# Flowering Gene Homologs Regulate Seasonal Growth Changes in Poplar

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## ABSTRACT

The adaptation of trees to temperate and boreal climates depends on their ability to respond to environmental signals that are markers of seasonal changes in order to survive winter and maximize growth. The genus *Populus* (poplars) is a model system for identifying the genes and molecular mechanisms that regulate growth and dormancy transitions. Poplar homologs of the flowering time genes *FLOWERING LOCUS T* (*FT*) and *FLOWERING LOCUS D* (*FD*) play important roles in photoperiod- and temperature-mediated control of growth and dormancy transitions and flowering. The distinct functions of the three poplar *FD-LIKE (FDL)* genes (*FDL1*, *FDL2*, and *FDL3*), including two *FDL2* splice variants), and the two *FT*s (*FT1* and *FT2*) were explored through gain of function, dominant repression, and CRISPR/CAS9-induced mutation. Dominant repression of each of the three poplar *FDL* homologs induced varying degrees of reduced shoot elongation, suggesting that their proteins share less than complete functional equivalency. Ectopic expression of *FDL2.2 and FDL3* further supported that their encoded proteins have diverged in function. Overexpression of *FDL2.2* induced early flowering within 6-months under long daylength conditions whereas it takes several years of growth before a wild-type tree to flower. In contrast, overexpression of *FDL3* did not promote early flowering but markedly delayed leaf development and the transition to secondary growth under long day conditions. Intriguingly, the growth of *FDL3* transgenics can be restored by exposure to short days. For the first time, we demonstrate differentiated functions of the two close *FT* paralogs using the CRISPR/CAS9 induced *ft1ft2* double mutants and *ft1*-specific mutants. WT-like *ft1*-specific mutants with delayed bud flush implicates that *FT1* promotes dormancy release. Double *ft1ft2* mutants with reduced shoot growth and budset in long days suggest that *FT2* is key in sustaining growth. Thus, our results reveal that poplar *FT*s and *FDLs* have different roles in controlling vegetative growth in addition to flowering.

# GENERAL AUDIENCE ABSTRACT

The adaptation of trees to temperate and boreal climates depends on their ability to respond to environmental signals that are markers of seasonal changes in order to survive winter and maximize growth. The genus *Populus* (poplars) is a model system for identifying the genes and molecular mechanisms that regulate growth and dormancy transitions.  Photoperiod and temperature regulate both vegetative and floral phenology. *FLOWERING LOCUS T* (*FT*) and *FLOWERING LOCUS D* (*FD*) are key regulators of flowering time in Arabidopsis and other plants. The distinct functions of three poplar *FD-LIKE (FDL)* genes and two *FT*s were explored through gain-of-function, dominant repression, and CRISPR/CAS9-mediated gene editing. We studied trees in controlled environments, including manipulation of daylength and temperature to mimic an annual seasonal growth and dormancy cycle. Our studies showed that the FDL proteins share less than complete functional equivalency.  Among the three paralogs, only *FDL2.2* promoted precocious flowering, whereas *FDL1* and *FDL3* appear to have distinct roles in vegetative growth and phenology. Whereas overexpression of any *FDL* gene delays short day-induced growth cessation and bud set, only *FDL3* coordinately altered leaf development and the transition to secondary growth in a photoperiod-dependent manner. For the first time, we demonstrate distinct functions of the two *FT* paralogs in vegetative phenology. Study of *ft1ft2* double mutants and *ft1*-specific mutants showed that *FT1* promotes dormancy release, whereas *FT2* is necessary to sustain growth. Collectively, our results reveal that poplar *FT*s and *FDLs* have distinct roles in controlling different aspects of vegetative phenology and woody shoot development.

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| | |
|---|---|
| aa | Amino acid(s) |
| AB | axillary bud |
| ABI3 | ABSCISIC ACID-INSENSITIVE3 |
| AP1 | APETALA1 |
| bp | base pair |
| bZIP | basic leucine zipper |
| CAL | CAULIFLOWER |
| CDL | critical day length |
| CDS | coding sequence |
| CO | CONSTANS |
| CEN1 | CENTRORADIALIS |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| FAC | floral activation complex |
| FDL | FLOWERING LOCUS D Like |
| FT | FLOWERING LOCUS T |
| FUL | FRUITFULL |
| GA | Gibberellic acid |
| gRNA | guide RNA |
| IN | internode |
| LD | long daylength |
| LPI | leaf plastochron index |
| NLS | nuclear localization signal |
| nt | Nucleotide |
| PD | plasmodesmata |
| PAM | protospacer-adjacent motif |
| PHYA | PHYTOCHROME A |
| qRT–PCR | quantitation Real Time–PCR |
| SD | short daylength |
| SAM | shoot apical meristem |
| SNP | Single nucleotide polymorphisms |
| TB | terminal bud |
| WT | Wild-type. |

# 1. GENERAL INTRODUCTION AND OVERVIEW OF RESEARCH

**Forest trees play significant roles in global ecology and economics**

Forest trees cover approximately 30 percent of the land area and provide global ecological and economic benefits (Bonan 2008). Global production and trade of forest products including timber, pulp, and paper were estimated to value $231 billion in 2012 (FAO). Forest biomass remains one of the largest renewable energy resources. Meanwhile, forests serve as immense native "carbon sinks" by storing over half of the carbon in terrestrial vegetation and soil (World Agriculture: Towards 2015/2030). Taken together, the world's forests, amounting to around 4 billion hectares, which corresponds to an average of 0.6 ha per capita (FAO, 2010), fulfill many roles such as providing renewable raw materials or energy and maintaining air and water quality. However, this natural privilege we have claimed for generations increasingly faces difficult challenges such as deforestation, exotic pests and pathogens and climate change. Therefore, it is very important to explore genomic networks and biotechnology to overcome the slow and labor-intensive traditional tree breeding process and to advance understanding of the genes and mechanisms important for adaptation in order to help improve future forest management strategies.

