Genetics, Genomics and Breeding of Crop Plants

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GENETICS, GENOMICS AND BREEDING OF POPLAR

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CRC Press
Taylor & Francis Group
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www.crcpress.com

6000 Broken Sound Parkway, NW
Suite 300, Boca Raton, FL 33487
270 Madison Avenue
New York, NY 10016
2 Park Square, Milton Park
Abingdon, Oxon OX14 4RN, UK

Science Publishers
Enfield, New Hampshire

Published by Science Publishers, P.O. Box 699, Enfield, NH 03748, USA
An imprint of Edenbridge Ltd., British Channel Islands

E-mail: info@scipub.net Website: www.scipub.net

Marketed and distributed by:

CRC Press
Taylor & Francis Group
an informa business
www.crcpress.com

6000 Broken Sound Parkway, NW
Suite 300, Boca Raton, FL 33487
270 Madison Avenue
New York, NY 10016
2 Park Square, Milton Park
Abingdon, Oxon OX 14 4RN, UK

ISBN 978-1-57808-714-3

Library of Congress Cataloging-in-Publication Data

Genetics, genomics and breeding of poplar / editors, Chandrashekhar P. Joshi, Stephen P. DiFazio, Chittaranjan Kole. -- 1st ed.
p. cm. -- (Genetics, genomics and breeding of crop plants)
Includes bibliographical references and index.
ISBN 978-1-57808-714-3 (hardcover : alk. paper)
1. Poplar--Genetics. 2. Poplar--Genome mapping. 3. Poplar--Breeding. I. Joshi, Chandrashekhar P. II. DiFazio, Stephen P.
III. Kole, Chittaranjan. IV. Series: Genetics, genomics and breeding of crop plants.
QK495.S16G46 2011
634.9'723--dc22

2010051279

Printed in the United States of America

Dedication

Dr. Gopi Krishna Podila

September 14, 1957–February 12, 2010

This book is dedicated to the loving memory of Dr. Gopi Krishna Podila who was a stellar scientist, excellent teacher, kind-hearted mentor, enthusiastic colleague, and dear friend to so many of us in the poplar community.

11

Regulation of Flowering Time in Poplar

Cetin Yuceer,[1,a,] Chuan-Yu Hsu,[1,b] Amy M. Brunner[2] and Steven H. Strauss[3]*

ABSTRACT

Trees have provided and will continue to provide shelter, energy, fiber, food, and numerous other benefits for society. However, the lengthy juvenile period is a major obstacle to early and frequent sexual reproduction for development of pedigreed offspring to accelerate tree domestication. Although much is known about the factors regulating the onset of sexual reproduction in the annual model plant *Arabidopsis*, far less is known about this transition in trees. Recent advances in poplar are beginning to provide a fundamental understanding of the signaling mechanism by which the onset of sexual reproduction is determined in trees. This chapter provides an overview of knowledge about the genetic, physiological, and environmental factors that regulate first time and seasonal reproduction poplar, making reference to insights from *Arabidopsis*. Furthermore, we discuss the potential for practical applications of knowledge in trees gained from fundamental flowering research.

Keywords: flowering, reproduction, development, juvenility, maturity, poplar, *Populus*

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11.1 Introduction

Compared to food crops, forest tree improvement is in its infancy. Innate features of trees provide major barriers to breeding progress, most significant of which is the lengthy juvenile phase of 5 to 20 years before they are developmentally capable of flowering. The long delay in flowering and typically high genetic load of trees makes it infeasible to use advanced methods such as inbreeding and introgression of rare or exotic alleles. The net result is a very slow rate of domestication for all breeding goals. Transgenic approaches can potentially advance tree domestication, but concerns over the dispersal of transgenic pollen or seed, in addition to a number of other social and technical factors, have prevented most commercial uses of transgenic forest trees in the world (Brunner et al. 2007). Thus, understanding the factors that regulate tree flowering and discovering ways to manipulate it could enhance tree improvement by speeding breeding and research to develop effective means for genetic containment. Moreover, because flowering time is an adaptive trait that is affected by global warming (Fitter and Fitter 2002), discovery of the genes important for control of tree flowering might also aid in the development of strategies for maintaining healthy forest tree populations in the world with rapidly changing climates.

Poplar (*Populus* spp.) is economically and ecologically important, and is a model system for deciphering the molecular and physiological processes that regulate flowering time in trees. The main advantages of poplar compared to other trees include the rich variety of genomic resources available for it (e.g., whole-genome sequence; Tuskan et al. 2006), its amenability to *Agrobacterium*-mediated transformation (Han et al. 2000; Song et al. 2006; Cseke et al. 2007), and its well-studied developmental processes. Poplar and the annual herbaceous plant *Arabidopsis thaliana* are both angiosperms and eudicots, facilitating comparative genomics between these two taxa (Soltis et al. 1999; Wikstrom et al. 2001). Comparison of flowering genes and gene function and pathways between poplar and *Arabidopsis* will advance our understanding of how changes in gene number, expression, and interactions have resulted in drastically different floral morphologies and flowering habits.

This chapter will provide an overview of current knowledge about the genetic and physiological factors that control flowering time in poplar. We also discuss the potential for practical applications of knowledge gained from molecular flowering research.

11.2 Development and Architecture

Poplar has a life span of more than 100 years and a juvenile phase of approximately five years to more than a decade prior to the onset of

flowering (Braatne et al. 1996), indicating slow maturation. Juvenile trees form vegetative buds, leaves, and internodes. A terminal bud is formed at the end of each shoot every season and is enclosed by several layers of bud scales that are formed by the enlargement of stipules to protect the foliage primordia of the following season's growth (Goffinet and Larson 1981). Juvenile trees exhibit rapid growth rates with long internodes, continuous shoot growth throughout the growing season and terminal bud formation at the end of the growing season when the critical daylength for bud set occurs. Following the first annual production of reproductive buds, seasonal production of both vegetative and reproductive buds occurs during the reproductive developmental phase. Thus, poplar has developed a shoot architecture that accommodates both vegetative and reproductive growth throughout its life cycle.

The developmental state of leaves, the positions of axillary buds, and seasonal timing of axillary meristem initiation on a shoot are important factors in flower initiation. Thus, a model for the development and architecture of axillary bud meristems and their temporal and spatial formation in shoots of mature *P. deltoides* was developed (Yuceer et al. 2003). Shoots with flower buds in mature trees tend to have short internodes and early cessation of primary vegetative growth. Consequently, shoots begin forming a terminal bud approximately two months following spring bud flush. It is currently unknown why, or how, shoots that produce flower buds cease growth prematurely.

Mature shoots possess a defined developmental pattern that includes specific locations for vegetative and reproductive buds and distinct leaf types (Critchfield 1960; Boes and Strauss 1994; Yuceer et al. 2003). Shoots on adult trees produce buds in a sequential manner, each with an associated leaf type. Early vegetative buds (Vegetative Zone I) are produced in axils of early preformed leaves, reproductive buds (Floral Zone) are produced in axils of late preformed leaves, and late vegetative buds (Vegetative Zone II) are produced in axils of neoformed leaves. During the first growing season (Year 1), the terminal bud forms and contains the early preformed leaves and the late preformed leaf primordia. Early preformed leaves are initiated early in the development of the terminal bud during Year 1 and have a long developmental period which is interrupted by a cold period (vernalization) prior to expansion in the second growing season (Year 2). The preformed buds that develop in the axils of the early preformed leaves (Vegetative Zone I) never develop into reproductive buds and form vegetative shoots with true leaf primordia. Late preformed leaf primordia develop during the advanced stage of terminal bud development and stay in a primordial stage during vernalization. The buds that develop in axils of these leaves are reproductive.

