sylleptic branching was observed in other 35S::*PtFLC2as* transgenic lines (AS2-1A, AS2-3A, AS3-3A, AS4-3A, and AS6a-1A) or wildtype plants under the same conditions (Figure 3-5). Unexpectedly, this phenotype was not observed in all 35S::*PtFLC2as1* transgenic lines plant grown either in LD greenhouse or the EGC chambers. The reason for this difference is unclear, however as shown in Figure 3-4, both light intensity and spectral quality differ from the greenhouse, EGC and Conviron chambers. This light difference may be the basis for the different branching phenotypes observed. Reduced leaf size with less serration of leaf margins and shortened shoot elongation under LD and SD growth conditions were observed in AS2-1A poplars (Figure 4-6B), but these phenotypes were not observed in other 35S::*PtFLC2as2* lines (AS2-2B, AS2-3A, AS2-4, and AS2-5). Then it might be due to the T-DNA insertion during *Agrobacteria* transformation has disrupted a gene involved in the regulation of these physiological development.

PtFLC2as1 and *PtFLC2as2* overexpression affects bud formation and chilling requirement in poplar

Vegetative apical buds formation and dormancy were induced under SD for wildtype and all 35S::*PtFLC2as* transgenic poplars (Figure 4-7). Vegetative apical bud initiation was delayed during the first 3 weeks of SD in 35S::*PtFLC2as1* poplars while it was induced in 35S::*PtFLC2as2* poplars when compared to all other transgenic and control poplars (Figure

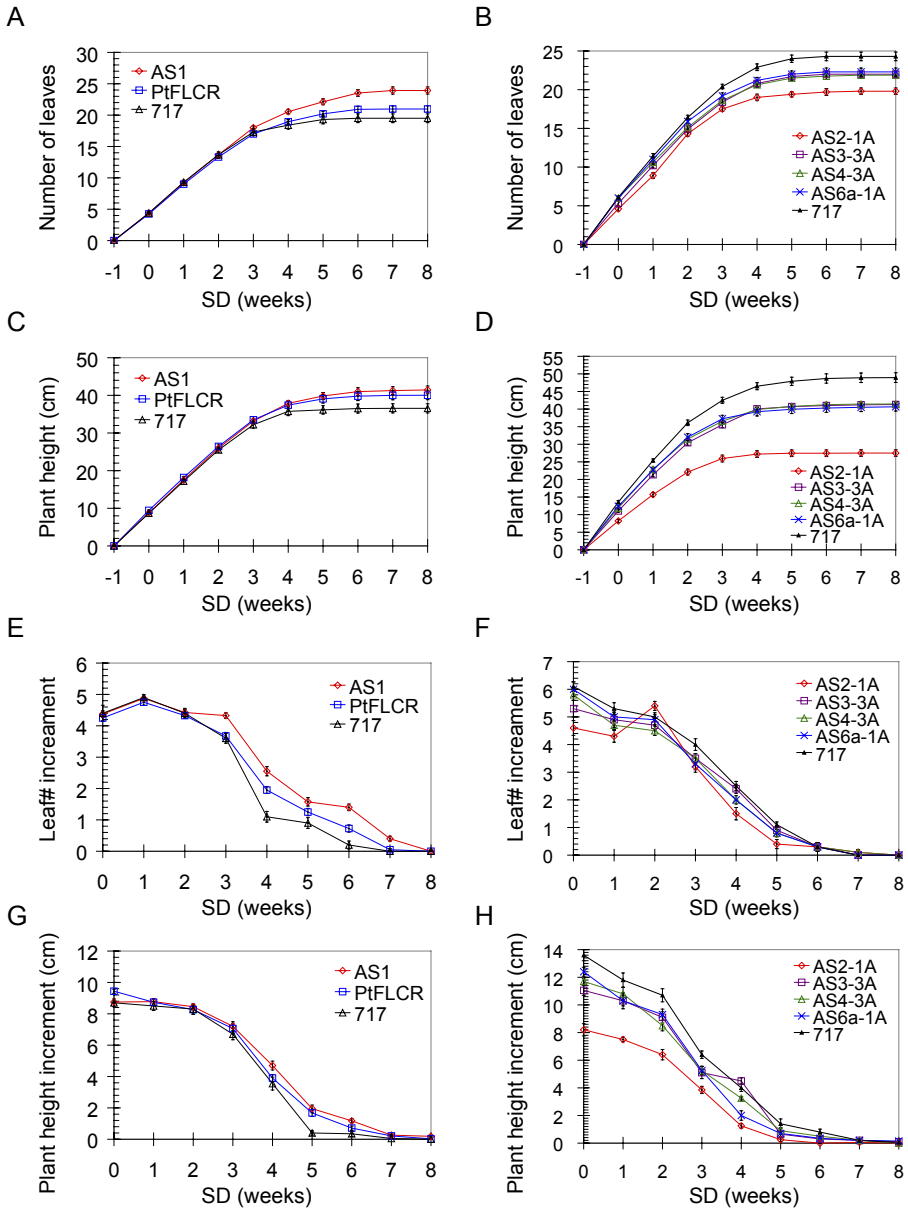


Figure 4-5. Growth cessation of wildtype 717-1B4, AS1, AS2, AS3, AS4, AS6a, and PtFLCR transgenic poplars in SD.
(A, B) Total leaf number
(C, D) Plant height (cm)
(E, F) Leaf number increment
(G, H) Plant height increment
Values given in A, C, E, and G are means+/-StdError of 40 plants with the same construct from 4 different lines. Values given in B, D, F, and H are means+/-StdError of 10 plants from same line. Plants were grown for 5 weeks (A, C, E, G) and 7 weeks (B, D, F, H) under LD after transplanting from tissue culture to potting soil before switching to SD.

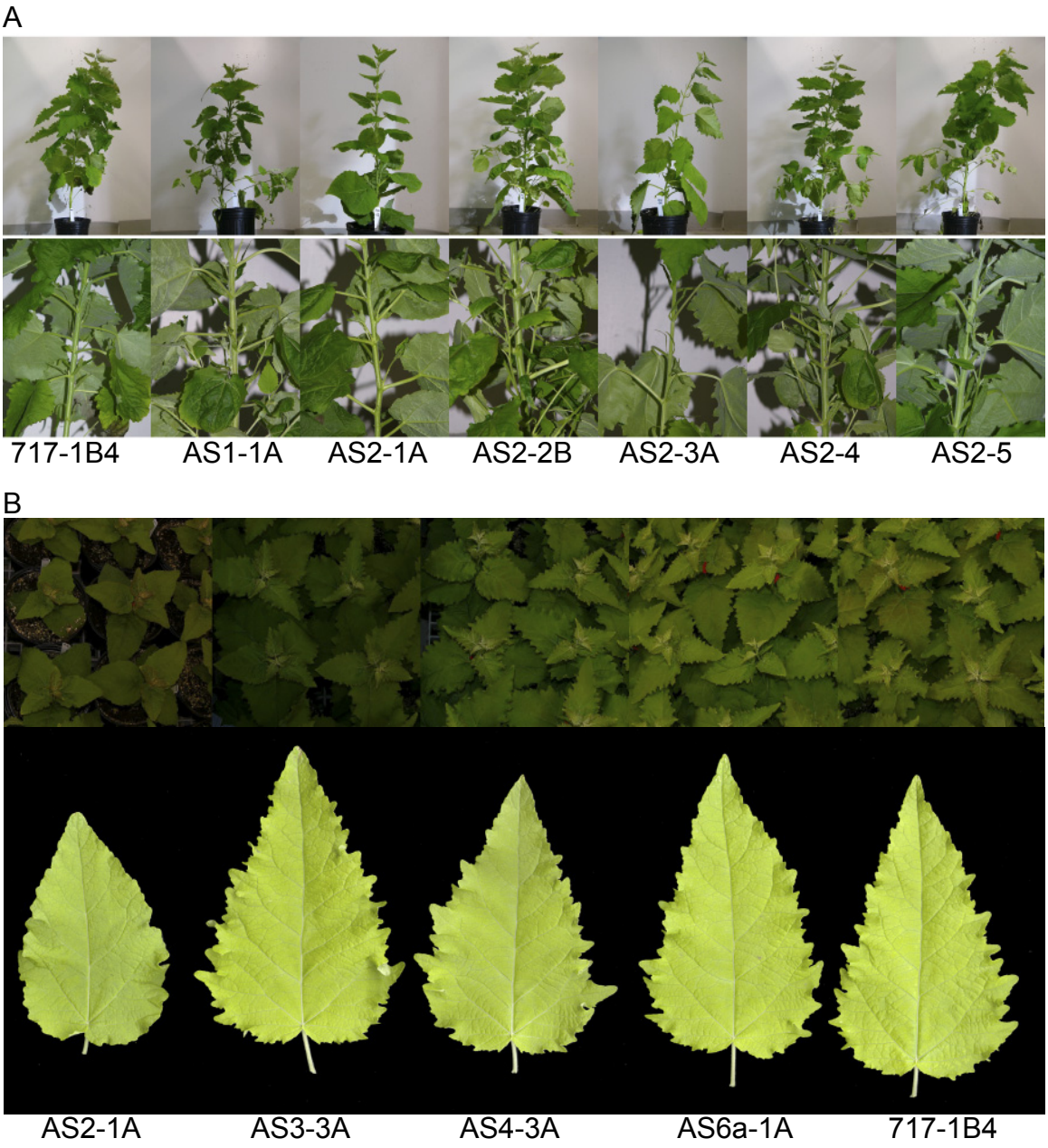


Figure 4-6. Morphology of *Populus alba* x *tremula* clone 717-1B4 and AS1, AS2, AS3, AS4, and AS6a transgenic poplars in LD.
(A) Sylleptic branching of 35S::*PtFLC2as1* (AS1) and 35S::*PtFLC2as2* (AS2) transgenic lines. Top photos are whole plant and bottom photos are closeup view of top photos.
(B) Leaf morphology of wildtype 717-1B4, 35S::*PtFLC2as2*-1A *(*AS2-1A), 35S::*PtFLC2as3*-3A (AS3-3A), 35S::*PtFLC2as4*-3A (AS4-3A), and 35S::*PtFLC2as6a*-1A (AS6a-1A) poplars under both LD and SD growth environments