**Poplar as a model for woody perennial biology**

The genus *Populus,* collectively known as poplars with some taxa commonly known as cottonwoods and aspens, contains more than 30 different species of deciduous flowering trees (Dickmann 2001). These members of the family of Salicaceae have natural distributions across the northern hemisphere. A number of *Populus* species and especially their hybrids are grown for commercial purposes such as wood products, pulp, and paper. Moreover, since the late 1970s, poplars have been listed as desirable short rotation biofuels woody crops in the U.S. Department of Energy's (DOE) Bioenergy Feedstock Development Program as they grow rapidly, and their biomass can be harvested after a short growing period. Additionally, the high cellulose (40%) and low lignin (22%) content make it easier to extract carbohydrates from the biomass (Hansen 1983).

Beside its enormous economic values, poplars are also selected as a model forest tree for molecular biology and biotechnology (Brunner et al. 2004). The diploid chromosome number in

*Populus* is 2n = 38. The physical size of *Populus* genome (~550 Mb) is relatively small (Tuskan et al. 2006), being only 4 times the size of the annual model plant Arabidopsis genome, while 40 times smaller than that of the loblolly pine. Furthermore, individual poplar clones bear either male or female flowers (catkins) on separate trees. The female clone INRA 717-1B4 *P. tremula* x *P. alba* is widely used for genetic transformation to study functions of genes without concern of pollen drift, which is a huge constraint on the commercial use of transgenic trees and also impedes long-term field tests (Brunner et al. 2007). More importantly, poplars can be relatively easy to regenerate from explants of excised leaf discs and stem cuttings, and grow rapidly after vegetative propagation.

**Key developmental events of seasonal growth cycle**
To maximize growth while limiting the potential for freezing damage, poplar trees in the temperate zone adjust their annual life cycle by responding to seasonal and environmental cues to align the timing of bud flush in spring, active growth in summer, bud set in autumn, and dormancy in winter. Key stages of an annual growth cycle of trees are outlined in Figure 1.1

*Dormancy in winter*

Dormancy of the shoot apical meristems (SAM) is essential for deciduous poplar trees in temperate zones to survive during the freezing temperatures of winter. Winter dormancy, also known as endodormancy, is a state defined by the inability to initiate growth from meristems even under favorable conditions (Rohde and Bhalerao 2007). At this stage, growth arrest is maintained by endogenous signals, and the dormant meristems are not responsive to external growth promotive signals such as warm temperatures and long days (LDs). Consequently, dormancy release by exposure to long enough chilling is required to restore the competency of meristems for growth (Brunner et al. 2014). After endodormancy is released, a bud transitions to a phase termed ecodormancy (Lang 1987), which allows for growth to resume as soon as trees are exposed to sufficient growth-promotive conditions such as warm temperatures and increasing daylengths. Thereafter, buds flush rapidly and growth continues during the growing season (Cooke et al. 2012). In addition, poplar trees also transition to ecodormancy during the growth season when they are under growth-limiting conditions such as poor light quality, nutrient

deficiency, and drought. However, the activity of an ecodormant meristem can be reactivated simply by supplying the growth-limiting factor (e.g., add fertilizer).
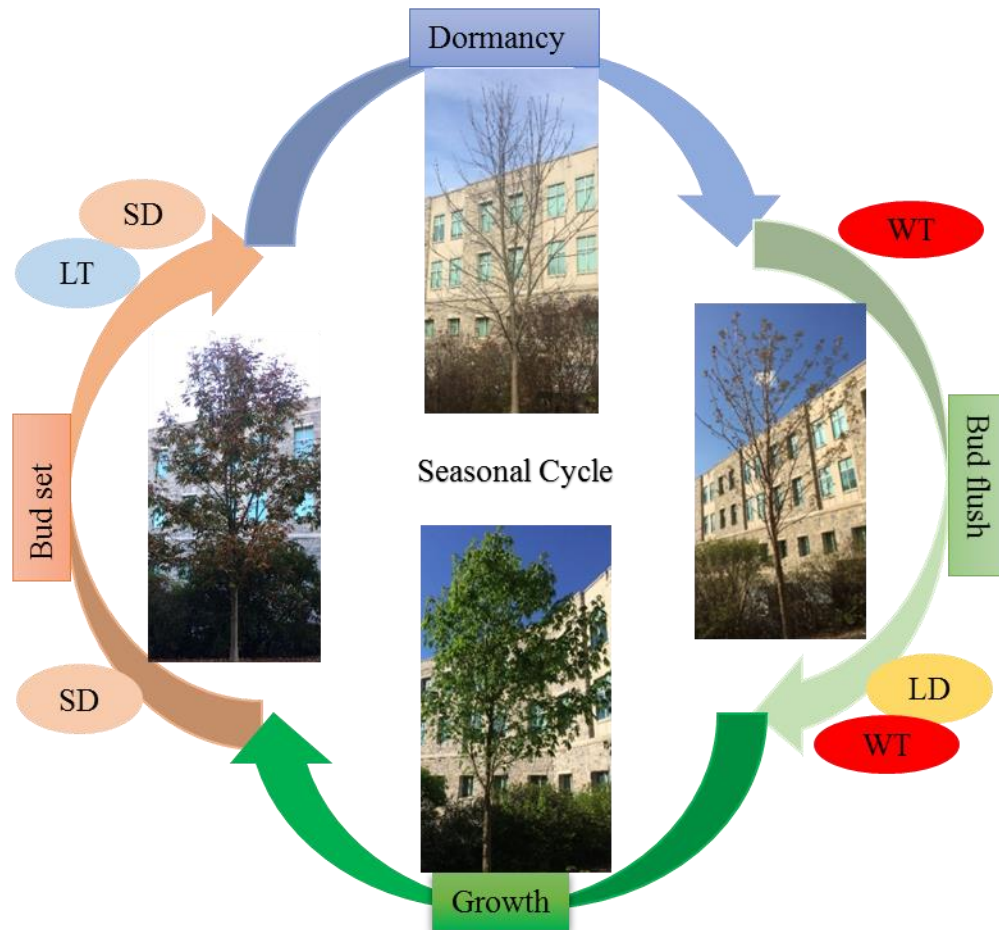