Spring flowering phenology varies among species, genotypes, and populations, but the sequence of events is the same in all cases. A typical phenology for *Populus deltoidies* in Mississippi, USA is described below. The terminal bud opens in late March of Year 2 following the formation in Year 1. Reproductive buds in the Floral Zone, numbering from two to 10 on shoots, subsequently become visible in late-leaf axils. Examination of the spring bud meristems in the Floral Zone indicates morphological changes that have led to inflorescence shoot formation, floral meristem development, and organ formation. On the developing inflorescence (catkin) beginning late spring (May), bracts and then axillary floral meristems develop acropetally. By the winter of Year 2, the floral meristems form a cup-like, reduced perianth with stigmas or tetrasporangiate anthers in the axils of fully-elongated bracts. As an adaptation to wind pollination, reproductive bud flush occurs before vegetative bud flush in March of Year 3; catkins rapidly elongate and floral anthesis occurs. Female trees continue to form seeds until May of Year 3.

After all preformed leaves have expanded in spring of Year 2, some shoots may produce neoformed leaves that initiate and expand entirely within the current growing season. Thus, the neoformed leaves have not undergone vernalization. These leaves comprise Vegetative Zone II and bear vegetative buds in their axils. Following the formation of reproductive buds, as many as 40 vegetative buds form in Vegetative Zone II.

Although it is unknown when exactly the floral induction occurs, the flowering process may begin as signal perception in early vernalized preformed leaves of the first growing season, prior to flower bud formation during the second growing season. Equally important is the question of whether the floral signal is translocated to the shoot apical meristem (SAM) where bud fate is determined or to developing axillary buds. It is possible that the floral signal translocates to the developing buds in the late-leaf axils through direct vascular connections, given that a specific repeating pattern of primary vascular tissues exists between leaves and the nodes where buds form (Larson and Pizzolato 1977; Pizzolato and Larson 1977; Dickson 1986). The primary vascular connections are formed in the primordial stem tissues of the overwintering terminal bud as a continuation of acropetal elongation of the shoot (Larson 1975).

11.3 Flowering-Time Genes

11.3.1 Arabidopsis thaliana

Arabidopsis is the best studied annual plant model, particularly for its reproductive biology. *Arabidopsis* completes its life cycle in two months, with a short juvenile period followed by the production of flowers (Somerville

and Koornneef 2002). The SAM initially gives rise to vegetative organs such as leaves, but at some point the SAM is transformed into an indeterminate inflorescence meristem that produces floral buds on its flanks (Levy and Dean 1998). The main inflorescence shoot and axillary buds continuously produce reproductive organs, and then the plant dies. *Arabidopsis* does not undergo stages of seasonal vegetative and floral development at the reproductive phase, nor does it revert to the vegetative phase once it begins the reproductive phase (Boss et al. 2004).

A combination of environmental and developmental signals trigger flowering in *Arabidopsis*. The four major linked pathways that control flowering time are the photoperiodic, developmental, vernalization, and gibberellin pathways (Fig. 11-1). They transmit signals that regulate the expression of floral meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*), which control the formation of floral meristems (Weigel et al. 1992; Mandel and Yanofsky 1995).

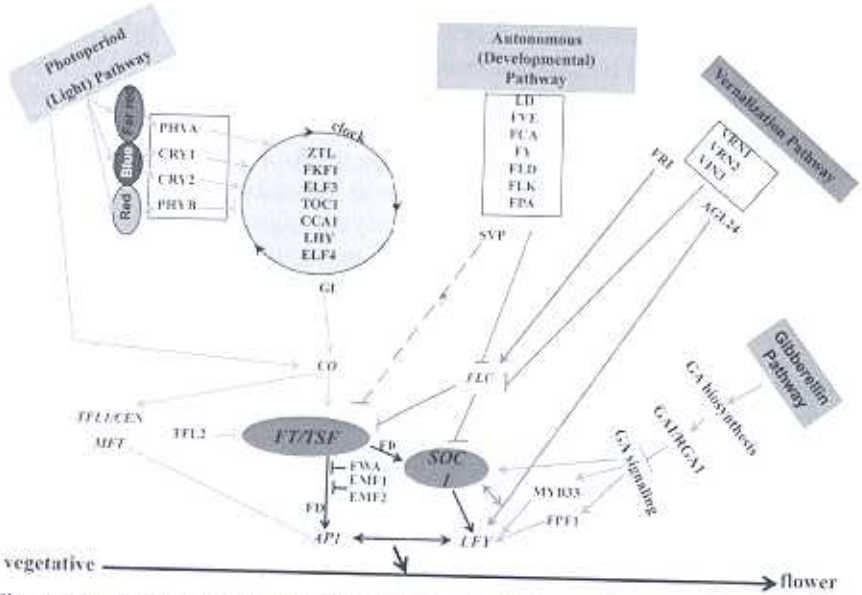

Figure 11-1 A simplified genetic network that controls flowering time in the annual plant *Arabidopsis*. *FT/TSF* and *SOC1* are floral integrators. Arrows indicate promotion and bars indicate repression.

Color image of this figure appears in the color plate section at the end of the book.

11.3.1.1 Photoperiodic Pathway

Duration of light period or photoperiod is one of the important environmental factors that control flowering in temperate plant species (Bernier et al. 1993;

Martinez-Garcia et al. 2002; Mouradov et al. 2002). Light signal is perceived by leaves and transported as a systemic signal or "florigen" to the shoot apex where floral development is induced (Knott 1934; Zeevaart 1976; Bernier and Perilleux 2005; Corbesier and Coupland 2005). *Arabidopsis* is a facultative long-day plant. Photoreceptors such as phytochromes and cryptochromes are involved in perception of light and mediate light input to the circadian clock (Goto et al. 1991; Johnson et al. 1994; Guo et al. 1998; Somers et al. 1998; Devlin and Kay 2000; Lin 2000). The far-red light sensor *PHYA* promotes flowering, but the red-light sensor *PHYB* inhibits flowering (Reed et al. 1993). PHYB is involved in degradation of CONSTANS (CO) protein early in the day (Valverde et al. 2004). Cryptochromes, CRY1 and CRY2, are blue light photoreceptors and encode flavoproteins (Lin et al. 1998; Cashmore et al. 1999). CRY2 is the main photoreceptor mediating day-length and flowering responses, perhaps by inhibiting *PHYB* signaling (Guo et al. 1998; Mockler et al. 1999; Mas et al. 2000).

The response of photoreceptors is integrated with clock entrainment factors such as *ZTL*, *FKF1*, and *ELF3* (Hicks et al. 1996; Zagotta et al. 1996; Somers et al. 2000). This results in the coordinated expression of the circadian-regulated genes such as *TOC1*, *CCA1*, *LHY*, and *ELF4*, which are the central components of the clock (Schaffer et al. 1998; Somers et al. 1998; Wang and Tobin 1998; Strayer et al. 2000). The clock then exerts its control of photoperiodic response by setting the rhythm of the flowering time genes *GIGANTEA* (*GI*) and *CO* (Putterill et al. 1995; Fowler et al. 1999; Park et al. 1999; Suarez-Lopez et al. 2001; Yanovsky and Kay 2002; Mizoguchi et al. 2005). Regulation of *CO* expression and activity is important for photoperiodic flowering. *Arabidopsis co* mutants are late flowering under long days, but flower at a similar time to wild-type under short days. Thus, *CO* promotes flowering under long days. High *CO mRNA* levels coincide with light in long days, but are largely confined to darkness in short days (Suarez-Lopez et al. 2001; Roden et al. 2002; Yanovsky and Kay 2002). Consequently, CO protein may not accumulate in darkness. Direct light activation of the encoded protein of *CO* also influences CO abundance or activity (Suarez-Lopez et al. 2001; Yanovsky and Kay 2002). CO protein is degraded in darkness, but light stabilizes it in the evening through cryptochromes and PHYA (Valverde et al. 2004). The promotion of flowering by *CO* requires *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*), previously described as AGL20) (Putterill et al. 1995; Borner et al. 2000; Lee et al. 2000; Onouchi et al. 2000; Samach et al. 2000; Wigge et al. 2005; Yoo et al. 2005).