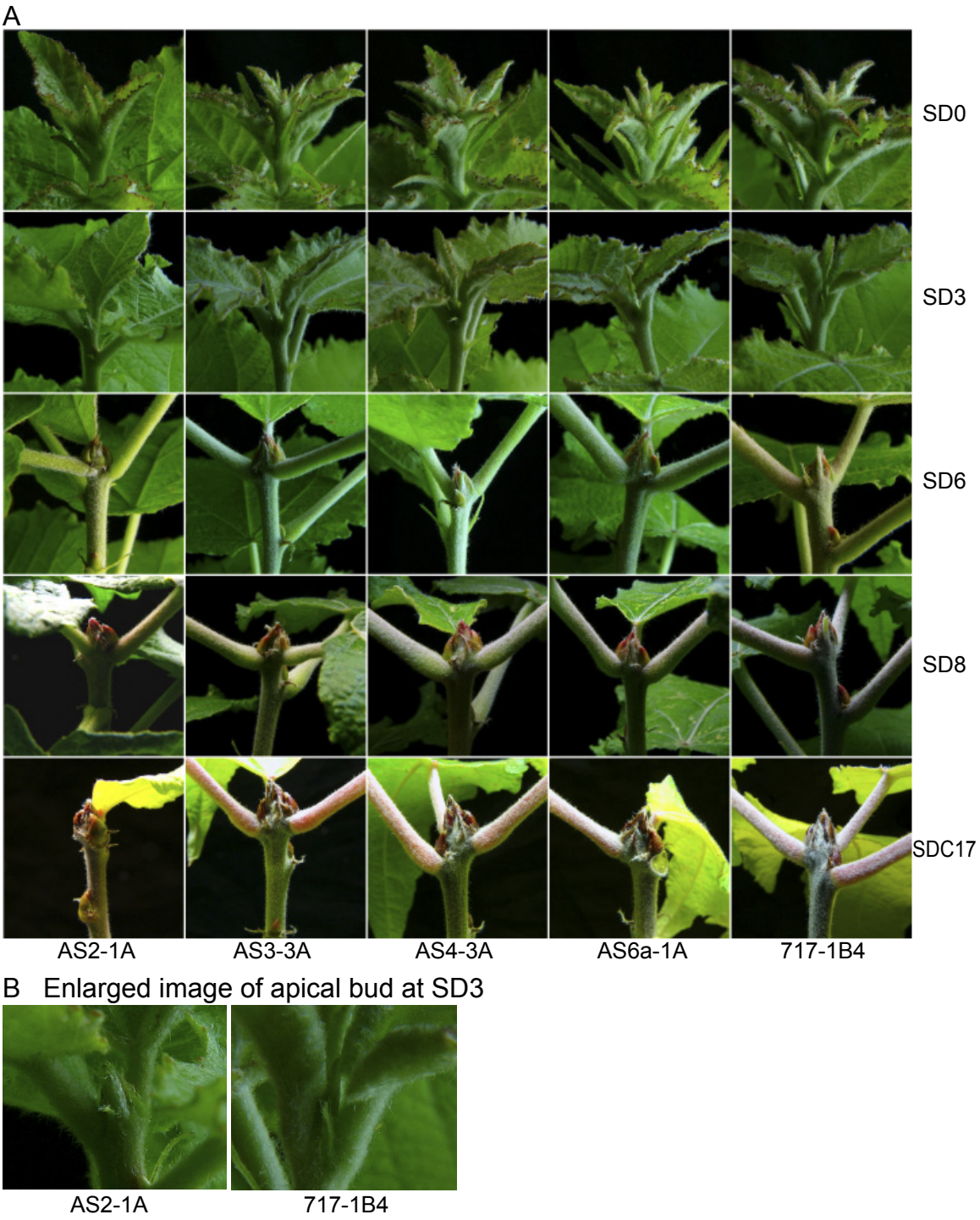


Figure 4-7. Apical bud formation in wildtype 717-1B4 and AS2-1A, AS3-3A, AS4-3A, and AS6a-1A transgenic poplars in SD

(A) Apical buds during SD or SD combined with LT treatment. SDC17 means that these plants were exposed to 17 weeks of SD combined with 1024 hrs LT beyond initial 8 weeks of SD.

(B) Enlarged view of apical buds of AS2-1A and 717-1B4 poplars at 3 weeks of SD.

4-7). Surprisingly 35S::*PtFLC2as2* poplars initiated the formation of apical bud earlier, but after 6 weeks of SD treatment the buds appeared smaller. SD treatment beyond 8 weeks resulted in little, if any, change in the morphology and size of buds for wildtype, 35S::*PtFLC2as2,* 35S::*PtFLC2as3,* 35S::*PtFLC2as4,* and 35S::*PtFLC2as6a* plants. Continued SD treatment beyond 8 weeks did result in continued development of buds for 35S::*PtFLC2as1* poplars and after 4.4 additional weeks of SD beyond 8 weeks combined with or without LT, the apical buds of these transgenic plants were comparable to wildtype plants (Figure 3-8).

Apical bud break in 35S::*PtFLC2as1,* 35S::*PtFLC2as2,* 35S::*PtFLC2as3,* 35S::*PtFLC2as4,* 35S::*PtFLC2as6a* lines and wildtype poplars treated with 8 wks of SD averaged 6.2, 17.8, 11.8, 11.4, 11.4, and 11.8 days respectively when transferred to LD permissive conditions. Apical buds begin to enter dormancy upon SD8 treatment as evidenced by a gradual increase in the time required for bud burst and shoot regrowth if plants are switched back to LD (Figure 4-8). After additional 2.3 weeks of SD combined with 256 hrs LT (10/4 ^{o}C day/night) beyond the initial 8 weeks of SD, 35S::*PtFLC2as1* transgenic poplar lines averaged 8.2 days for bud break in LD conditions while the average number of days required for bud break in the other transgenic poplars and wildtype control plants ranged from 134.6–143 days. Endodormancy was induced in 35S::*PtFLC2as1* with an additional 4.4 weeks of SD treatment when combined with 496 hrs LT beyond the initial 8 week SD treatment. Apical buds remained endodormant until after 14.7 weeks of SD combined with 752 hrs of LT for all lines except 35S::*PtFLC2as2* plants. 35S::*PtFLC2as2* poplars began to be released from endodormancy with 496 hrs LT treatment. Axillary buds showed a similar dormancy pattern to apical buds (Figure 4-8C). Continued SD and LT treatment resulted in a decline in the number of days required for bud break to occur for all transgenic and wildtype poplars. These results suggest that overexpression of *PtFLC2as2* has opposite effect on the onset of endodormancy by SD than overexpression of *PtFLC2as1* whereby *PtFLC2as2* appears to accelerate the onset of bud formation and dormancy while *PtFLC2as1* appears to delay these events. There was no significant effect on bud dormancy status for overexpression of *PtFLC2as3*, *PtFLC2as4*, and *PtFLC2as6a* poplars.

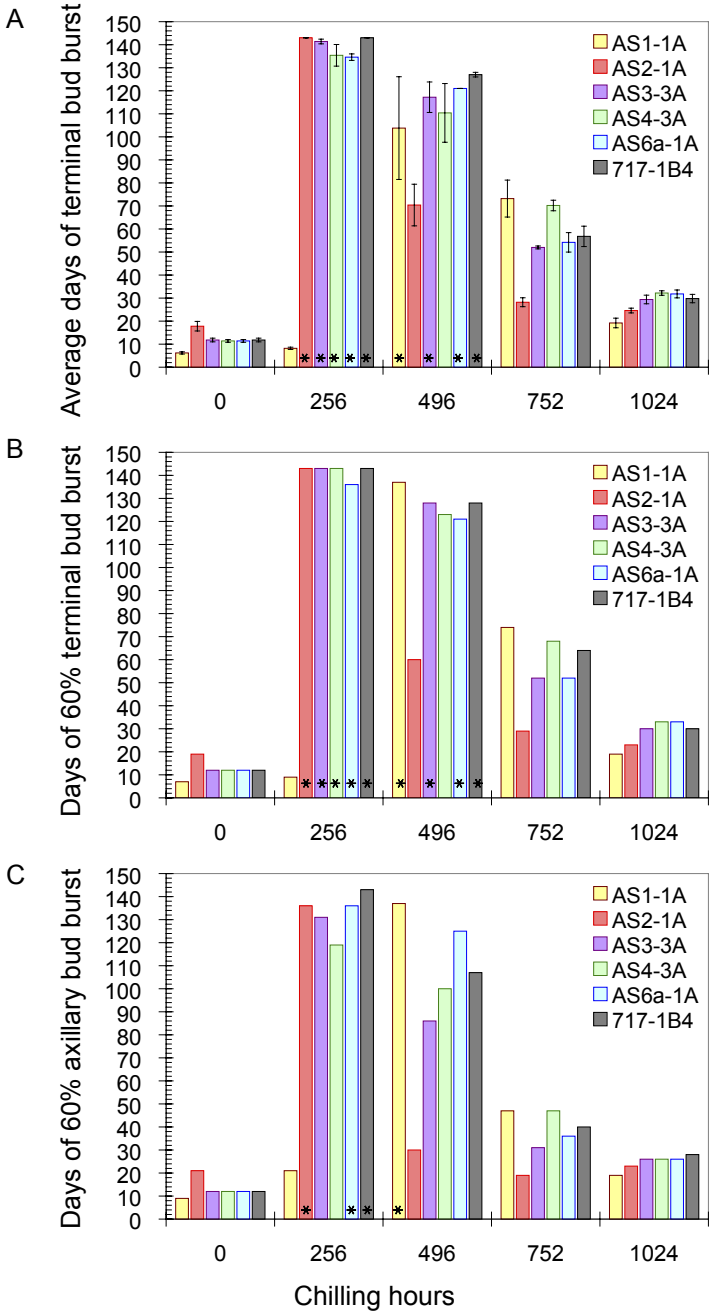


Figure 4-8. Dormancy of transgenic poplars overexpressing *PtFLC2as1* and *PtFLC2as2*.
(A) Average days of terminal bud burst in wildtype 717-1B4, AS1-1A, AS2-1A, AS3-3A, AS4-3A, and AS6a-1A poplars. Values are mean+/-StdError.
(B) Days to 60% terminal bud burst in wildtype 717-1B4, AS1-1A, AS2-1A, AS3-3A, AS4-3A, and AS6a-1A poplars
(C) Days to 60% axillary bud burst in wildtype 717-1B4, AS1-1A, AS2-1A, AS3-3A, AS4-3A, and AS6a-1A poplars
*: more than 50% buds were still dormant when the experiment was terminated