*Figure 1.1 Seasonal cycle of dormancy-growth in deciduous trees.*

To survive freezing temperatures in the winter, apical buds of temperate trees such as poplars stay dormant. In a dormant state, shoot apical meristem (SAM) enclosed in the bud becomes insensitive to growth-promoting signals. Prolonged exposure to chilling temperatures is required to break dormancy. After release from dormancy, warm temperature in spring promotes bud flush. Short days during early autumn induces growth cessation and bud set. As daylength and temperature reductions continue during autumn, dormancy of the bud is established before the risk of frost damage in winter. LD, long daylength; WT, warm temperature; SD, short daylength; LT, low temperature.

### Bud flush in spring

In early spring, after dormancy is released, shoot apical buds and lateral buds flush after exposure to sufficient warm temperatures, typically quantified as accumulated heat sum. After leaves preformed within the bud in the previous year protrude from the opening bud scales and unfold, new shoots grow out quickly (Rinne et al. 2011). Bud flush of temperate-zone forests is primarily driven by warm temperature in spring (Cooke et al. 2012). Earlier bud flush is more favorable for optimal light harvest and to more effectively compete for nutrient and soil moisture, however, most freezing injuries in trees are caused by late spring frosts after bud-break (Timmis et al. 1994; Ningre and Colin 2007).

### Leaf development and secondary growth

*Populus* is a free-growing taxa. When conditions are favorable, shoots are capable of continuous growth; and thus, produce neoformed leaves and axillary buds. However, the proportion of shoots that exhibit this growth pattern declines with tree age or size. More importantly, the morphology of leaf development determines its secondary growth, the wood production of poplar trees (Dickmann 2001).

Leaf maturation is closely associated with the stem transition from primary to secondary growth in cottonwood trees (*P. deltoides*). Primary growth, or internode elongation, takes place only within the zone of the first 4 to 6 internodes beneath the shoot apex (Larson and Isebrands 1974). Leaf development from a partially unfolded young leaf to fully expanded leaf also coordinately occurs within the zone. Simultaneously, the transition of primary growth to secondary growth occurs after leaves at least begin to transition from sink to source (Larson 1971). Secondary growth is initiated from cell divisions of the vascular cambium, a thin cylinder of meristematic tissue between bark and wood. All the cambial derivatives from the cambial periclinal division undergo further division and ultimately differentiate to become secondary xylem cells (wood) or secondary phloem cells (bark) (Larson 1994). Thereby, stems of trees grow thicker and produce wood.

*Bud set in fall*

In late summer or early fall, as the day length reduction passes a threshold termed critical day length (CDL), poplar trees immediately activate a process for dormancy, starting with growth cessation and followed by bud formation, cold adaptation, and finally entry of dormancy within 6 to 8 weeks (Nitsch and JP 1957; Weiser 1970; Cooke et al. 2012; Maurya and Bhalerao 2017). After leaves perceive the CDL, internode elongation stops and no more new leaves emerge from the shoot apex. Consequently, a visible apical bud forms after 4 to 6 weeks in SDs (Goffinet and Larson 1981). A mature bud consists of the SAM surrounded by a leaf priomoridia/leaflets enclosed by dark brown protective bud scales (Rohde 2002).

**Photoperiod and Temperature are the key environmental cues controlling seasonal growth**
Dormancy is a very important local adaptation trait of poplar trees in the boreal and temperate regions in order to live through severe cold winter temperatures as low as − 40 °C (Weiser 1970). As described above, induction of growth cessation and dormancy establishment is mainly controlled by photoperiod in poplar trees since it is a reliable environmental cue (Ruttink et al. 2007; Cooke et al. 2012; Kumar et al. 2017). Furthermore, population genomic studies support that seasonal changes in photoperiod largely determines the geographical distributions of natural populations of poplar trees (Evans et al. 2014). The length of the CDL for growth cessation and dormancy varies with latitude-of-origin. More northern poplar populations display earlier growth cessation and bud set compared to southern populations because their CDL is comparatively longer. This adaptation ensures that dormancy has been induced before frost occurs (Bohlenius et al. 2006).

In addition to photoperiod, seasonal temperature changes also control the annual growth rhythm of poplar trees. Similar to vernalization requirements of seed germination and the floral transition in many herbaceous plants, an extended chilling period is required to break bud dormancy in woody plants adapted to temperate climates (Junttila and Hanninen 2012; Brunner et al. 2014). For many species of the temperate deciduous forest, this prolonged exposure to cold temperatures is naturally obtained during the coldest time of the year in December and January (Murray et al. 1989; Heide 1993). Under control environments, hybrid aspen (*P. tremula × P. tremuloides*) requires a total of approximately 6 weeks or ~1000 h of chilling temperatures (5

°C) for subsequent bud burst, which leads to internode elongation and new leaf initiation after preformed leaves are unfolded (Rinne et al. 2011).