The *FT* gene is activated by CO only under long days at the end of the day and promotes the transition from vegetative to reproductive phase (Kardailsky et al. 1999; Kobayashi et al. 1999; Samach et al. 2000; Suarez-Lopez et al. 2001). *FT* encodes a protein with similarity to mammalian

phosphatidylethanolamine binding proteins, indicating that *FT* plays a role in signaling (Kardailsky et al. 1999; Kobayashi et al. 1999). CO protein activates *FT* in the leaf phloem companion cells (Takada and Goto 2003; An et al. 2004; Ayre and Turgeon 2004). The FT protein then moves out of the phloem to the SAM where floral development is induced (Corbesier et al. 2007; Jaeger and Wigge 2007; Mathieu et al. 2007). In the nucleus of the SAM, FT forms a complex with FD (bZIP transcription factor), which upregulates the MADS-box transcription factor *AP1* to induce floral development (Abe et al. 2005; Wigge et al. 2005).

TWIN SISTER of FT (*TSF*) is the closest homolog of *FT* in *Arabidopsis* (82% amino acid similarity), and perhaps being products of a duplication event. *TSF* acts redundantly with *FT* in the same molecular pathway (Yamaguchi et al. 2005) and both are involved in flower induction, show similar patterns of mRNA diurnal oscillation, and respond to long-day photoperiods (Kardailsky et al. 1999; Kobayashi et al. 1999; Suarez-Lopez et al. 2001; Yanovsky and Kay 2002; Yamaguchi et al. 2005). However, *TSF* and *FT* do not appear to affect each other's transcription.

Although *TERMINAL FLOWER1* (*TFL1*) is closely related to *FT*, it determines the potential for continuous growth of the shoot apex, prolonging the vegetative stage (Alvarez et al. 1992; Bradley et al. 1997). Loss-of-function mutation in *TFL1* promotes earlier flowering, whereas constitutive overexpression (Pro_{35S}:*TFL1*) delays flowering under long days with a prolonged vegetative stage (Ohshima et al. 1997; Ratcliffe et al. 1998). CO upregulates *TFL1* in the inflorescence meristem in the center of the shoot apex (Simon et al. 1996). However, the TFL protein also moves into other parts of the meristem (Conti and Bradley 2007). Given that CO activates both *FT* and *TFL1*, and that both genes are highly similar, the function of *TFL1* may be to compete with *FT* in the shoot apex to prevent the conversion of the apex into a source of floral meristems (Ahn et al. 2006). This might occur via competitive binding of FT and TFL1 to FD.

TFL1 inhibits the activity of meristem identity genes *LFY* or *AP1* at the center of the shoot apex by delaying their upregulation and preventing the meristem from responding to *LFY* or *AP1* (Shannon and Meeks-Wagner 1991; Alvarez et al. 1992; Weigel et al. 1992; Bradley et al. 1997; Ratcliffe et al. 1998, 1999). In contrast, *LFY* and *AP1* prevent *TFL1* transcription in floral meristems on the apex periphery. *TFL2* represses *CO*-dependent activation of *FT* to restrict flowering in response to transient changes in *CO* activity if the long-day signal has not yet been perceived. The *SOC1* gene encodes a MADS-box protein and integrates the photoperiodic, autonomous, vernalization, and gibberellin pathways (Borner et al. 2000; Lee et al. 2000; Samach et al. 2000).

11.3.1.2 Autonomous (Developmental) Pathway

The autonomous pathway mediates flowering by monitoring the developmental stages of the plant. The homeodomain protein *LUMINIDEPENDENS* (*LD*) promotes flowering by reducing the levels of the floral repressor and MADS-box transcription factor *FLOWERING LOCUS C* (*FLC*) (Lee et al. 1994; Michaels and Amasino 1999). Other genes in the developmental pathway that primarily target *FLC* and that positively regulate flowering include *FVE*, *FCA*, *FY*, *FPA*, *FLOWERING LOCUS D* (*FLD*), and *FLOWERING LATE KH MOTIF* (*FLK*). *FCA*, *FPA*, and *FLK* are all RNA binding proteins (Macknight et al. 1997; Schomburg et al. 2001), whereas FY is a polyadenylation factor (Simpson et al. 2003; Lim et al. 2004; Mockler et al. 2004; Henderson et al. 2005; Metzger et al. 2005). *FCA* and *FY* regulate RNA processing of *FLC* (Simpson et al. 2003). FLD and FVE might play a role in histone deacetylation, because *FLD* is similar to the lysine-specific histone demethylase LSD1 (He et al. 2003; Ausin et al. 2004; Shi et al. 2004).

11.3.1.3 Vernalization Pathway

The vernalization pathway mediates low temperature signals that alter gene expression and induce flowering by reducing the levels of the floral repressor *FLC* (Michaels and Amasino 1999; Sheldon et al. 1999; Sheldon et al. 2000, 2002; Bastow et al. 2004; Searle et al. 2006). *FLC* appears to act at the shoot apex and in leaves to delay flowering, and is downregulated by *VIN3*, *VRN1*, and *VRN2* (Gendall et al. 2001; Levy et al. 2002; Sung and Amasino 2004). Conversely, *FRIGIDA* (*FRI*) upregulates *FLC*, which in turn delays flowering by reducing the expression of *FT* (Michaels and Amasino 2001). FLC protein directly binds to the regulatory regions of *FT* and *SOC1* prior to vernalization (Searle et al. 2006). This interaction appears to inhibit the formation of the systemic signal that is required to activate *SOC1*, which initiates the switch from vegetative to floral development. These observations indicate that flowering signals from vernalization and photoperiod pathways are integrated through the regulation of *FT* and *SOC1*.

11.3.1.4 Gibberellin Pathway

Arabidopsis eventually flowers under non-inductive short days, despite an absence of *FT* signaling. Genetic studies indicate that gibberellins (GA) control flowering under short days, therefore compensating for the absence

of *FT* signaling. For example, the *GA1* gene is involved in GA biosynthesis, and a mutation in this gene (*ga1-3*) results in plants that are severely dwarfed, unable to flower under short days, and strongly enhances the *co2* mutation under long days because the *co2 ga1-3* double mutant never flowers (Wilson et al. 1992; Reeves and Coupland 2001). *ga1-3* mutants carry a deletion of the gene encoding the enzyme *ent*-copalyl diphosphate synthase (formerly *ent*-kaurene synthetase A) that catalyzes the first step in GA biosynthesis (Sun and Kamiya 1994). Overexpression of *LFY* and *SOC1* restores flowering of the *ga1-3* mutants under short days (Blazquez et al. 1998; Moon et al. 2003). This suggests that GAs promote flowering in *Arabidopsis* through a pathway that controls *LFY* and *SOC1* transcription (Blazquez et al. 1998). The *LFY* promoter contains *cis*-elements (e.g., the 8-base-pair CAACTGTC motif) involved in GA_3 response (Balazquez and Weigel 2000). *GAMYB*-like genes (e.g., *AtMYB33*) bind to the *LFY* promoter.

GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GA1-3 (RGA), RGA-LIKE 1 (RGL1), RGL2, and *SPINDLY (SPY)* negatively regulate the GA signaling pathway and play a role in control of flowering (Jacobsen and Olszewski 1993; Dill and Sun 2001; Cheng et al. 2004; Tyler et al. 2004). RGL1 is predicted to function in repressing GA responses in the inflorescence, given that in the absence of the DELLA domain of RGL1, sepals, petals, and stamens are underdeveloped and the flowers are male sterile (Wen and Chang 2002). The DELLA domain is a conserved sequence near the N-termini of RGA, GAI, and RGL1, and plays a role in GA response (Wen and Chang 2002). If the DELLA domain is removed, GAI is insensitive to GA (Peng et al. 1997). This causes repression of shoot growth and flowering in the presence of GA. The *spy* mutant shows an early flowering phenotype (Jacobsen and Olszewski 1993), possibly because of the increased activity in the GA signaling pathway. The *SPY* gene is highly similar to Ser/Thr O-linked N-acetylglucosamine transferases in rats and humans (Olszewski et al. 2002). This suggests that SPY may play a role in post-translational modification of unknown downstream proteins.

11.3.2 Poplar

The molecular basis of "first-time" and "seasonal" reproduction is poorly understood in poplar. Using the protein sequences of *Arabidopsis* flowering-time genes, a search in the poplar genome database was conducted (*http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html*). Each of these *Arabidopsis* genes was found to have at least one corresponding poplar homolog (Table 11-1) and in some cases many poplar homologs. For example, some transcription factor homologs such as *FWA, GAI/RGA1, CO,* and MADS-box proteins consist of large families of genes in poplar. To help resolve the phylogenetic relationships between well characterized flowering control

Table 11-1 Genes involved in transition to flowering in *Arabidopsis thaliana* (At) and their closest homologs in *Populus trichocarpa* (Pt).

Annotation	At (gene ID)	Pt (protein ID)	Pt (gene ID)
PHYTOCHROME A (PHYA)	At1g09570	729311	estExt_Genewise1_v1.C_LG_XIII0395
PHYTOCHROME B (PHYB)	At2g18790	832686	estExt_fgenesh4_pm.C_LG_VIII0434
		1091155	estExt_Genewise1Plus.C_LG_X3762
CRYPTOCHROME 1 (CYR1)	At4g08920	559103	eugene3.00050718
		830225	estExt_fgenesh4_pm.C_LG_II0442
CRYPTOCHROME 2 (CYR2)	At1g04400	1119034	estExt_Genewise1Plus.C_2730024
		803751	fgenesh4_pm.C_LG_VIII000706
ZEITLUPE (ZTL)	At5g57360	809263	fgenesh4_pm.C_LG_XVIII000281
		580505	eugene3.01210044
FLAVIN-BINDING KELCH DOMAIN F BOX PROTEIN (FKF1)	At1g68050	822050	estExt_fgenesh4_pg.C_LG_X0958
		564672	eugene3.00081267
ELF3	At2g25920	1099051	estExt_Genewise1Plus.C_LG_XIV1951
		409304	gw1.II.6391.1
PSEUDO-RESPONSE REGULATOR 1 (TOC1)	At5g61380 (TOC1; APRR1)	784463	fgenesh4_pg.C_scaffold_129000038
	At5g60100 (APRR3)	824063	estExt_fgenesh4_pg.C_LG_XIV1968
	At5g24470 (APRR5)	832516	estExt_fgenesh4_pm.C_LG_VIII0151

Table 11-1 contd....

Table 11-1 contd....

Annotation	At (gene ID)	Pt (protein ID)	Pt (gene ID)
	At2g46790 (APRR9)	755476	fgenesh4_pg.C_LG_II001656
	At5g02810 (APRR7)	227771	gw1.X.2468.1
	At4g31920 (ARR10)	422771	gw1.XII.1231.1
	At3g16857 (ARR1)	574629	eugene3.00150024
		725513	estExt_Genewise1_v1.C_LG_X3573
		231691	gw1.X.6388.1
		419669	gw1.VIII.1097.1
		825773	estExt_fgenesh4_pg.C_LG_XVIII0466
		732723	estExt_Genewise1_v1.C_LG_XV0053
		575747	eugene3.00151142
		230318	gw1.X.5015.1
		419184	gw1.VIII.612.1
		763573	fgenesh4_pg.C_LG_VII001883
		415998	gw1.VI.371.1
		262782	gw1.XVIII.3323.1
CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)	At2g46830 (CCA1)	552368	eugene3.00021683
LATE ELONGATED HYPOCOTYL (LHY)	At1g01060 (LHY)	731468	estExt_Genewise1_v1.C_LG_XIV1950
		552367	eugene3.00021682
		198884	gw1.IV.3973.1
		569412	eugene3.00120065
		784079	fgenesh4_pg.C_scaffold_122000043
		256693	gw1.XVI.2632.1
		289426	gw1.44.639.1
		281820	gw1.28.394.1
		409823	gw1.II.1158.1
		572303	eugene3.00140348
		198916	gw1.IV.4005.1
GIGANTEA (GI)	At1g22770	818606	estExt_fgenesh4_pg.C_LG_VI131
		551288	eugene3.00020603
FLOWERING LOCUS T (FT)	At1g65480 (FT)	582519	eugene3.14090001
	At4g20370 (TSF)	765657	fgenesh4_pg.C_LG_VIII000671
	At1g18100 (MFT)	805395	fgenesh4_pm.C_LG_X000701
		790700	fgenesh4_pg.C_scaffold_1444000001
		775913	fgenesh4_pg.C_LG_XV000341
		573457	eugene3.00141502
TERMINAL FLOWER 1 (TFL1)	At5g03840	827246	estExt_fgenesh4_pg.C_660171
	At2g2755 (ATC)	648937	grail3.0001004901
	At5g62040 (BFT)	575797	eugene3.00151192
TERMINAL FLOWER 1 (TFL2)	At5g17690	738591	estExt_Genewise1_v1.C_LG_XIX1329
		571324	eugene3.00130688
FD	At4g35900	424191	gw1.57.158.1
	At2g17770	818828	estExt_fgenesh4_pg.C_LG_VI569
		642918	grail3.0003013801
SUPPRESSION OF OVEREXPRESSION OF CONSTANS 1 (SOC1)	At2g456609 (SOC1)	730942	estExt_Genewise1_v1.C_LG_XIV0937

Table 11-1 contd....

Table 11-1 contd....