Discussion

Identification of *PtFLC2* alternatively spliced transcripts

The *PtFLC2* transcript is alternatively spliced with thirteen different splicing variants detected in poplar. The expression of transcript *PtFLC2as1* declines while several *PtFLC2* splicing variants (*PtFLC2as2, as3, as4, as5a, as5b, as6a*, and *as6b*) are up-regulated during bud dormancy. These changes in gene expression appear to be associated with exposure to SD photoperiod and cold. The cold treatment does not result in maintaining repression of *PtFLC2as1* and up-regulation of other *PtFLC2* splicing variants into the next growth cycle in poplar. After release from dormancy resulting from prolonged cold exposure and subsequent growth, their expression appears to be reset to the pre-dormancy state. These results indicate that *PtFLC2* post-transcriptional regulation is specific to developmental stages and may be an important mechanism involved in the dormancy-associated process including bud formation, leaf senescence, leaf abscission, and dormancy release. This resetting of expression of *PtFLC2* is similar to what has been observed in biennial sugar beet (Reeves et al. 2007). In nature, a resetting of the *PtFLC2* response may prevent plants from initiating dormancy following transient cold periods. In *Arabidopsis FLC* is regulated by a mitotic stable repression that is reset during meiosis (Sung et al. 2004a; Sung et al. 2004b). This does not appear to be the case for poplar *PtFLC2.* Such differences might be a consequence of the perennial vs. annual nature of poplar vs. *Arabidopsis*.

All of the *PtFLC2* transcript variants contain the MADS-box domain which is responsible for DNA binding and protein dimerization. An alternatively spliced transcript of *Arabidopsis FLC,* observed after 15 days of vernalization, encodes a shorter protein with a DNA-binding/dimerization MADS-box domain and C-terminal region (Caicedo et al. 2004). Similar to the *Arabidopsis FLC* variant, *PtFLC2as2* encodes a smaller protein which contains a complete MADS-domain and C-terminal and a partial K-domain that appears to be up-regulated by cold. It is possible that the splice variant from *Arabidopsis FLC* and the *PtFLC2as2* from poplar share comparable roles in the response to cold. Because PtFLC2as2

still contains the C-terminal which is responsible for transcription regulation or the formation of higher order complexes, it is possible that PtFLC2as2 may also function in different roles than PtFLC2as1 or act as a dominant negative regulator to compete with PtFLC2as1.

Because the expression of the other *PtFLC2* splice variants (*PtFLC2as3, PtFLC2as4, PtFLC2as5a, PtFLC2as5b, PtFLC2as6a*, and *PtFLC2as6b*) also appears to be associated with cold and dormancy and since the truncated proteins may still have DNA-binding or protein-dimerization activities but impaired protein-protein interactions, it is possible that these proteins may also act as dominant negative regulators. This mechanism could involve competing for target gene promoter-binding sites or dimerization with a functional PtFLC2 protein. This competition by shorter or truncated PtFLC2as3–PtFLC2as9 proteins on functional PtFLC2as1 might be weak because there is no significant phenotype observed in 35S::*PtFLC2as3,* 35S::*PtFLC2as4*, and 35S::*PtFLC2as6a* plants. Alternatively, these splice variants may function in attenuating or fine tuning the expression of *PtFLC2as1* during cold-mediated dormancy release as opposed to complete repression.

Effect of *PtFLC2as* on *Arabidopsis*

Overexpression of *FLC* in *Arabidopsis* is sufficient to delay flowering even in genotypes without an active *FRI* allele (Michaels et al. 1999). On the other hand, the extreme late flowering of *Arabidopsis* FRI-Sf2 (Col) is eliminated in the *flc-3* null mutant (Michaels et al. 1999). If the function of *FLC* as a floral repressor is conserved across species and other *PtFLC2* splice variants act as a dominant negative repressor on *FLC* and *PtFLC2as1*, then overexpression of *PtFLC2as1* in early-flowering *Arabidopsis* LER would have been expected to generate late flowering transgenic plants. Conversely, overexpression of those *PtFLC2* alternatively spliced variants in late-flowering *Arabidopsis* FRI-Sf1 would have been expected to result in early flowering. Surprisingly, overexpression of *PtFLC2as1* or *PtFLC2as2* in the late-flowering *Arabidopsis* FRI-Sf2 resulted in early-flowering phenotypes while having no effect on the early-flowering *Arabidopsis* LER genotype. The effect of overexpressing *PtFLC2as1* or *PtFLC2as2* was not as great as that reported for *flc-3* (Michaels et al. 1999), yet reduced flowering time by approximately 50%.

Two possible explanations could account for the early flowering phenotype. PtFLC2as1 or PtFLC2as2 may serve as a dominant negative repressor which reduces FLC repression on flowering time by competing with FLC. Alternatively, PtFLC2as1 or PtFLC2as2 may by itself induce flowering. As discussed in chapter 3, the inclusion of PtFLC2as1 or PtFLC2as2 into a multimeric protein complex could result in a nonfunctional complex. Furthermore, the other splice variants of *PtFLC2* that failed to result in early flowering may be due to lack the domains necessary for recruitment or participation in these multimeric protein complexes and therefore failed to give an early flower response. Another possibility is related to the retention of A class gene functions. It is possible that both PtFLC2as1 and PtFLC2as2 retain the functions while the proteins encoded by the other splice variants no longer contain the domains necessary to function as AP1/CAL/FUL. A recently published paper on *BpMADS4* in birch (Hoenicka et al. 2008) suggests this may be the case for this gene. The other splice variants could then act as negative regulators of PtFLC2as1 and PtFLC2as2 possibly by interfering with protein complex interactions. Whatever the mechanism might be, it is clear that *PtFLC2* regulation is complex.

The type II MADS-box proteins contain 4 major domains - a MADS-box domain with DNA-binding domain and dimerization motif, an I domain influencing DNA binding specificity and dimerization, a K domain containing a protein-protein interaction region, and a variable length C-terminal involved in transcriptional activation, posttranslational modification, or protein-protein interaction (Alvarez-Buylla et al. 2000b; Hileman et al. 2006; Kaufmann et al. 2005; Nam et al. 2003; Vandenbussche et al. 2003). It is possible that the different effect on flowering time is based on the specific domain(s) of the protein (Figure 4-1B). All *PtFLC2as* transcripts contain a MADS-box domain. The most significant difference between PtFLC2as1 & PtFLC2as2 proteins compared to PtFLC2as3, PtFLC2as4, & PtFLC2as6a proteins is that PtFLC2as1 & PtFLC2as2 proteins contain the C-terminal. PtFLC1as1 has all 4 domains (MADS, I, K, and C-terminal) while PtFLC2as2 lacks the I domain and only has a partial K domain. This could affect protein-protein interaction and affect the ability of PtFLC2as2 to form protein complexes with other proteins compared to

PtFLC2as1. Interestingly the early flowering phenotype has a correlation with the predicted protein structure with the earliest flowering phenotype associated with 35S::*PtFLC2as1* plants, followed by 35S::*PtFLC2as2* plants. This suggests that a PtFLC2 protein with all domains is more effective in accelerating flowering, possibly by its greater ability to compete with a FLC protein to form a multimeric protein complex to repress downstream genes. It could also serve as a flowering meristem identity protein to promote flowering. PtFLC2as3 and PtFLCas4 lack part of the K-domain and entire C-terminal which may result in the loss of the ability to regulate downstream gene expression even if they can form a protein complex with other proteins. PtFLCas6a lacks a complete K-domain and entire I-domain and C-terminal which might affect the ability to form a protein complex.

In this study, constitutive expression of *PtFLC2as1, PtFLC2as2,* or *PtFLC2as6a* had little if any effect on the transcript abundance of *Arabidopsis FLC,* suggesting that cosuppression is likely not the cause of the early flowering phenotype. In addition, little change in the expression of *SOC1* or *FT* was observed in the early flowering transgenic *Arabidopsis*. The up-regulation of *AP1* and *SOC1* during transition from vegetative to flowering in *Arabidopsis* takes place in the shoot apical meristem after bolting with a slight delay in *AP1* activation relative to *FT* activation (Abe et al. 2005; Wigge et al. 2005). Since this study sampled leaf tissue long before bolting, it is possible that the effect on gene expression of *AP1* and *SOC1* will not be significant.