**Molecular mechanisms in the synchronization of growth with seasonal cycle**
*Regulatory module of CONSTANS/FLOWERING LOCUS T (CO/FT)*

Plants sense photoperiod changes to regulate important developmental transitions during their life cycle. The CONSTANS/FLOWERING LOCUS T (CO/FT) regulon is the core of the photoperiodic pathway that promotes flowering in Arabidopsis (Andres and Coupland 2012). Photoperiodic control of *FT* expression through *CO* is illustrated in Figure 1.2. In summary, for many LD plants such as Arabidopsis under inductive LD conditions, *CO* transcription is repressed by CYCLING DOF FACTORs (CDFs) in the morning, while the repression of *CO* is released as the CDFs are degraded by the afternoon. Thereby, the expression of *CO* peaks late in the day (Song et al. 2013). At the same time, the CO protein is stabilized by photoreceptors when there is light. CO is a *FT* activator, inducing *FT* in the leaf at the end of the light phase (Putterill et al. 1995; Shim et al. 2017). FT protein is then transported to the shoot apex, where it activates floral genes to promote flowering (Taoka et al. 2013). By contrast, when plants are grown in SD conditions, despite *CO* being expressed in the afternoon, the CO protein is unstable and does not accumulate due to the earlier onset of the dark phase. Consequently, *FT* expression is restricted, which leads to late flowering (Suárez-López et al. 2001).

During the transition from vegetative growth to dormancy in poplar trees, daylength sensing and at least some of the regulatory genes and pathways are analogous to photoperiod-controlled flowering (Cooke et al. 2012; Maurya and Bhalerao 2017). The initial breakthrough was the discovery that a CO/FT regulon could be linked to the time of growth cessation in four European aspen (*P. tremula*) clones from different latitudes (Bohlenius et al. 2006). Under 19-hour day-length conditions, expression of *FT* was not induced, resulting in growth cessation and bud set in the trees from high-latitudes in Sweden, as the photoperiod was shorter than their CDL of 21 hours. Whereas, the transcript of *FT* accumulated, and growth was maintained in the trees from lower latitudes in Germany, as the photoperiod was longer than their CDL of 15 hours (Bohlenius et al. 2006). In addition, various circadian clock components and photoreceptors have been shown to be involved in growth cessation and bud set. For example, overexpression of the

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oat *PHYTOCHROME A* (*PHYA*) gene in poplar promoted growth and delayed bud set in SDs (Olsen et al. 2002); whereas, downregulation of *PttPHYA* in poplar trees (*P. tremula* × *P. tremuloides*) led to earlier growth cessation and bud formation under SD conditions (Kozarewa et al. 2010).
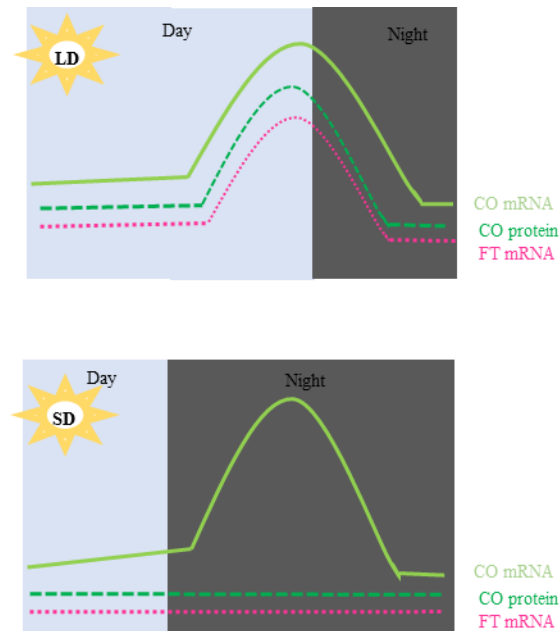


***Figure 1.2 Photoperiodic regulation of FLOWERING LOCUS T (FT) expression under long day (LD) and short day (SD) conditions.***

In long day (LD, top panel), *CONSTANS* (*CO*) transcription is repressed in the morning, and gradually upregulated in the afternoon, and reaches a peak at the end of light phase. Meanwhile, CO protein is stable when there is light. The accumulations of CO proteins can activate *FLOWERING LOCUS T* (*FT*) expression. In short day (SD, bottom panel), *CO* expression peaks in the dark phase, while CO protein is rapidly degraded in the dark; and thus, cannot induce expression of *FT*.