Annotation	At (gene ID)	Pt (protein ID)	Pt (gene ID)
	At5g62165 (AGL42)	644373	grail3.0033033502
	At4g11880 (AGL14)	640573	grail3.0008056401
	At4g22950 (AGL19)	554289	eugene3.00030922
	At5g51870 (AGL71)		
	At5g51860 (AGL72)		
LUMINIDEPENDENS (LD)	At4g02560	572730	eugene3.00140775
FVE	At2g19520	592758	eugene3.00440093
	At4g29730	271079	gw1.145.113.1
	At2g16780	589194	eugene3.02850001
	At4g35050	200694	gw1.IX.1159.1
	At5g58230	818199	estExt_fgenesh4_pg.C_LG_IV1464
	At2g19540	653315	grail3.0112007001
		816770	estExt_fgenesh4_pg.C_LG_II1945
		824422	estExt_fgenesh4_pg.C_LG_XIV1179
		207387	gw1.V.2788.1
		217471	gw1.VII.1776.1
FCA	At4g16280	225956	gw1.X.653.1
	At2g47310	755673	fgenesh4_pg.C_LG_II001853
		799273	fgenesh4_pm.C_LG_II000962
		246020	gw1.XIV.2763.1
		572009	eugene3.00140054
		829855	estExt_fgenesh4_pm.C_LG_II1075
FY	At5g13480	178488	gw1.I.7088.1

		415574	gw1.III.2677.1
		725686	estExt_Genewise1_v1.C_LG_X3939
		835833	estExt_fgenesh4_pm.C_LG_XVIII0164
		561983	eugene3.00061942
		803047	fgenesh4_pm.C_LG_VIII000002
FPA	At2g43410	292764	gw1.64.416.1
	At4g12640	802569	fgenesh4_pm.C_LG_VII000055
	At1g27750	247179	gw1.XIV.3922.1
		243881	gw1.XIV.624.1
SHORT VEGETATIVE PHASE (SVP)	At2g22540 (SVP)	719775	estExt_Genewise1_v1.C_LG_VII4001
MADS-box protein (AGL24)	At4g24540 (AGL24)	643871	grail3.0003092401
		651191	grail3.0083000102
		871682	e_gw1.VII.1214.1
		838041	APOLLO_200
		763951	fgenesh4_pg.C_LG_VII000296
		258177	gw1.XVII.168.1
		778182	fgenesh4_pg.C_LG_XVII000061
REDUCED VERNALIZATION RESPONSE 1 (VRN1)	At3g18990	267709	gw1.130.31.1
	At4g33280	267711	gw1.130.33.1
		791723	fgenesh4_pg.C_scaffold_2789000001
		649491	grail3.0001051501
		784500	fgenesh4_pg.C_scaffold_130000008
		744205	estExt_Genewise1_v1.C_1300033

Table 11-1 contd....

Table 11-1 contd....

Annotation	At (gene ID)	Pt (protein ID)	Pt (gene ID)
		770235	fgenesh4_pg.C_LG_X001725
		783236	fgenesh4_pg.C_scaffold_86000096
		591695	eugene3.00400040
		581458	eugene3.01300012
		764626	fgenesh4_pg.C_LG_VII000971
		779701	fgenesh4_pg.C_LG_XVIII001069
		583676	eugene3.01520080
		286795	gw1.40.555.1
		755009	fgenesh4_pg.C_LG_II001189
		781471	fgenesh4_pg.C_scaffold_40000221
		551891	eugene3.00021206
		551892	eugene3.00021207
REDUCED VERNALIZATION RESPONSE 2 (VRN2)	At4g16845 (VRN2)	830729	estExt_fgenesh4_pm.C_LG_III0191
	At5g51230 (EMF2)	814985	estExt_fgenesh4_pg.C_LG_I0694
	At2g35670 (FIS2)	412944	gw1.III.47.1
	At4g16810	554592	eugene3.00031225
	At4g16807		
FRIGIDA (FRI)	At4g00650	253112	gw1.XV.2548.1
		710332	estExt_Genewise1_v1.C_LG_III377
		664960	grail3.0012039001
		552423	eugene3.00021738
FLOWERING LOCUS C (FLC)	At5g10140 (FLC)	647039	grail3.0047013603
	At1g77080 (FLM)	680171	grail3.0690000103

	At5g65050 (AGL31)	647042	grail3.0047013701
	At5g65060 (AGL70)	840315	e_gw1.1.7774.1
	At5g65070 (AGL69)	288710	gw1.4342.4.1
	At5g65080 (AGL68)	294451	gw1.690.3.1
FWA	At4g25530 (FWA)	774292	fgenesh4_pg.C_LG_XIV000202
	At5g52170 (HDG7)	552123	eugene3.00021438
	At3g61150 (HDG1)	575801	eugene3.00151195
	At4g00730 (ANL2)	773381	fgenesh4_pg.C_LG_XII001156
		591580	eugene3.04030001
EMBRYONIC FLOWER 1 (EMF1)	At5g11530	763364	fgenesh4_pg.C_LG_VI001674
		825684	estExt_fgenesh4_pg.C_LG_XVIII0291
EMBRYONIC FLOWER 2 (EMF2)	At5g51230 (EMF2)	830729	estExt_fgenesh4_pm.C_LG_III0191
	At4g16845 (VRN2)	814985	estExt_fgenesh4_pg.C_LG_I0694
	At2g35670 (FIS2)	412944	gw1.III.47.1
	At4g16810	554592	eugene3.00031225
	At4g16807		
GA INSENSITIVE (GAI)	At1g14920 (GAI)	803616	fgenesh4_pm.C_LG_VIII000571
REPRESSOR OF GA1 (RGA1)	At2g01570 (RGA1)	726855	estExt_Genewise1_v1.C_LG_X6481
	At1g66350 (RGL1)	411207	gw1.II.2542.1
	At3g03450 (RGL2)	831148	estExt_fgenesh4_pm.C_LG_IV0241
	At5g17490 (RGL3)		
APETALA 1 (AP1)	At1g69120 (AP1)	719996	estExt_Genewise1_v1.C_LG_VIII0399
	At1g26310 (CAL)	659188	estExt_Genewise1_v1.C_LG_VIII0399

Table 11-1 contd....

Table 11-1 contd....

Annotation	At (gene ID)	Pt (protein ID)	Pt (gene ID)
	At5g60910 (FUL)	661810	grail3.0042013901
	At3g30260 (AGL79)	1076378	estExt_GenewisePlus.C_LG_IV3240
		745710	estExt_Genewise1_v1.C_1550090
LEAFY (LFY)	At5g61850	835248	estExt_fgenesh4_pm.C_LG_XV0337
CONSTANS (CO)	At5g15840 (CO)	266027	gw1.123.49.1
	At5g15850 (COL1)	831202	estExt_fgenesh4_pm.C_LG_IV0339
	At3g02380 (COL2)		

genes and their poplar homologs, we conducted phylogenetic analyses. Based on the Neighbor-Joining method (Saitou and Nei 1987), three genes closely cluster with *FWA* in *Arabidopsis*, but five genes are present in the same clade with *FWA* in poplar (Fig. 11-2). RGA1 and GAI belong to the GRAS family of transcription factors and cluster with three other RGL proteins in *Arabidopsis*, whereas there are four DELLA domain poplar proteins in this cluster (Fig. 11-3). Interestingly, a group of six poplar proteins form a sister group to the DELLA protein group, but lack a DELLA domain. A total of 16 CO-like (COL) proteins (including CO) are present in *Arabidopsis*, and COL1 and COL2 closely cluster with CO (Fig. 11-3). Two zinc finger-containing proteins in poplar show high similarity to the *Arabidopsis* CO protein in the same clade (Yuceer et al. 2002; Fig. 11-4). An analysis of the evolutionary relationship among the MADS-box proteins in poplar (Leseberg et al. 2006) showed that many poplar gene families have expanded due in part to gene duplications occurring after the divergence of *Arabidopsis* and poplar (Tuskan et al. 2006). The closest poplar homologs of *Arabidopsis* FLC, SVP, SOC1, and AP1 proteins are individually grouped in Fig. 11-5.