Effect of *PtFLC2as2* on poplar

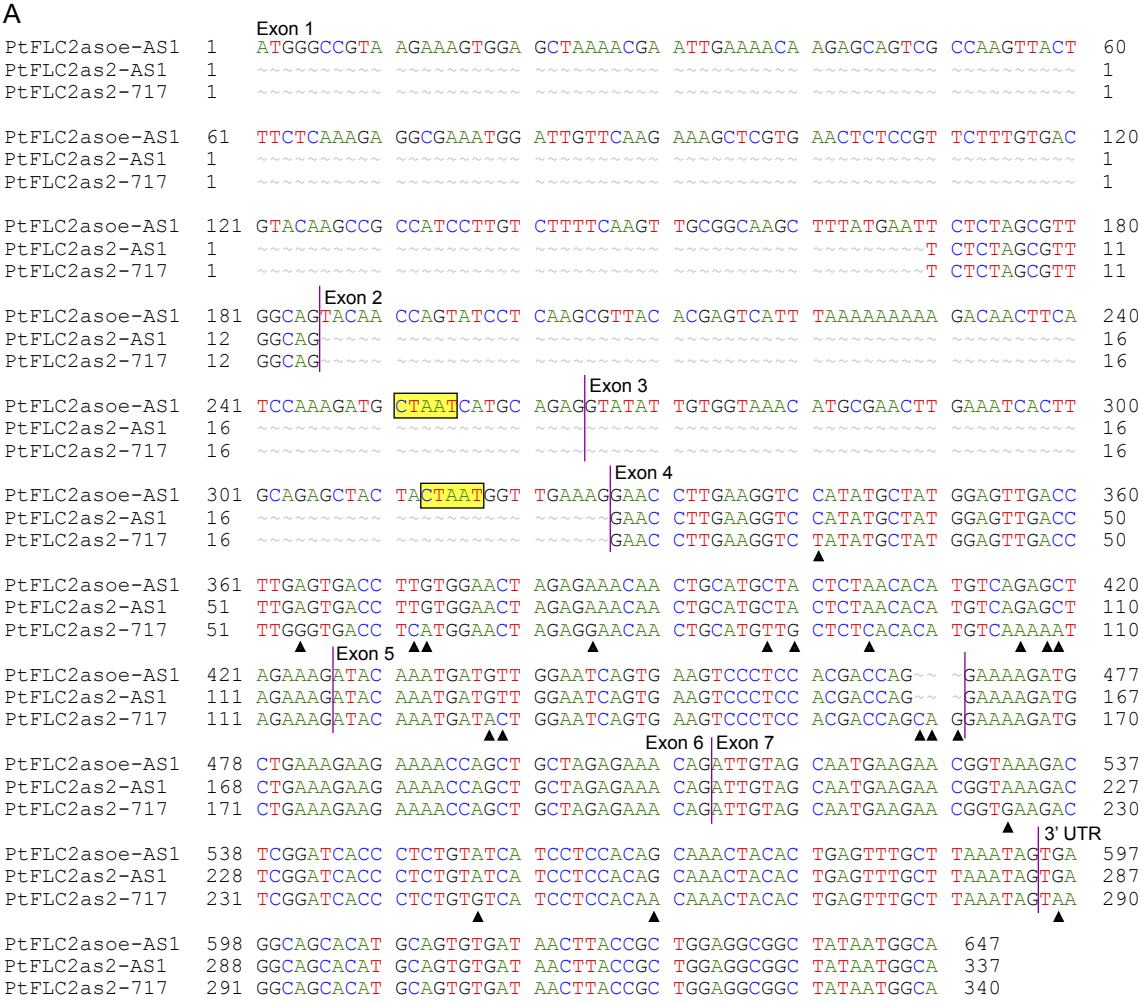
DNA sequencing of cloned PCR products revealed that the higher expression level of *PtFLC2as2* in 35S::*PtFLC2as1* poplars (Figure 4-2C) during dormancy originates from the transgene 35S::*PtFLC2as1*. There are two possible explanations. The first is associated with technical issues associated with PCR, where the *PtFLC2as2* specific primer may amplify both *PTFLC2as1* and *PtFLC2as2* transcripts. The difference between *PtFLC2as1* and *PtFLC2as2* is the presence or absence of exons 2 and 3. The forward primer used in *PtFLC2as2*, 5'-CTCTAGCGTTGGCAGGAACCTTG-3', spans across exon 1 (primer sequence in exon 1: CTCTAGCGTTGGCAG) and exon 4 (primer

sequences in exon 4: GAACCTTG) to avoid amplifying *PtFLC2as1* (Figure 4-9B). The sequence difference between the 3' end of exon 1 (CTCTAGCGTTGGCAG) and exon 3 (ACTAATGGTTGAAAG) is 8 out of 15 nucleotides. If this difference is not sufficient to distinguish both splicing variants under 64°C annealing temperature, then the expression pattern from the *PtFLC2as2* specific primer would include *PtFLC2as1* plus *PtFLC2as2*.

It is also possible that recursive splicing (or resplicing) with the overexpressed *PtFLC2as1* transcript has happened in 35S::*PtFLC2as1* transgenic poplar. It has been shown that recursive splicing could remove subfragments or nested introns either co-transcriptionally or uncoupled from transcription efficiently and accurately in yeast, mouse and *Drosophila* ((Burnette et al. 2005; Grellscheid et al. 2006; Lopez et al. 2000). It has been proposed that many large introns could be removed by multiple recursive splicing steps, involving sequential excision of smaller subfragments and regenerating a new splicing site after the previous splicing, instead of a one-step direct splice (Burnette et al. 2005). There is a splice acceptor (AG) at the end of exon 3 and a possible branch point (CTAAT) in exon 2 and 3 (Figure 4-9A). When evaluating intron 2 (170–188 bp) and intron 3 (76–87 bp) sequences of 5 *PtFLC* genes, we found that only half of them contained branch points (CURAY) and about half of them contained U-rich motifs. It is not necessary to have all splice recognition sites for the spliceosome to recognize an intron. Thus it is possible that recursive splicing happens on the overexpressed *PtFLC2as1* transcript even without a splice donor (a.k.a. 5' splicing site) and polypyrimidine tract (or U-rich sequences).

Multiple lines of 35S::*PtFLC2as* transgenic poplars (35S::*PtFLC2as2*, 35S::*PtFLC2as3*, 35S::*PtFLC2as4*, and 35S::*PtFLC2as6a*) have been regenerated. It is difficult to suggest how *PtFLC2* alternatively spliced variants affect poplar dormancy given only the initial results from of 35S::*PtFLC2as2*, 35S::*PtFLC2as3*, 35S::*PtFLC2as4*, and 35S::*PtFLC2as6a* transgenic poplars. In chapter 3, it was shown that *PtFLC2as1* overexpression promotes sylleptic branching under certain light quality, delays bud formation, leaf senescence, and leaf abscission, and alters dormancy status. In this study, *PtFLC2as2* overexpression also appears to promote sylleptic branching, induces bud formation, shortens

A



B

PtFLC2as2 forward primer (23mer): CTCTAGCGTTGGCAGGAACCTTG

3' end of exon 1: CTCTAGCGTTGGCAG

3' end of exon 3: ACTAATGGTTGAAAG

Figure 4-9. DNA Sequence alignment of *PtFLC2as2* splice variant in 35S::*PtFLC2as1* transgenic poplar..

(A) DNA sequence alignment of *PtFLCas2* splice variant with RNA from 35S::*PtFLC2as1* and wildtype *Populus alba* x *tremula* clone 717-1B4. The PtFLC2asoe primers were used to amplify full-length *PtFLC2* transcript. The forward primer of *PtFLC2as2* primer combination is spanned from exon 1 to exon 4 in order to specifically amplify the *PtFLC2as2* splice variant. AS1 represents RNA that was isolated from 35S:: *PtFLC2as1* transgenic poplars. 717 represents the RNA was isolated from wildtype *Populus alba* x *tremula* clone 717-1B4. The sequence for PtFLC2asoe-AS1 is the consensus from 8 independent clones from 4 AS1 lines with *PtFLC2asoe* primer combination. Sequence in PtFLC2as2-AS1 represents 8 cloned sequencing results from 4 AS1 lines. Sequence in PtFLC2as2-717 represents the consensus of 4 independent clones from wildtype 717-1B4. ▲ indicates the SNP in *PtFLC2* transcript between transgene from *Populus deltoides* x *trichocarpa* clone 545-4183 and the endogenous gene in wildtype *Populus alba* x *tremula* clone 717-1B4. CTAAT in exon 2 and exon 3 is possible branch point.

(B) Sequence comparison between *PtFLC2as2* forward primer and the 3' end of exon 1 and exon 3. Blue color GAACCTTG in *PtFLC2as2* forward primer is located in the 5' end of exon 4.

chilling requirement, and alter dormancy status in poplars, but there seems to be no effect on leaf senescence and leaf abscission. No obvious phenotypes have been observed in 35S::*PtFLC2as3*, 35S::*PtFLC2as4*, and 35S::*PtFLC2as6a* transgenic poplars.

The results from 35S::*PtFLC2as1* poplars suggest that overexpression *PtFLC2as1* may maintain active growth in poplars which delays bud formation and releases buds from dormancy quicker than wildtype poplars. The initial results of 35S::*PtFLC2as2* suggest that PtFLC2as2 may impair the regulatory function of PtFLC2as1 in bud formation, but enhance cold-mediated dormancy release. It is possible that *PtFLC2* participates in bud formation and dormancy release through independent pathways. Since bud formation is induced by photoperiod, it is possible that the full-length PtFLC2as1 may promote active growth through a multimeric protein complex which inhibits SD induced bud formation. The formation of this protein complex may be inhibited by a long period of short days. In contrast PtFLC2as2 may induce early bud formation by competing with PtFLC2as1.