***Two Poplar FT paralogs (FT1 and FT2)***

*FT* belongs to the small phosphatidylethanolamine-binding protein (PEBP) gene family, which is conserved in all eukaryote kingdoms (Pin and Nilsson 2012) . There are two *FT* orthologs in the *Populus* genome, *FT1* and its paralog *FT2* (Hsu et al. 2006; Hsu et al. 2011). They control two important aspects of the woody perennial growth habit: flowering time (the first time of flowering in the life cycle, and seasonal reproductive onset within a year); and seasonal vegetative growth and dormancy transitions (Hsu et al. 2011).
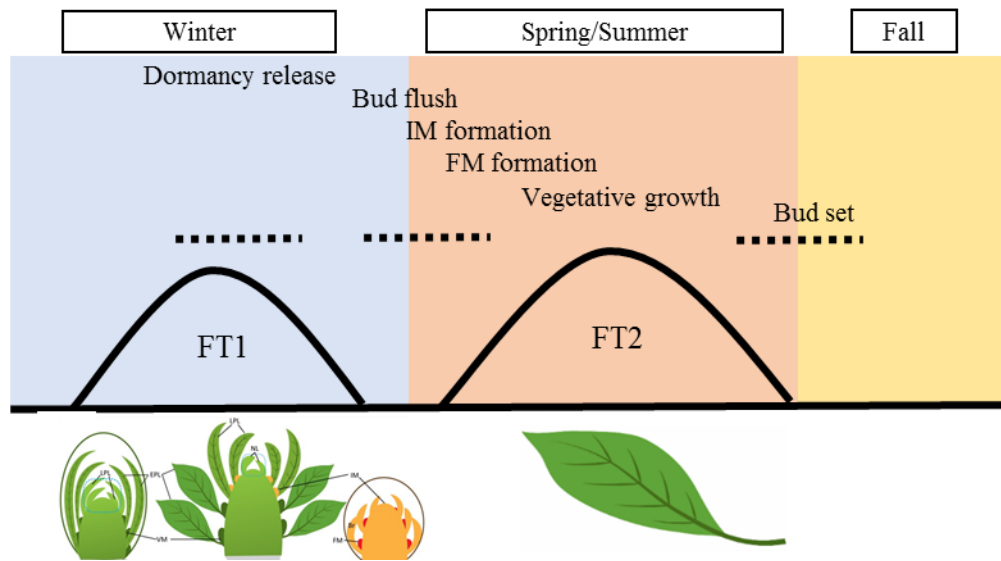


***Figure 1.3 Environmental regulation of phases of the annual growth, dormancy cycle and flowering and seasonal expression of FT1 and FT2.***

Decreasing photoperiods in fall induce terminal bud set and dormancy, a prolonged exposure to chilling temperatures in winter releases dormancy, followed by bud flush, inflorescence meristem (IM) and floral meristem (FM) formation, and subsequent warm temperatures promote resumption of growth. Within a winter bud, *FT1* is upregulated in the shoot apex, preformed leaves and preformed stem by low temperatures, while *FT2* is upregulated in leaves following bud flush by increased day length and warm temperatures during spring and summer. *FT2* is rapidly downregulated in responding to decreased day length in fall. (The figure is adapted from Hsu et al. 2011; Brunner et al. 2014).

Although the two *FT*s share some functional redundancy in promoting early flowering in LDs and delayed bud set in responding to SDs when either one of them is overexpressed (Bohlenius et al. 2006; Hsu et al. 2006; Hsu et al. 2011), *FT1* and *FT2* have distinct seasonal expressional profiles and diverged functions. In adult *P. deltoides* trees, *FT1* is predominantly upregulated in the shoot apex, preformed leaves, and preformed stem packed within a winter bud after exposure to low temperatures for a prolonged period to induce the floral transition in some of the axillary meristems, while *FT2* is upregulated in leaves after bud flush under favorable conditions of warm temperatures and long day length in spring to promote vegetative growth (Figure 1.3). Furthermore, in controlled environments, the expression of *FT1* is only induced by chilling temperatures (4 ºC) regardless of lighting conditions, whereas expression of *FT2* requires both warm temperatures and LDs (25 ºC and 16 hr/8hr light/dark) (Hsu et al. 2011).

Although a number of experiments support these contrasting roles, it is important to note that these have not included gene-specific downregulation or knock out (e.g., both *FT1* and *FT2* were downregulated in RNAi transgenics). Expression directed by the 35S or heat-shock inducible promoter showed that the encoded proteins differ in their ability to induce flowering in juvenile trees (Hsu et al. 2011). These results supported that *FT1* was a strong promoter of the floral transition, whereas only abnormally high levels of *FT2* induced some flower development but not the wild-type-like catkins observed with increased *FT1* expression. Downregulation of *FT2* expression occurs shortly after the CDL is perceived and initiates growth cessation and bud set before dormancy is established in poplar trees. Overexpression of *FT2* could override the induction of growth cessation by SDs; whereas, downregulation of both *FT2* and *FT1* led to earlier SD-induced growth cessation and bud set (Bohlenius et al. 2006). In addition, a role for *FT1* in dormancy release and/or bud flush has been hypothesized (Rinne et al. 2011; Brunner et al. 2014; Evans et al. 2014) based on a number of lines of evidence. In controlled experiments with juvenile trees, *FT1* upregulation in a dormant vegetative bud occurs during chilling and correlates with the opening of plasmodesmata, which is necessary for SAM reactivation (Rinne et al. 2011). Single nucleotide polymorphisms (SNPs) in the *FT1* locus were also associated with bud flush time in a genome wide association study (Evans et al. 2014). Finally, the previously demonstrated role of conserved *FT* antagonist, poplar *CEN1*, in repressing dormancy

release suggested that *FT1* promotes dormancy release (Mohamed et al. 2010; Brunner et al. 2014).