11.3.2.1 LFY May Play a Role in Poplar Flowering Time

Overexpression of the *Arabidopsis LFY* gene regulated by the CaMV 35S promoter (Pro_{35S}:*LFY*) caused early flowering in a male poplar (*P. tremula* x *P. tremuloides*) (Weigel and Nilsson 1995). However, Pro_{35S}:*LFY* did not consistently produce early flowering in other poplar genotypes (Rottmann et al. 2000), nor did it produce normal inflorescences and viable gametes. Four of seven lines flowered within six months, but flowering was observed primarily in males (*P. tremula* x *P. tremuloides*). Only two of 19 lines of a female poplar clone (*P. tremula* x *P. alba*) transformed with this construct flowered, doing so after two years of growth. Single flowers in these lines also formed anthers, suggesting that *LFY* may promote male flowering in poplar. A *LFY-like* gene, *PTLF*, is the only copy of a gene with substantive resemblance to *LFY* in the poplar genome (Rottmann et al. 2000). A construct with the native poplar *LFY* homolog under the 35S promoter (Pro_{35S}:*PTLF*) did not cause early flowering in the female clone, and only one of 16 transformed males produced unitary flowers without evidence of viable pollen production (Rottmann et al. 2000).

11.3.2.2 FT1 and FT2 Control Flowering Time

FLOWERING LOCUS T1 and *T2* (*FT1* and *FT2*) are major players in "first-time" and "seasonal" reproduction in poplar, and their transcription is controlled by developmental and environmental factors

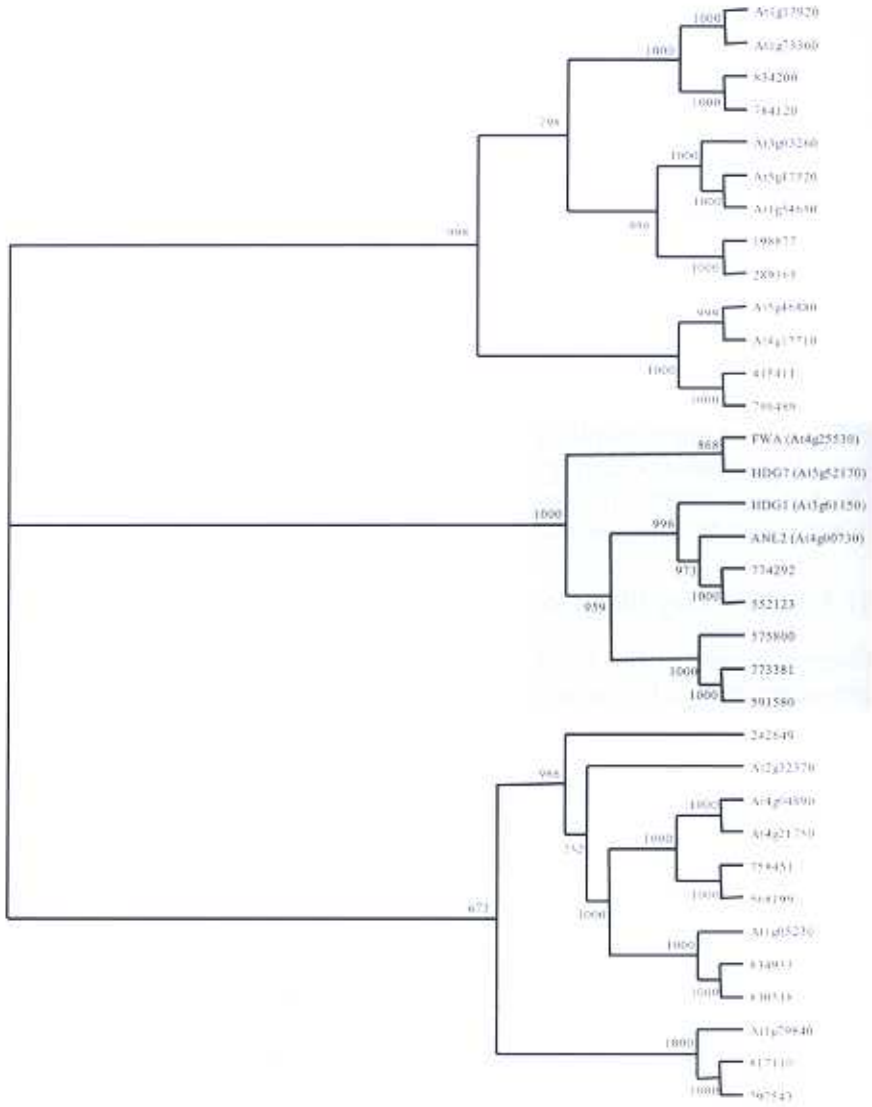

Figure 11-2 Phylogenetic analysis of the FWA family proteins in *Arabidopsis thaliana* (At) and *Populus trichocarpa* using the Neighbor-Joining method. Gray shading indicates the clade with close homologs of FWA. Bootstrap analysis was conducted to estimate nodal support using 1,000 replicates.

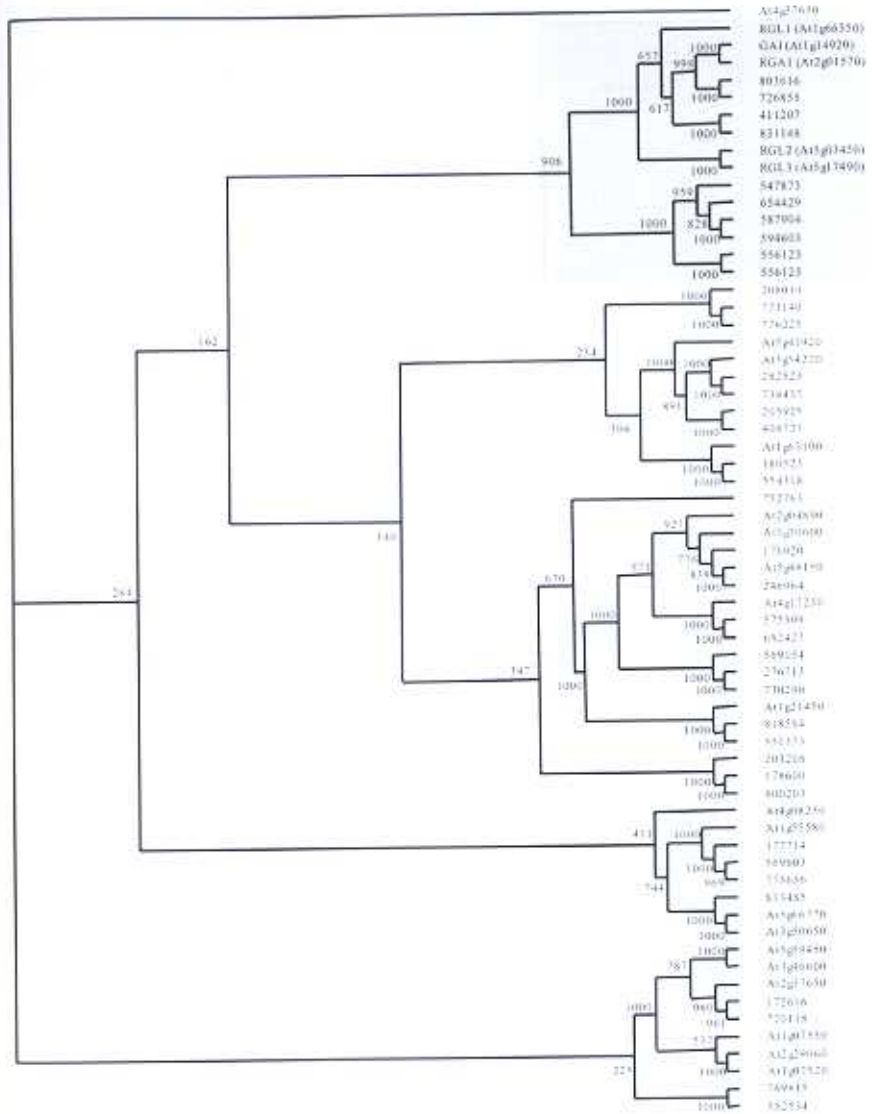

Figure 11-3 Phylogenetic analysis of GRAS family proteins in *Arabidopsis thaliana* (At) and *Populus trichocarpa* using the Neighbor-Joining method. Gray shading indicates the clade with close homologs of GAI/RGA1. Bootstrap analysis was conducted to estimate nodal support using 1,000 replicates.