In cold-mediated dormancy release, a certain amount of chilling is required to switch plants from dormant to active status. All wildtype, PtFLCR, *35S::PtFLC2as2*, *35S::PtFLC2as3*, *35S::PtFLC2as4*, and *35S::PtFLC2as6a* poplars established dormancy during 8–10.3 weeks of SD combined with 0–256 hrs LT. *35S::PtFLC2as1* poplars did not established dormancy until 10.3–12.4 weeks of SD combined with 256–496 chilling hours. All wildtype, PtFLCR, *35S::PtFLC2as1*, *35S::PtFLC2as3*, *35S::PtFLC2as4*, and *35S::PtFLC2as6a* poplars started gradually to be released from dormancy before 14.7 weeks of SD combined with less than 752 hrs LT and were completely released from dormancy at 17 weeks of SD with 1024 chilling hours. *35S::PtFLC2as2* poplars started to be released from dormancy gradually before 12.4 weeks of SD combined with 496 chilling hours and were completely released from dormancy around 752 chilling hours which is earlier than wildtype, PtFLCR, *35S::PtFLC2as1*, *35S::PtFLC2as3*, *35S::PtFLC2as4*, and *35S::PtFLC2as6a* poplars. If chilling hours accumulate from the time plants are dormant to the time the plants are released from dormancy, then *35S::PtFLC2as1* and *35S::PtFLC2as2* poplars require less chilling (least 256 hours) than wildtype, *35S::PtFLC2as3*, *35S::PtFLC2as4*, and *35S::*

PtFLC2as6a poplars. Therefore, it seems that both 35S::*PtFLC2as1* and 35S::*PtFLC2as2* reduces the chilling requirement and promotes dormancy release. Because the expression of *PtFLC2as1* is down-regulated by cold in wildtype plants, it is hard to believe that *PtFLC2as1* is actually promoting dormancy release instead of repressing it. If chilling hours are calculated as how many hours of cold exposure are required for dormancy release regardless of the dormancy status of the plants, about 1024 hours of chilling are sufficient for wildtype, *35S::PtFLC2as1*, *35S::PtFLC2as3*, *35S::PtFLC2as4*, and *35S::PtFLC2as6a* poplars and 752 hours for *35S::PtFLC2as2* poplars. It could be argued then that cold-mediated dormancy release may involve only PtFLC2as2 and PtFLC2as1 may not have an effect on dormancy release. *35S::PtFLC2as1* poplars require more LD at warm temperatures for bud burst than wildtype poplars after switching from SD plus cold to LD at warm temperatures after 496 and 752 hours of chilling (Figure 4-8). This might suggest that 35S::*PtFLC2as1* weakly represses dormancy release. Once this chilling requirement is satisfied after 1024 chilling hours, the greater levels of PtFLC2as1 and PtFLC2as2 promote faster regrowth than wildtype, *35S::PtFLC2as3*, *35S::PtFLC2as4*, and *35S::PtFLC2as6a* poplars.

Materials and Methods

Plant materials and growth conditions

Plant materials and growth conditions for 35S::*PtFLC2as* transgenic poplars (abbreviation as AS1, AS2, AS3, AS4, and AS6a) and *Arabidopsis* were as described in chapter 3. Only one line for each construct (35S::*PtFLC2as2*, 35S::*PtFLC2as3*, 35S::*PtFLC2as4*, and 35S::*PtFLC2as6a*) has been tested because the transgenic lines were just established in tissue culture in late 2007 and the time required to propagate sufficient numbers for experiments is long.

Isolation of *PtFLC*2 alternative splicing variants and RT-PCR analysis

The details of RNA extraction, reverse transcription reaction, and PCR analysis are as described in chapter 2.

PtFLC2 and the splice variants (abbreviation as AS1, AS2, AS3, AS4, AS5a/b/c/d, AS6a/b, AS7, AS8, and AS9) (Figure 4-1) were identified and verified using 3 different sets of primer combinations. The *PtFLC2* specific primer combination amplifies one major product (478 bp, AS1) and several minor products (AS3=603 bp, AS4=595 bp, AS7=690 bp, and AS8=565 bp). The full-length cDNA *PtFLC2asoe* primers amplifies one major product (647+4 bp, AS1) and several minor products (AS2=506+4, AS3=772+4, AS4=764+4, AS6a=631+4, AS6b=794+4, AS7=859+4, AS8=734+4, and AS9=568+4). The *PtFLC2oeT* specific primers (5'-CACCATGGGCCGTAAGAAAGTGGAG-3' as the forward primer and 5'- TAGGTATGGAAGAAGACGCCCTAA-3' as the reverse primer) amplifies two major products (AS3=464+4, AS6=323+4 bp) and several minor products. The CACC nucleotides in the beginning of *PtFLC2asoe* and *PtFLC2oeT* forward primers were added to the original sequences to enable directional cloning into the pENTR plasmid using the Directional TOPO Cloning kit (Invitrogen). The "+4" in the size of PCR product represents this CACC nucleotide. The reverse primer of *PtFLC2* and *PtFLC2asoe* is located in 3'UTR. The reverse primer of *PtFLC2oeT* is located in the alternative internal exon (E_{AI}). The reason for using the *PtFLC2oeT* primer combination instead of the *PtFLC2asoe* primer combination is that the amount of splicing variants with E_{AI} is much lower than those without it; hence, those clones sent for sequencing did not result in any containing E_{AI} when using primers *PtFLC2asoe*. The PCR products were cloned as described in previous chapters and sequenced. The sequencing results were used for sequence comparisons in BioEdit to determine the structure of splice variants. A functional study of *PtFLC2as1* was conducted in chapter 3. The annealing temperature for all 3 primer combinations was 62°C. The primer combinations specific for several alternative splicing variants were designed such that one primer spanned the splicing site (Figure 4-1C). The cycle numbers ranged from 31–40, depending on the specific primer combinations. RT-PCR procedures were as described in chapter 2.

Overexpression *PtFLC2as* constructs and *Agrobacterium* transformation

The *PtFLC2asoe* primers were used to amplify the cDNA used for 35S::*PtFLC2as1* and 35S::*PtFLC2as2* constructs. The *PtFLC2oeT* primers were used to amplify the cDNA used to construct 35S::*PtFLC2as3,* 35S::*PtFLC2as4* and 35S::*PtFLC2as6a*. Construction of chimeric genes was as described in chapter 3. Total RNA from shoot apices of *Populus deltoides* x *trichocarpa* clone 545-4183 was used for RT-PCR. The detail of *Agrobacterium* transformation of *Populus alba* x *tremula* clone 717-1B4 and *Arabidopsis* ecotypes LER and FRI-Sf2 were as described in chapter 3.

Segregation and RNA analysis of transformed *Arabidopsis*

Segregation analysis was applied to 5 early flowering 35S::*PtFLC2as1*, five early flowering 35S::*PtFLC2as2*, and four early flowering 35S::*PtFLC2as6a* transformed T2 generations of *Arabidopsis* in the FRI-Sf2 background. RNA and segregation tests were carried out as described in chapter 3. Multiple comparisons of means among construct within the same cluster were carried out by one-way ANOVA Post Hoc tests with Tamhane's T2 and Games-Howell methods because the assumption of equal variance was rejected. Multiple comparisons of means among lines within the same cluster with equal variance were tested with Bonferroni, Tukey HSD and Scheffe methods as described in chapter 3.

Measurement of growth cessation, chilling requirement and dormancy status

Ten plants per line from each 35S::*PtFLC2as* transgenic poplars (AS2-1A, AS3-3A, AS4-3A, AS6a-1) were used. All the experimental conditions are as described in chapter 3.

Chapter 5: Summary

A number of fundamental questions have guided my Ph.D. project. First, what genes are involved in the regulation of bud initiation, development, dormancy and the release from dormancy? Do analogous regulatory pathways control flowering and bud dormancy? Do homologs to the flower-related type II MADS-box genes play a role in the morphological development of shoot apices in poplars? To begin to answer these questions, my study first focused on the relationship between type II MADS-box genes and plant dormancy, followed by functional studies aimed at characterizing one candidate gene, *PtFLC2*.

The similarities between the morphological structures of vegetative and floral buds as well as aspects of common environmental and hormonal regulation suggest that it is possible that these important plant processes may share analogous regulatory pathways (Figure 1-2). Since many type II MADS-box genes (e.g. *AP1, AP3, PI, AG, SOC1, FLC, AGL24,* and *SVP*) were known to be involved in the transition of a vegetative to flowering phase and play roles in the development of the floral bud in *Arabidopsis* (Figure 1-2, Figure 1-3), I decided to select this group of genes for further study. After identifying 119 MADS-box genes in poplar (45 type I, 57 type II, 2 S, and 15 possible pseudo-genes), 57 putative type II MADS-box genes were classified into 14 functional classes using phylogenetic analysis (Table 1-1). Among them, homologs of 5 *PtA*, 3 *PtAP3*, 2 *PtPI*, 4 *PtCD*, 5 *PtF*, 6 *PtFLC* and 9 *PtSVP* genes have been suggested to play regulatory roles in flowering time control and floral organ identification in *Arabidopsis*. Because of the time required to establish transgenic poplar lines and since each dormancy experiment requires at least 7 months to complete, it is a daunting task to use poplar to study the roles of all these genes in bud development and dormancy. Therefore it was reasoned that by using RT-PCR to examine gene expression of these 57 genes in shoot apices, it might be possible to narrow down the pool of candidate genes to study. One candidate gene, *PtFLC2*, was chosen for further study.