### *The FT–FD complex and its downstream components participate in control of growth development*

Although FT is a key regulator in controlling many vital developmental transitions in the photoperiodic pathway, it is not able to trigger the transitions by itself due to its lack of DNA binding activity. Instead, FT depends on transcriptional factors as partners to control the transitions. One of its most important and well-known partners is FLOWERING LOCUS D (FD), a bZIP transcription factor (Abe et al. 2005; Wigge et al. 2005). After the FT protein is transported to the shoot apical meristem (SAM), it forms a FT-FD complex that acts as a master flowering promotor. The FT-FD complex can activate many downstream floral identity genes, including *APETALA1* (*AP1*) and its closely related paralogs *FRUITFULL* (*FUL*) and *CAULIFLOWER* (*CAL*) (Abe et al. 2005; Wigge et al. 2005; Taoka et al. 2011). Despite the canonical FT–FD complex being most prominently described in the context of flowering, different FT–FD complexes containing combinations of diverse FT-like and FD-like proteins play significant roles in diverse development process and certain functions may only be apparent under specific environmental conditions (Pin and Nilsson 2012; Tsuji et al. 2013). For example, leaf development was severely inhibited by overexpression of *FT* or *Tomato FT* (*TFT*) in Arabidopsis plants in SDs because abnormally high levels of FT in the presence of FD promoted mis-expression of the targets *SEPALLATA 3* and *FUL* in leaves (Teper-Bamnolker and Samach 2005). Overexpression of *OsFD2* in rice resulted in abnormal branching of shoots and small leaves (Tsuji et al. 2013). In hybrid aspen, overexpression of one *FDL2* splice variant led to precocious early flowering in poplar under LDs and also delayed growth cessation and budset under SDs (Parmentier-Line and Coleman 2016). However, overexpression of *FDL1* delayed bud set and growth cessation in response to SD signals, but did not induce flowering under LDs (Tylewicz et al. 2015).

### Overview of this research
In annual plants, the decision to flower is an irreversible developmental transition from vegetative to reproductive growth followed by the production of seeds and the end of its life cycle before unfavorable growth conditions arrive. It is a critical strategy for plants to preserve

their genetic information in the next generation and to enable germination at an appropriate time. In addition, flowering leads to production of seeds and fruits, which represent an important food source for humans. Therefore, the underling mechanisms of controlling flowering time in many annual plants have been extensively studied.

Perennial plants must maintain indeterminate vegetative identity of a portion of their shoot meristems. Adult poplar trees contain indeterminate vegetative shoot apical meristems, and both vegetative and reproductive axillary buds on flowering branches. Therefore, both vegetative and reproductive growth coexist within certain shoots of adult poplar trees (Yuceer et al. 2003). Thus, in addition to age-maturation transitions, adult poplar trees undergo seasonal cycles of growth and dormancy of vegetative meristems, maintain apical meristems as vegetative and limit the number of axillary meristems that transition to flowering. To date, a few studies on poplar flowering and photoperiod- mediated dormancy induction have been reported and our understanding of the molecular mechanisms underlying such complex phenotypes remains limited. In this study, I will focus on the investigation of flowering gene homologs in regulating growth changes in response to photoperiod and temperature changes.

The understanding of how trees adjust their life cycles by integrating endogenous signals and environmental cues will allow us to gain more insights into the critical ecological and evolutionary tradeoff between survival and growth in trees, as well as its adaptation to new geographical areas and the effects of global climate change. Knowledge concerning these mechanisms will therefore be instrumental for future tree breeding programs and the management of native forest populations.

The specific objectives of this study are:
    I.  To functionally characterize the three poplar *FD*-like genes in the regulation of vegetative growth and development and flowering.
    II.  To differentiate the roles of *FT1* and *FT2* in seasonal growth transitions through gene-specific editing using the CRISPR/CAS9 system.

**Chapter II** presents the seasonal expression profiles and functional characterization of three *FD*-like genes in regulation of vegetative growth and development in poplar. Dominant negative

repression each of the three *FD*-like genes inhibited shoot growth to different extents. Overexpression of *FDL2.2* induced precocious flowering, whereas overexpression *FDL3* did not. Moreover, *FDL3* overexpression affected leaf development as well as secondary growth. In summary, three *FD*-like genes have diversified functions in regulating vegetative growth and flowering.

**Chapter III** presents a study of *FT1* in dormancy release using CRISPR-directed *ft1*-specific mutants. Although a role for *FT1* in vegetative growth has been hypothesized, this has yet to be demonstrated. Thus, we generated *ft1*-specific mutants using CRISPR/CAS9 system. Both *ft1* mutants showed WT-like growth under LD conditions, as well as in response to SD signals. However, they showed significantly delayed bud flush after 6 weeks of chilling temperatures. Our results show that *FT1* has a key role in vegetative phenology, by promoting dormancy release.

**Chapter IV** presents a summary of this study, final discussions and future perspective of this research.

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# 2. FUNCTIONAL DIVERSIFICATION OF FD TRANSCRIPTION FACTORS IN POPLAR

**Sheng X,** Hsu CY, Cetin Y, Ma C, Strauss SH and Brunner AM.

**Abstract**

*FLOWERING LOCUS D* (*FD*) homologs, which encode bZIP transcription factors, have been implicated in the regulation of many developmental processes apart from flowering. The functional diversification of three poplar *FD-like* (*FDL*) homologs were explored using a combination of a year-round gene expression analysis and study of transgenic poplars overexpressing wild-type genes or dominant repressor versions. Dominant repression of each of the three poplar *FDL* homologs showed different degrees of reduced shoot elongation. Consistent with its ability to induce precocious flowering under long daylengths (LDs), in adult trees, *FDL2.2* was highly upregulated in newly developing reproductive buds. In contrast, overexpression of *FDL3* did not induce flowering under LDs, but altered shoot development. Specifically, it prolonged the primary growth phase with a greater rate of leaf formation, but delayed leaf expansion. Similar to its paralogs, *FDL3* overexpression delayed growth cessation under SDs. In addition, SDs induced changes in leaf development and secondary growth in transgenic plants overexpressing *FDL3*. Phytomers that initiated under SDs exhibited leaf expansion and primary to secondary growth transitions similar to wild-type trees under LDs. Our results show that the three *FDL* genes have diverged in regulation and indicate that their encoded proteins share only partial functional equivalency.