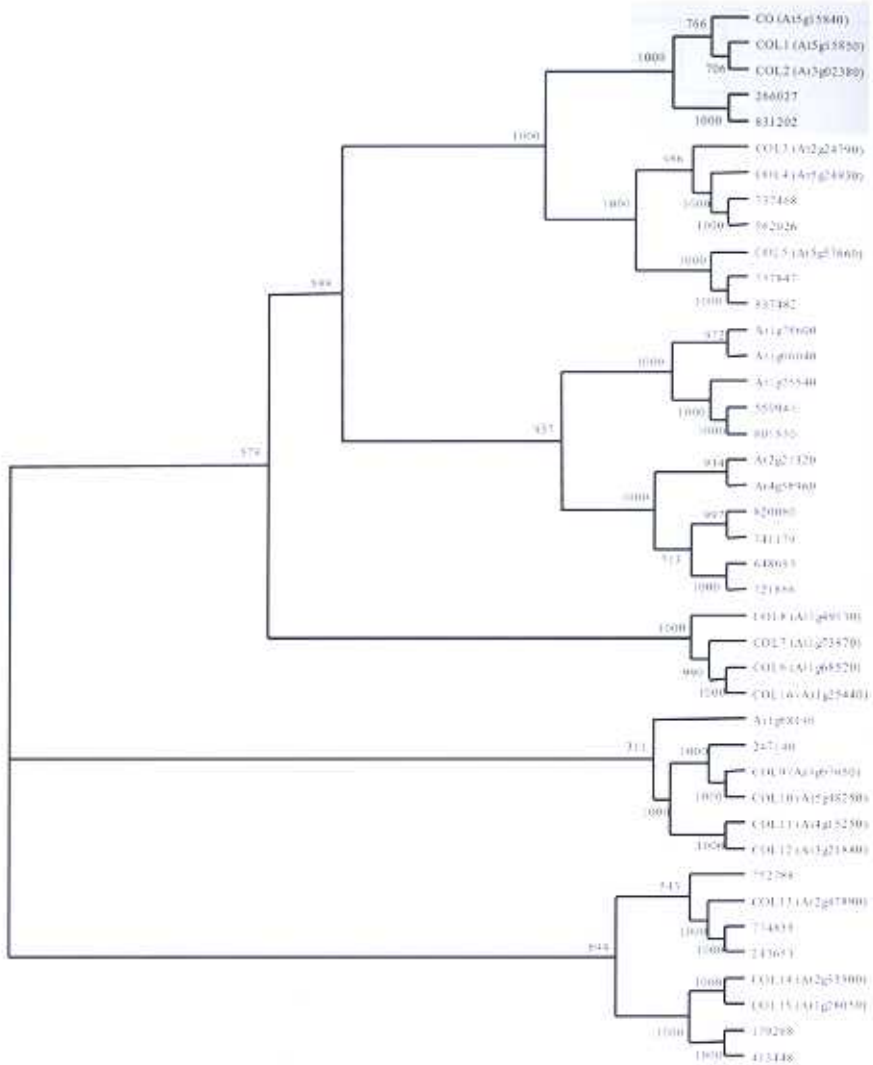

Figure 11-4 Phylogenetic analysis of the CONSTANS (CO) family proteins in *Arabidopsis thaliana* (At) and *Populus trichocarpa* using the Neighbor-Joining method. Gray shading indicates the clade with close homologs of CO. Bootstrap analysis was conducted to estimate nodal support using 1,000 replicates.

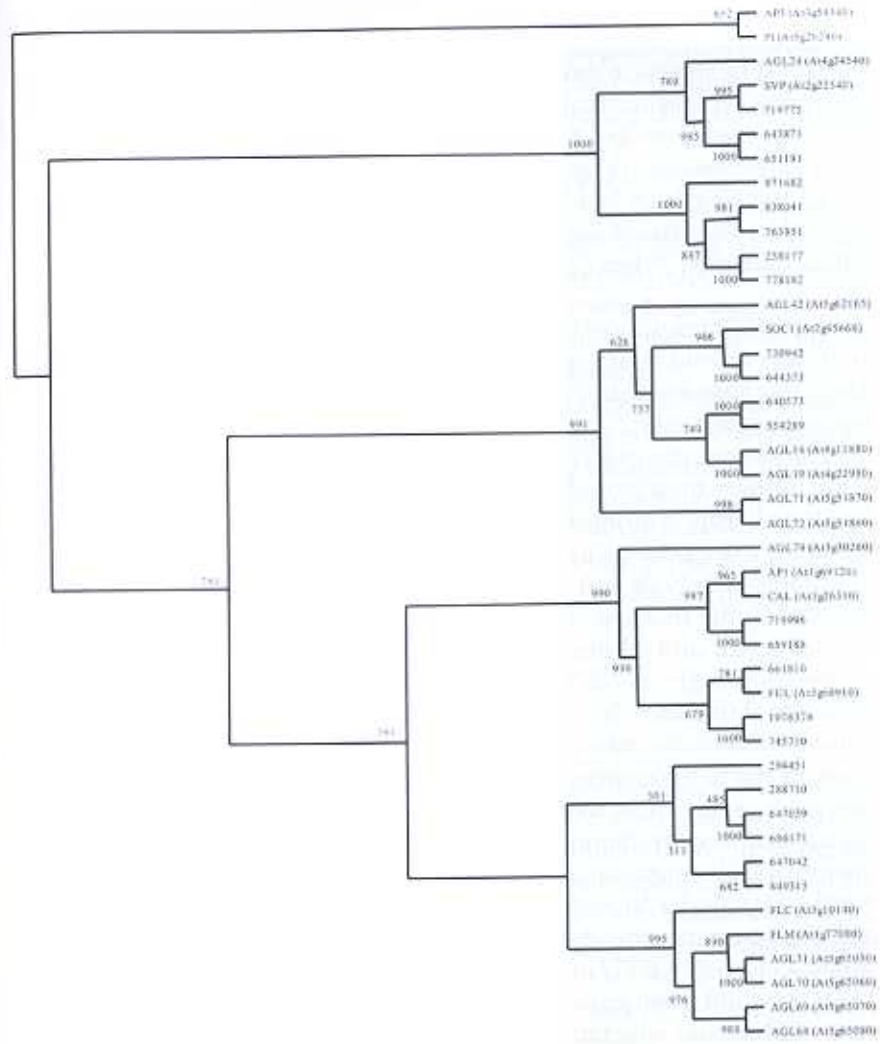

Figure 11-5 Neighbor-Joining phylogenetic analysis of *Arabidopsis thaliana* (At) and *Populus trichocarpa* MADS domain family proteins involved in flowering. The clades with close homologs of FLC, SVP, SOC1, and AP1 subfamilies in both *Arabidopsis* and poplar are individually gray-boxed. Bootstrap analysis was conducted to estimate nodal support using 1,000 replicates.