In this dissertation, I present the first comprehensive analysis of expression of type II MADS-box genes in various vegetative tissues (shoot apices, leaf, bark, xylem, and

root), spring floral buds, and shoot apices during bud development and dormancy (Figure 2-4, Table 2-4). Because of the difficult of producing and screening mutants by forward genetic approaches in tree species such as poplar, knowledge of gene expression patterns could be useful for selecting candidate genes for functional studies in poplar development. Several *PtCD* and *PtE* genes were found to have little if any expression in shoot apices in LD or during SD induced dormancy even when PCR cycles were extended to more than 40 cycles. This suggests that the vegetative shoot apex and apical bud have a gene expression profile similar to an *agamous* and/or leaf-like organ (Figure 1-2A). A number of differences between the exon-intron structures from the gene predictions in JGI poplar genome database and sequenced cDNA were discovered from DNA sequence analysis as well as the identification of a number of alternatively spliced transcripts for these poplar type II MADS-box genes (Table 2-1, Table 2-2). These results provide an experimental basis for transcript structure as opposed to predicted structure of poplar MADS-box genes. As a result, this information will be of value in improving the annotation accuracy of current JGI poplar genome database.

Multiple alternatively spliced variants were discovered during the *PtFLC2* functional study (Figure 4-1). The major splicing variant, *PtFLC2as1*, encodes a protein which consists of all 4 known domains (MADS, I, K, and C) of type II MADS-box genes. It was down-regulated during dormancy (Figure3-2) while most other splicing variants were up-regulated during dormancy (Figure 4-2). This expression pattern is reminiscent of the vernalization-mediated repression of *Arabidopsis FLC* and upregulation of its splicing variant (Caicedo et al. 2004). Alternatively splicing variants are known to play important roles during the development of mammals and *Drosophila*. However, little is known about the functional regulation of splicing variants in plant development. To study the regulation of alternatively spliced variants of *PtFLC* genes in various stages of the dormancy cycle, *PtFLC2* was selected as the candidate gene to begin to understand its regulatory role. Functional studies using *Arabidopsis* LER and FRI-Sf2 ecotypes and *Populus alba* x *tremula* clone 717-1B4

revealed the relationship between five alternative splicing variants (*PtFLC2as1–6a*) and flowering time control, apical bud development, as well as cold-mediated dormancy release.

The results from poplar suggest that *PtFLC2as1* and *PtFLC2as2* promote active vegetative growth. Also, it revealed that overexpression of either *PtFLC2as1* or *PtFLC2as2* repress apical dominance and promotes axillary bud growth under LD (Figure 3-5 and 4-6). Overexpression of only *PtFLC2as1* delays all dormancy responses, including growth cessation, bud formation, leaf senescence, and abscission (Figure 3-6, 3-9, and 3-10). Prolonged SD and/or SD with cold eventually overcomes the effects of overexpression of *PtFLC2as1*. Analysis of bud dormancy showed that overexpression *PtFLC2as1* only delays the acquisition or entry into dormancy and has little or no effect on releasing buds from dormancy (Figure 3-9). Overexpression of *PtFLC2as2* induced bud formation, as well as accelerated dormancy induction and release (Figure 4-7 and 4-8).

From this I hypothesize that active growth, bud formation, dormancy induction, and dormancy release are possibly regulated by three pathways, which involve both *PtFLC2as1* and *PtFLC2as2*. This could involve the formation of three different protein complexes with other proteins (Figure 5-1). Protein complex 1 promotes active growth in long day conditions and may also be involved in sylleptic growth. In contrast, protein complex 2 inhibits short day photoperiod induced transition from active growth to bud formation, growth cessation, and leaf senescence and abscission. Finally, protein complex 3 promotes cold-mediated dormancy release. PtFLC2as1 is present in complex 1 and 2, but not in protein complex 3. On the other hand, PtFLC2as2 is a key component of protein complex 1 and 3. However, by overexpressing *PtFLC2as2*, PtFLC2as2 competes with PtFLC2as1 in the formation of complex 2 and results in early formation of the apical bud. Both PtFLC2as1 and PtFLC2as2 proteins promote regrowth of the shoot apical meristem when the chilling requirement is satisfied. The challenge that now awaits is to determine if indeed these complexes are formed and what the interacting partners might be.

In the early flowering phenotype in 35S:*PtFLC2as1* and 35S::*PtFLC2as2* *Arabidopsis* (FRI-Sf2), PtFLC2as1 and PtFLC2as2 are assumed to serve as dominant

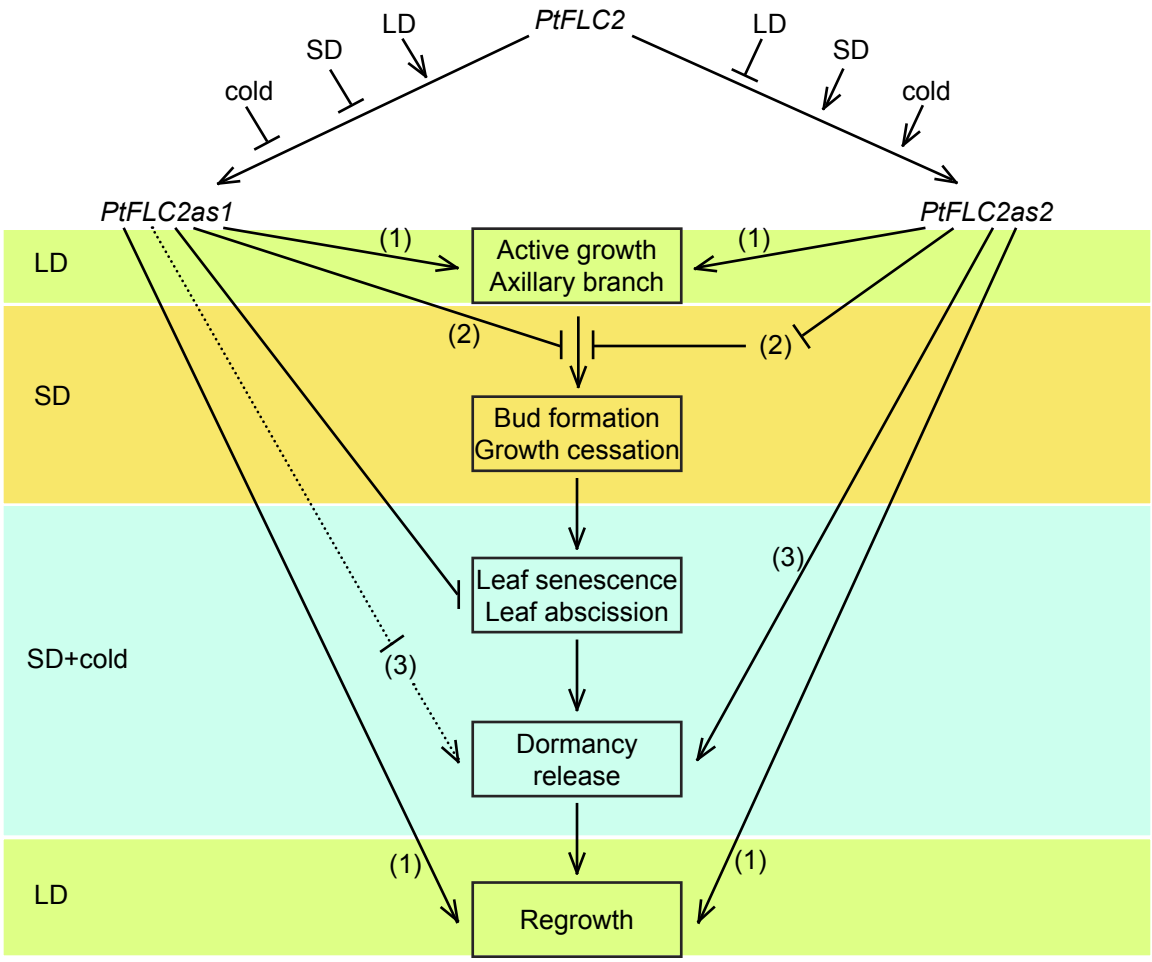


Figure 5-1. Model for the interaction of *PtFLC2* and environmental cues (photoperiod and chilling temperature) in the regulation of apical bud dormancy.
PtFLC2 proteins form 3 different protein complexes. The protein complex 1 promotes active growth in long day growth plants and results in axillary bud branching. The protein complex 2 inhibits the short day photoperiod induced transition from active growth to bud formation, growth cessation, and leaf senescence and abscission. The protein complex 3 promotes cold-mediated dormancy release. PtFLC2as1 forms functional complex 1 and 2, but not a protein complex 3. PtFLC2as2 are capable to form a functional protein complex 1 and 3, but an impaired protein complex 2. When overexpression *PtFLC2as2*, it competes the formation of complex 2 with PtFLC2as1 and induces the formation of apical bud earlier. Both PtFLC2as1 and PtFLC2as2 proteins promote regrowth of shoot apical meristem when chilling requirement satisfied and symplasmic organization restored poplars are switched to LD growth environment.
(1), (2), and (3) represent protein complex 1, 2, and 3.

negative repressors which compete with endogenous FLC and interfere with the formation of a functional multiple protein complex. In principle it results in reduction of *FLC* repression and induces early flowering. On the other hand, my results showed that 35S::*PtFLC2as1* poplars (Figures 3-9 and 3-10) have similar phenotypes such as maintaining active growth, delaying dormancy induction, and delaying leaf senescence to that of poplars overexpressing the birch gene *BpMADS4*, a homolog of *FRUITFULL* (Hoenicka et al. 2008). In addition, *BpMADS4* is the homolog of *PtA1* and *PtA*5 but not *PtFLC2*. This could be partially explained if *PtFLC2* still retains some A class gene function while evolving the neofunction of *FLC* class during evolution of *A* and *FLC* classes. Since PtFLC2as1 and PtFLC2as2 proteins possess the function of meristem identity proteins (e.g. AP1, CAL, and FUL) which stimulates the transition from vegetative to inflorescence phase, this might explain why overexpression of *PtFLC2as1* or *PtFLC2as2* induced early flowering in *Arabidopsis* (FRI-Sf2) (Tables 3-12 and 4-2). Based on the results of 35S::*PtFLC2as1* in poplar and all of the other 35S::*PtFLC2as* in *Arabidopsis* (FRI-Sf2) and the preliminary results of other 35S:: *PtFLC2as* in poplars, it might be that in *Arabidopsis*, PtFLC2as1 and PtFLC2as2 proteins will either (1) compete with an endogenous functional FLC protein to form a multimeric protein complex that is unable to repress downstream gene expression, or (2) they can serve as a flowering meristem identity protein to upregulate the expression of downstream floral organ identity genes (*AP3, PI,* and *AG*) and convert the vegetative meristem to a floral fate. The second model can initially be tested easily by checking the expression of *AP3, PI*, and *AG*.