**Introduction**

Plants precisely time key developmental transitions, such as flowering and endodormancy, by sensing changes in photoperiod and temperature. In photoperiodic-responsive poplar trees, leaf development and the transition of the stem from primary to secondary growth are coordinated and these developmental transitions are altered by growth cessation-inducing short daylengths (SDs). Under LDs, primary growth is limited to the leaf development zone, which is defined as the zone from the shoot apex to the first fully expanded leaf (Larson 1971; Larson and Isebrands 1974). Leaf maturation is closely associated with secondary growth. The transition from primary to secondary growth occurs below a leaf that is at least partially mature, though the exact

relationships between leaf development stage and the stem transition to secondary growth vary depending on factors such as shoot height (Larson 1971). SDs induce growth cessation, budset and dormancy over several weeks in controlled conditions (Rohde 2002; Bohlenius et al. 2006; Ruttink et al. 2007). In the first few weeks of SD exposure, the shoot's leaf and stem developmental gradients are also altered. The last leaves formed under LDs, that are directly beneath the base of the apical bud, expand and the internodes directly below the apex transition to secondary growth (Isebrands and Larson 1973). A number of studies (Kumar et al. 2017; Maurya and Bhalerao 2017; Shim et al. 2017) have shown that homologous genes act in photoperiodic pathways controlling vegetative phenology in poplar and flowering in Arabidopsis. The conserved *CONSTANS/FLOWERING LOCUS T FT* (*CO/FT*) regulon plays a central role in SD-mediated growth cessation (Bohlenius et al. 2006). In poplar, expression *of FT2* in leaves is rapidly downregulated after exposure to SDs and its overexpression delays SD-induced growth cessation (Bohlenius et al. 2006; Hsu et al. 2011).

To promote flowering, FT forms a complex with FLOWERING LOCUS D (FD), a bZIP transcription factor, to activate floral meristem and organ identity genes, including *APETALA1* (*AP1*) and the closely related genes, *FRUITFULL* (*FUL*) and *CAULIFLOWER* (*CAL*) (Abe et al. 2005; Wigge et al. 2005). Beyond flowering, FT-FD complexes consisting of different FT-like and FD-like proteins have roles in other developmental processes. For example, under certain environmental conditions, leaf development was severely inhibited by the overexpression of *FT* or *Tomato FT* (*TFT*) in *Arabidopsis* plants, because abnormally high levels of the *FT* promoted misexpression of the targets *SEPALLATA3* and *FUL* in leaves (Teper-Bamnolker and Samach 2005). Moreover, leaf phenotypes induced by *FT* overexpression were dependent on *FD*. Overexpression of a rice *FD* homolog, *OsFD2,* in rice resulted in more branches and small leaves (Tsuji et al. 2013). These studies suggest that, FT-FD complexes have a broader functionality. However, the mechanisms underlying the functional diversification of the FT-FD complexes and how they can participate in the control of other developmental pathways remain poorly understood.

The *P. trichcocarpa* genome contains three *FD-like* genes (Parmentier-Line and Coleman 2016). Two of these were previously studied (Tylewicz et al. 2015; Parmentier-Line and Coleman

2016). The growth and morphology of *35S::FDL1* poplar transgenics was similar to wild-type (WT) under LDs, but growth cessation and bud set was greatly delayed under SDs (Tylewicz et al. 2015). Two splice variants of *FDL2* have been studied. *FDL2.1* (referred to as *FDL2* in (Tylewicz et al. 2015) encodes a protein with 29 amino acid insertion in the middle of the conserved bZIP domain and overexpression transgenics were dwarf, but their SD growth response did not differ from WT. Under LDs, *35S::FDL2.2* (referred to as *FD1* in (Parmentier-Line and Coleman 2016) poplar transgenics flowered precociously and had small leaves and increased branching, but similar to *FDL1* overexpression, SD-induced growth cessationa and bud set was delayed. Here, we use detailed seasonal gene expression and transgenic manipulation to study *FDL3* and also provide new details on *FDL1* and *FDL2* and the functional divergence of the three paralogs.

**Materials and methods**
*Binary constructs and plant transformation*
The coding regions for *P. deltoides FD-like (FDL)* genes, *FDL2.2* and *FDL3,* were cloned into pGEM-T Easy vector (Promega) using Pfu DNA polymerase (Stratagene). For the dominant repression constructs, the coding regions of *FDL1, FDL2.1*, *FDL2.2,* and *FDL3* were translationally fused to dominant repressor domain, SRDX (LDLDLELRLGFS) by designing SRDX sequence and stop codon (CTCGATCTGGATCTCGAACTGAGACTCGGATTCTCCTGA) in the 3'-end of the reverse primer. The inserts were subsequently digested with BamHI/KpnI and cloned into the pBI121 binary vector (BDBiosciences) between the cauliflower mosaic virus (CaMV) 35S promoter and the *nos* 3′ transcriptional terminator (Supplemental Figure 2.1a and 2.1b). The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 using the freeze-thaw method. Transformation of poplar using wild-type (WT) clone INRA 717-1B4 (*P. tremula* x *P. alba*), was carried out as previously described (Meilan and Ma 2006). The primer sequences used for gene amplification are presented in the supporting information (Supplemental Table2.3).