(Bohlenius et al. 2006; Hsu et al. 2006). *FT1* and *FT2* are in the same gene family with 91% amino acid sequence similarity. *FT2* mRNA was detected at background levels in roots and the shoot apex (Hsu et al. 2006). However, its expression was most abundant in leaf 11 (from the base of the shoot) and in the bud in its axil that was destined to be reproductive, suggesting that *FT2* expression is upregulated in leaves and buds. The abundance of *FT2* transcripts in leaf 11 increased from the juvenile to reproductive developmental phases, suggesting that *FT2* might play a role in juvenile to mature transition. When Pro_{35S}:*FT1* and Pro_{35S}:*FT2* constructs were separately inserted into juvenile poplar, trees produced flowers within several months. The Pro_{35S}:*FT1* trees were not, however, induced to enter dormancy under short days or cold temperatures such as in wild type trees (Bohlenius et al. 2006). This suggests that flowering and dormancy induction share common regulatory elements.

The abundance of *FT2* transcript in leaf 11 was low from February to April, but was high in mid-May (Hsu et al. 2006). During this time, leaves developed from a primordial preformed leaf to a fully expanded leaf. Beginning in mid-May, *FT2* transcript was abundant in bud 11 which formed an inflorescent shoot and floral meristems on its flanks. Potential factors involved in the increase of *FT2* transcript in leaves include temperature, development, and photoperiod. Poplar trees were treated under two temperature regimes (23°C and 38°C) to determine if this affected *FT2* transcript abundance. No change, however, was observed in the expression pattern of *FT2* under either temperature regime, suggesting that temperature is not a factor in the expression pattern of *FT2* (Hsu et al. 2006). When poplar trees were grown under long (14 hours) and short (8 hours) days for 14 days, *FT2* transcripts were abundant under long days throughout the experiment, whereas they were either at background levels (first 7 days) or undetectable after 14 days under short days (Hsu et al. 2006). These results suggest that long days promote the abundance of *FT2* transcript. The poplar genome contains at least two *FD* orthologs and all transgenic lines overexpressing *PtFD1* flowered when grown in a long day-length greenhouse, but flowering was not observed when transgenics were grown under short day-lengths (G. Coleman, pers. comm.). The *FT2* and *PtFD1* results suggest that long photoperiods promote floral bud formation in poplar.

Photoperiod controls many aspects of poplar growth and development including growth cessation and winter dormancy (Pauley and Perry 1954; Howe et al. 1995, 1996; Olsen et al. 1997). Reports indicate that photoperiod is a physiological stimulus that triggers flower bud initiation in woody perennial plants (Junttila 1980; Rivera and Borchert 2001). In the related species, *Salix pentandra*, flower bud formation was maximally promoted by photoperiods of 18 to 22 hours (Junttila 1980). However, detailed molecular studies have yet to be conducted to complete understanding of

how photoperiod controls flowering in poplar. A major barrier has been the lack of naturally occurring early-flowering poplar genotypes that can be easily moved and studied in various controlled environments, but as the *FT* and *PtFD1* results show, use of early-flowering transgenics is likely to be useful in circumventing this problem.

Study of *PopCEN1*, a poplar homolog of snapdragon *CENTRORADIALIS (CEN)* and *TFL1* from *Arabidopsis* revealed a conserved role in repressing flowering (Mohamed et al. 2010). Downregulation of *PopCEN1* via RNAi did not induce the extreme early flowering seen in poplar transgenics overexpressing *FT1*, *FT2*, or *PtFD1*, but a multi-year field study revealed that suppression of *PopCEN1* did promote an earlier onset of flowering and a markedly increased number of lateral inflorescences.

These few examples show the power of transgenic manipulation in poplar and of comparative genomics, especially when whole genome sequences are available. Moreover, combining transgenesis, microarray expression analysis, protein-protein interaction studies, and other -omics approaches should reveal the transcription-based regulatory networks controlling flowering in poplar and thus, how these genes and pathways are modified to yield the dramatically different flowering habits of poplar and *Arabidopsis*.

11.4 Practical Applications

The rationale, projected benefits, and mechanisms for the manipulation of flowering via genetic engineering have been widely discussed, most recently in an extensive review by Brunner et al. (2007). They are: 1) Improved vegetative growth by removal or reduction of inflorescences, floral organs, and fruits as sinks for carbon and nutrients. The evidence that this could be substantial in some species and circumstances was discussed in depth by Strauss et al. (1995); 2) Containment of genes or exotic organisms by suppression of floral onset, floral organ or fruit function, or by transgene removal during gametogenesis. A very wide variety of options have been shown to work in *Arabidopsis*, tobacco, or other model annual plants. The only evidence that these kinds of genes can be effective in substantially reducing fertility in a field environment was presented by Brunner et al. (2007) with reference to poplars containing a gene for male sterility (Pro_{TA29}:*barnase*); Finally, 3) Acceleration of flowering to speed breeding or research has been a long sought goal in tree breeding, for which hormone treatments have been highly effective in conifers and some other woody species, but not in poplars (Meilan 1997). However, as discussed above, the transgenic approaches attempted to date have given unsatisfying results with respect to consistent production of viable gametes and seeds. The more normal appearance of catkins with *FT1* induction of flowering in poplar

(Bohlenius et al. 2006), and the graft-transmissibility of the *FT* signal protein, have inspired hope that transgenic rootstocks might be useful for inducing rapid flowering of grafted scions. The *FT*-associated inductive signal can be transmitted from leaves to shoot apical meristems, as demonstrated using intra- and inter-specific grafting experiments (Imaizumi and Kay 2006; Zeevaart 2006). Such a tactic could avoid the regulatory or environmental concerns of transgene deployment in production forests. However, graft induction of *FT*-associated flowering has yet to be demonstrated in woody species, and at least in Germany—where labeling of transgenic associated products is required by the EU whether transgenes persist or not—such a tactic would not be likely to obviate regulatory oversight of derived non-trangenic seeds and forests (M. Fladung, pers. comm.).

The process-based regulatory oversight of all transgenic products in the USA and most other countries, where transgenes are assumed to be dangerous until proven otherwise on a case by case basis, makes it extremely difficult to do the required field research evaluations to assess the level of fertility reduction, postponement, or precocious induction under conditions relevant to commercial forestry programs. This is because genetic dispersal of even minute amounts of as little as fertility-reducing genes is not permitted, yet it is very difficult to fully guarantee this during the course of multiple year research in large, flowering trees. Until there is substantial regulatory reform that takes into account the risks of specific classes of genes, as has been proposed earlier many times and in many ways (e.g., Hancock 2003; Strauss 2003a, b; Bradford 2005)—and is now under active consideration in the USA (USDA 2007)—research to develop practical applications for trees that have been genetically engineered for modified flowering characteristics will proceed very slowly and at great expense, if it can proceed at all.

Acknowledgments

The authors thank the NSF Plant Genome Research Program, USDA-NRI Plant Biology Program, USDA Biotechnology Risk Assessment Grants Program, NSF Industry/University Centers, and the industrial members of the Tree Biosafety and Genomics Research Cooperative based at Oregon State University for supporting our research on flowering.

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