From either case, the results from all *PtFLC2as* transgenic lines in poplar and *Arabidopsis* suggest that only *PtFLC2as1* and *PtFLC2as2* encode functional proteins while the other splicing variants (*PtFLC2as3, PtFLC2as4,* and *PtFLC2as6a*) appear to contain premature termination codons (PTCs) which are subjected to degradation by nonsense-mediated mRNA decay (NMD). Since *PtFLC2as3–PtFLC2as9* transcripts were detected, it might be possible that they still encode shorter or truncated PtFLC2as3–PtFLC2as9 proteins and may function in attenuating or fine tuning the expression of *PtFLC2as1* during cold-mediated dormancy release as opposed to complete repression. If it is

assumed that all *PtFLC2* alternatively spliced transcripts encoded proteins, they can be separated into 4 groups based on the protein domains they possess: (1) MADS+I+K+C: PtFLC2as1, (2) MADS+partial K+C: PtFLC2as2, (3) MADS+I+partial K: PtFLC2as3, PtFLC2as4, PtFLC2as7, and PtFLC2as8, and (4) MADS: PtFLC2as5a/b/c/d, PtFLC2as6a, and PtFLC2as9. Only plants transformed with 35S::*PtFLC2as1* or 35S::*PtFLC2as2* show phenotypes different from wildtype, suggesting that MADS domain and C-terminal together are essential to perform the regulatory function of *PtFLC2*. The difference in structure between proteins encoded by *PtFLC2as1* and *PtFLC2as2*, i.e. with or without complete I and K domains, are likely to be the basis for the different regulatory roles in 35S::*PtFLC2as1* and 35S::*PtFLC2as2* poplars. I hypothesize that both I+K-domains and MADS+C-terminal are the key components for the PtFLC2 protein to perform its regulatory function. Moreover, cold-mediated down-regulated *PtFLCas1* expression and up-regulation of possible dominant negative *PtFLCas2* may work together to regulate bud morphogenesis and mediate cold-temperature mediated bud dormancy release.

Based on my results, *PtFLC2* is a component of the integration point of both photoperiod and low temperature signals, which regulate bud development, dormancy, and release. The *PtFLC2* transcript is alternatively spliced with only one isoform encoding a complete MADS-box protein. Overexpression of *PtFLC2as2* isoform in poplar accelerates cold-mediated dormancy release while the *PtFLC2as1* isoform had little if any effect on dormancy release when overexpressed. The amount of *PtFLC2* transcript decreases during SD treatment and continues to decline with further SD treatment combined with low temperature suggesting that photoperiod contributes to the regulation of *PtFLC2*. Furthermore, the cold-mediated increase of the abundance of several alternative splicing variants especially *PtFLC2as2* and reduction of the major splice variant *PtFLC2as1* suggests that post-transcriptional regulation of mRNA splicing may play a regulatory role in bud dormancy. These suggest that the both transcriptional and post-transcriptional regulatory mechanisms may play important roles in bud development and dormancy.

In the future, a detailed functional study of *PtFLC4* and *PtFLC2* splicing variants beside full-length *PtFLC2as1*, especially *PtFLC2as2*, will help to clarify their regulatory role. Fusion of the whole *PtFLC2* gene with a reporter gene will give a clearer picture about their spatial and/or temporal expression pattern while keeping all the *cis*-elements and splicing sites, and therefore the regulatory mechanisms on *PtFLC2* intact. Several reports have shown that FLC complex protein binds to a region with CArG boxes to repress the expression of *FT*, *SOC1* and *FD* in *Arabidopsis* (Helliwell et al. 2006; Searle et al. 2006). The similar expression pattern of type II MADS-box genes among wildtype 717-1B4, 35S:: *PtFLC2as1*, and *PtFLC2*-RNAi poplars suggests that *PtFLC2as1* may not regulate the expression of *PtF, PtA*, and *PtFT* (Figure 3-11). This is different from *Arabidopsis FLC*. To find out what the target gene(s) of PtFLC2as1 and PtFLC2as2 are, it will be valuable to conduct an experiment using chromatin immunoprecipitation (ChIP) and see if they bind to CArG boxes in the region of the promoter or intron 1 of those target gene(s). A yeast-2-hybrid experiment to identify the possible protein-protein interactions and protein complexes will further dissect the story of *PtFLC2*.

Appendices

Table A-1. PCR conditions used for specific primer combinations of poplar type II MADS-box genes. *: unable to get a clear expression or no expression under these PCR cycles. N/A: no PCR has performed in that sample. The size of PCR product: ### -> ### represents the changes from size with JGI prediction -> actual size from sequencing results.

			717-1B4	545-4183	Nisqually-1	545-4183	Nisqually-1	717-1B4
			2007 Tissue	2007 tissue	2006 tissues	2006 dormancy	2006 dormancy	2007dormancy
Gene	Size (bp)	Ta (°C)	cycle	cycle	cycle	cycle	cycle	cycle
PtA1	591	64	35	35	35	35	35	35
PtA2	433	64	34	34	34	31	34	34
PtA3	334	64	33	33	33	35	36	36
PtA4	458	64	24	24	24	26	24	24
PtA5	176->203	58	40	40	40	40	38	38
PtAGL12a	247	64	38	29	29	30	29	40
PtAGL12b	297	64	29	29	29	30	31	31
PtAGL15a	149	64	38	38	38	38	40*	40
PtANR1c	as1=165,as2=641	60	37	37	37	37	36	36
PtANR1d	481->603	60	31	31	31	35	35*	35
PtANR1e	528->543	64	29	29	29	29	29	29
PtANR1f	529	60	36	36	36	38	40*	40
PtAP3a	345	60	33	33	33	33	33	33
PtAP3b	680->761	64	32	32	32	32	32	28
PtAP3c	618	64	35	35	35	35	35	35
PtPI1	399->403	62	35	35	35	29	33	33
PtPI2	338	60	41	41	41	44	45	46*
PtBs2	210	60	42	42	42	42	40	40
PtBs3	134	64	38	38	38	40	38	38
PtCD1	132	58/60	40	40	40	44	42*	48(58)*
PtCD2	229	60	41	44	40	44	38	40
PtCD3	570	60	40	40	40	44	40	40
PtCD4	as1=323,as2=742	58/60	40	40	40	44	40	42(58)*
PtE1	136	64	36	36	36	35	36	33
PtE2	599	62	44	44	44	42	44	48*
PtE3	217	58/60	44	44	44	44	44*	48(58)*
PtE4	470	58/64	41	32	32	30	32	42(58)
PtE5	477	58/64	35	35	35	33	33	44(58)*
PtF1	185	64	28	28	28	27	28	28
PtF2	344	64	29	29	29	27	29	29
PtF3	418	64	28	26	28	26	28	23
PtF4	559	64	33	33	33	33	33	34
PtF5	325	64	28	28	28	26	28	28
PtFLC1	as1=720,as2=638	64	36	36	36	30	36	34
PtFLC2	as1=478	62	32	30	32	31	35	32
PtFLC3	517	64	37	37	37	37	37	33
PtFLC4	as1=213,as2=320	60	39	39	39	40	37,41	37
PtFLC2asoe	651,510,776,768,635,798,863,738,572	62	30	30	32	33	36	30
PtFLC2oeT	as3=468,as6a=327	62	N/A	40	N/A	N/A	N/A	N/A
PtG1	234	64	35	35	35	33	35	35
PtG2	337	62	38	40	38	38	38	34
PtG3	181	64	36	36	36	36	35	33
PtSVP1	432	64	21	21	21	22	21	23
PtSVP2	340->350	64	28	25	25	26	25	30
PtSVP3	407,527	64	26	26	26	24	26	24
PtSVP4	473	62	28	28	28	27	31	35*
PtSVP5	190	60	30	30	30	28	29	29
PtSVP6	298	60	39	39	39	37	39	27
PtSVP7n1	as1=384,as2=526	60	36	36	36	34	36	36
PtSVP7n2	380->300	60	38	31	31	36	31	40
PtSVP8	206	64	36	36	36	34	36	36
PtU1	207	64	38	38	38	38	38	38
PtU2	332	64	28	26	28	28	28	28
PTFRI	349	64	N/A	N/A	N/A	28	28	28
PtUBQL	207	58-64	22	22	22	22	22	23
PtFT1	103	62	38	38	38	38	38	40
PtFT2	700	60	N/A	N/A	N/A	41	N/A	N/A
PtFLC2as1	274	62	N/A	N/A	N/A	35	37	35
PtFLC2as2	337	64	N/A	N/A	N/A	31	N/A	31
PtFLC2as3	287	64	N/A	N/A	N/A	33	N/A	39
PtFLC2as4	158	62	N/A	N/A	N/A	37	N/A	39
PtFLC2as6	as1=151,as2=314	64	N/A	N/A	N/A	35	N/A	39
PtVIN3a	697	60	22	22	22	22	22	22

Table A-2. ANOVA tables of growth cessation, leaf senescence, and dormancy measurement.
(A) Growth cessation: height increment and number of leaf increment per week
(B) Leaf senescence: concentration of chlorophyll a and carotenoids
(C) Dormancy measurement: average days of apical buds to burst

A

Height Increment (SD0-SD8): Exp 1					
Source	Type III SS	df	MS	F	Sig.
Model	6234.331	26	239.782	121.220	0.000
Intercept	5617.628	1	5617.628	2839.943	0.000
Construct	66.759	2	33.380	16.875	0.000
SD	3795.287	8	474.411	239.834	0.000
Const*SD	252.140	16	15.759	7.967	0.000
Error	1548.834	783	1.978		
Total	17159.690	810			
Corr. Total	7783.166	809			

Leaf# Increment (SD0-SD8): Exp 1					
Source	Type III SS	df	MS	F	Sig.
Model	2185.960	26	84.075	80.512	0.000
Intercept	2128.119	1	2128.119	2037.934	0.000
Construct	43.943	2	23.472	22.477	0.000
SD	1359.540	8	169.942	162.741	0.000
Const*SD	95.118	16	5.945	5.693	0.000
Error	817.650	783	1.044		
Total	6422.000	810			
Corr. Total	3003.610	809			

Height Increment (SD0-SD8): Exp 2					
Source	Type III SS	df	MS	F	Sig.
Model	15121.302	26	581.589	587.014	0.000
Intercept	7608.757	1	7608.757	7679.744	0.000
Construct	18.008	2	9.004	9.088	0.000
SD	9579.193	8	1197.399	1208.570	0.000
Const*SD	60.005	16	3.750	3.785	0.000
Error	775.763	783	0.991		
Total	27957.250	810			
Corr. Total	15897.065	809			

Leaf# Increment (SD0-SD8): Exp 2					
Source	Type III SS	df	MS	F	Sig.
Model	2894.236	26	111.317	358.687	0.000
Intercept	1767.646	1	1757.646	5695.749	0.000
Construct	15.502	2	7.751	24.976	0.000
SD	1845.845	8	230.731	743.465	0.000
Const*SD	23.820	16	1.489	4.797	0.000
Error	243.000	783	0.310		
Total	5941.000	810			
Corr. Total	3137.236	809			

Height Increment (SD0-SD8): Exp 3					
Source	Type III SS	df	MS	F	Sig.
Model	10793.269	26	415.126	275.703	0.000
Intercept	10318.445	1	10318.445	6852.926	0.000
Construct	21.831	2	10.916	7.250	0.001
SD	7242.809	8	905.351	601.283	0.000
Const*SD	45.054	16	2.816	1.870	0.020
Error	1178.963	783	1.506		
Total	28190.750	810			
Corr. Total	11972.231	809			

Leaf# Increment (SD0-SD8): Exp 3					
Source	Type III SS	df	MS	F	Sig.
Model	2724.573	26	104.791	290.448	0.000
Intercept	3072.119	1	3072.119	8514.934	0.000
Construct	27.934	2	13.967	38.712	0.000
SD	1854.231	8	231.779	642.417	0.000
Const*SD	24.933	16	1.558	4.319	0.000
Error	282.500	783	0.361		
Total	7901.000	810			
Corr. Total	3007.073	809			

B

Chlorophyll a (LPI3,6): Exp 1					
Source	Type III SS	df	MS	F	Sig.
Model	393.340	5	78.668	34.079	0.000
Intercept	835.251	1	835.251	361.830	0.000
Construct	365.585	2	182.793	79.186	0.000
LPI	22.922	1	22.922	9.930	0.004
Const*LPI	6.097	2	3.049	1.321	0.286
Error	55.402	24	2.308		
Total	1531.023	30			
Corr. Total	448.742	29			

Carotenoids (LPI3,6): Exp 1					
Source	Type III SS	df	MS	F	Sig.
Model	15.623	5	3.125	3.991	0.009
Intercept	246.296	1	246.296	314.588	0.000
Construct	9.514	2	9.514	12.152	0.002
LPI	5.065	1	2.533	3.235	0.057
Const*LPI	1.439	2	0.719	0.919	0.413
Error	18.790	24	0.783		
Total	303.933	30			
Corr. Total	34.413	29			

Chlorophyll a (SDC8, 10.3, 12.4, 14.7): Exp 2					
Source	Type III SS	df	MS	F	Sig.
Model	1449.548	9	161.061	94.354	0.000
Intercept	4303.032	1	4303.032	2520.829	0.000
Construct	156.632	2	78.316	45.879	0.000
chilling hrs	1110.450	3	370.150	216.577	0.000
Const*chill	239.835	4	59.959	35.125	0.000
Error	141.680	83	1.707		
Total	10113.027	93			
Corr. Total	1591.229	92			

Carotenoids (SDC8, 10.3, 12.4, 14.7): Exp 2					
Source	Type III SS	df	MS	F	Sig.
Model	54.479	9	6.053	20.232	0.000
Intercept	611.883	1	611.883	2045.138	0.000
Construct	6.691	2	3.346	11.182	0.000
chilling hrs	36.242	3	12.081	40.378	0.000
Const*chill	9.595	4	2.399	8.018	0.000
Error	24.833	83	0.299		
Total	1178.530	93			
Corr. Total	79.312	92			

Chla (SDC123.4, 14.7): Exp 3					
Source	Type III SS	df	MS	F	Sig.
Model	921.271	5	184.254	72.333	0.000
Intercept	1335.111	1	1335.111	524.129	0.000
Construct	657.957	2	328.979	129.148	0.000
chilling hrs	161.565	1	161.565	63.426	0.000
Const*chill	1.646	2	0.823	0.323	0.726
Error	122.270	48	2.547		
Total	3581.331	54			
Corr. Total	1043.541	53			

Carotenoids (SDC123.4, 14.7): Exp 3					
Source	Type III SS	df	MS	F	Sig.
Model	17.566	5	3.513	24.944	0.000
Intercept	72.921	1	72.921	517.734	0.000
Construct	8.665	2	4.332	30.759	0.000
chilling hrs	0.028	1	0.028	0.202	0.655
Const*chill	8.169	2	4.084	28.999	0.000
Error	6.761	48	0.141		
Total	145.829	54			
Corr. Total	24.327	53			

C

Dormancy (SDC8, 10.3, 12,4, 14.7, 17, 20): Exp 1					
Source	Type III SS	df	MS	F	Sig.
Model	311678.083	17	18334.005	35.605	0.009
Intercept	314571.842	1	314571.842	610.899	0.000
Construct	26668.087	2	13334.043	25.865	0.000
Chilling	169668.442	5	33933.688	65.899	0.000
Const*Chill	68698.497	10	6869.850	13.341	0.000
Error	89598.229	174	514.932		
Total	805894.000	192			
Corr. Total	401276.313	191			

Dormancy (SDC8, 10.3, 12.4, 14.7, 17): Exp 2					
Source	Type III SS	df	MS	F	Sig.
Model	7536.644	14	538.332	46.824	0.000
Intercept	19763.333	1	19763.333	1719.004	0.000
Construct	1889.644	2	944.822	82.180	0.000
SD	3691.000	4	922.750	80.260	0.000
Const*SD	1268.633	8	158.579	13.793	0.000
Error	1897.000	165	11.497		
Total	34686.000	180			
Corr. Total	9433.644	179			

Dormancy (SDC8, 10.3, 12.4, 14.7, 17): Exp 3					
Source	Type III SS	df	MS	F	Sig.
Model	380602.949	14	27185.925	35.649	0.000
Intercept	452321.127	1	452321.127	593.127	0.000
Construct	20650.909	2	10325.454	13.540	0.000
SD	190654.707	4	47663.677	62.501	0.000
Const*SD	101874.236	8	12734.279	16.698	0.000
Error	160146.900	210	762.604		
Total	1136528.000	225			
Corr. Total	540749.849	224			

Dormancy (SD10.3, SD12.4, SDC10.3, SDC12.4): Exp 3					
Source	Type III SS	df	MS	F	Sig.
Model	471570.128	11	42870.012	27.570	0.000
Intercept	1097679.408	1	1097679.408	705.937	0.000
Construct	361448.028	2	180724.014	116.227	0.000
Chilling Temp	29.008	1	29.008	0.019	0.892
Chilling Hrs	21200.208	1	21200.208	13.634	0.000
Const*Temp	38190.028	2	19095.014	12.280	0.000
Const*Hrs	8290.961	2	4145.481	2.666	0.072
Temp*Hrs	848.008	1	848.008	0.545	0.461
Const*Temp*Hrs	12798.028	2	6399.014	4.115	0.018
Error	261227.400	168	1554.925		
Total	1991981.000	180			
Corr. Total	732797.528	179			

Dormancy (SD10.3, SD12.4, SDC10.3, SDC12.4): Exp 3					
Source	Type III SS	df	MS	F	Sig.
Model	519376.328	35	14839.324	10.012	0.000
Intercept	1259183.472	1	1259183.472	849.599	0.000
Line	373639.078	8	46704.885	31.513	0.000
Chilling Temp	920.272	1	920.272	0.621	0.432
Chilling Hrs	43090.139	1	43090.139	29.074	0.000
Line*Temp	54093.078	8	6761.635	4.562	0.000
Line*Hrs	18392.211	8	2299.026	1.551	0.145
Temp*Hrs	6832.672	1	6832.672	4.610	0.033
Line*Temp*Hrs	22408.878	8	2801.110	1.890	0.066
Error	213421.200	144	1482.092		
Total	1991981.000	180			
Corr. Total	732797.528	179			

Chilling temperature is 18 °C for SD10.3 and SD12.4

Chilling temperature is 10/4 °C (day/night) for SDC10.3 and SDC12.4

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