*Plant material growth conditions*
All transgenic and non-transgenic WT plants were propagated in vitro. Rooted plantlets were transferred from tissue culture to soil (Promix B, Canada), grown in growth chamber or

transferred to a greenhouse. We set LD growth chamber conditions as: 16 hr light/8 hr dark, with light intensity of 100 µ mol m$^{-2}$ s$^{-1}$ (at plant level), temperatures of 20-22°C, and 65% RH. Greenhouse conditions were set like growth chamber conditions except with ambient light during the daytime and supplemental incandescent light extended the daylength to 16 hours. Plants were normally grown for 2-6 months to a height of 30-60 cm under LD conditions before starting a SD treatment except as otherwise specifically described. SD conditions were 8hr light/ 16 hr dark.

*Morphological parameters for measurements and gradient sampling*

Leaf plastochron index (LPI) was adopted to take measurements and collect samples (Larson and Isebrands 1971). LPI1 was defined as the first leaf below the shoot apex with a lamina length of at least 1 cm. The internode (IN) directly beneath the LPI1 leaf was designated as IN1.

*Gene expression*

The samples used to study seasonal gene expression in leaf, reproductive bud, shoot apex, vegetative bud and shoot from *P. deltoids* trees were described previously (Hsu et al. 2011). RNA extraction, cDNA synthesis, and parameters for qRT-PCR were also performed as previously described except that qRT-PCR results were analyzed as described below.

Tissues for spatial expression studies of poplar *FDL* genes were collected from 4-month-old WT plants grown in LD greenhouse conditions. For the shoot tip (ST) samples, all leaves visible by the naked eye were removed. Axillary buds (ABs) were collected from the zone between IN10 and IN20. In addition, the young leaf (YL) was obtained from LPI2 (LPI1 >= 1 cm), and the mature leaf (ML) was the recently mature leaf from LPI6. Internode 2 (IN2) indicates the internode beneath LPI2 in the primary growth zone. IN6 is beneath LPI6, representing the transitional zone from primary to secondary growth. Phloem (Ph) and xylem (Xy) were scraped from the stem undergoing secondary growth below IN6. Furthermore, we collected active non-woody lateral roots (approximately 1 cm sections including root tips) as the sample of root (Rt).

For expression of *FT2* in the leaf under LD and SD conditions, we collected leaves at the position of LPI6 (LPI1 >= 1 cm) of WT and two independent events of *FDL3ox* plants. These

plants were grown in a LD growth chamber for 2 months before the SD treatment, and LPI6 of WT in LDs was chosen as it is usually the most recently mature leaf. LD samples were collected on the day before the SD treatment, and SD samples were collected after 3 weeks SD treatment. All samples were collected 2 hours after lights were turned on to begin the light period.

All samples were immediately frozen in liquid nitrogen and stored at -80˚C. We extracted total RNA and treated it with DNase I digestion and cleanup procedures using the RNeasy Plant Mini Kit (Qiagen) and RNase-Free DNase Set (Qiagen) as described in Brunner et al. (2004). Each cDNA reaction of 20 µl was synthesized from 2.0 µg total RNA and an oligo (dT) primer using the High Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. In this procedure, we used the Power SYBR Green PCR Master Mix kit (Applied Biosystems) and the ABI PRISM™ 7500 Real-Time PCR system (Applied Biosystems) for quantitative Real Time-PCR (qRT-PCR) reactions with three technical replications per RNA sample. Each qPCR reaction mixture of 25 µL contained 0.5 µL of cDNA template, 12. 5 µL of SYBR Green Mix, 1.0 µL of 10 µM forward primer, 1.0 µL of 10 µM reverse primer, and 10.0 µL of ddH2O. The PCR program was set up to perform an initial incubation at 95 °C for 10 min, followed by 95 °C for 15 s, and 60 °C for 1 min, for a total of 40 cycles. Additionally, the gene-specific primers (Supplemental Table2.3) were designed to produce amplification products of 100–150 nucleotides. We used an aspen ubiquitin gene (aUBQ2) as an internal reference gene (Mohamed et al. 2010).

Data from replicated samples in different plates were exported for further analysis. We normalized the Ct values across plates, and determined relative quantities using comparative Ct method (2^(-Delta Delta Ct)), in accordance with the method (Livak and Schmittgen 2001). Relative fold change was calculated by normalizing each sample expression to the sample showing the lowest expression.

*Microscopic analysis*

For analysis of primary growth internodes and internodes undergoing transition to secondary growth, internodes were immobilized in 5% agarose. We cut 60 µm thick sections with a Vibratome (Leica VT1200). In addition, we sectioned internodes from the secondary growth

zone into 60 μm thick sections with a GSL1-microtome (Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Switzerland). Sections were then stained in a drop of the following solution: 1 g phloroglucinol (Sigma-Aldrich) in 100 mL 95% EtOH and 16 ml 37% HCL. For more detailed images of stem anatomy, samples were fixed and embedded in resin. For fixation, we used 2.5% glutaraldehyde in 0.5 M potassium phosphate buffer at a pH of 7.2. Samples were fixed in the buffer at room temperature for 6 hours and then washed with 0.5 M potassium phosphate buffer (2 x 10 minutes). We then dehydrated the specimens through a graded ethanol series (30%, 50%, 70% and 90% ethanol, 2 x 10 minutes each), followed by 100% ethanol (3 x 10 minutes). After ethanol was gradually replaced with LR White resin over three days, samples were placed in gelatin capsules with 100% LR White resin. The resin was then polymerized at 55°C for 24 hours. For these resin embedded samples, we sliced transverse sections of 2 μm thick with glass knives on a Microtome (Leica RM2265). Sections were stained with Toluidine blue/boric acid (0.05% w/v) for one minute. All images were taken with a Zeiss Axio Imager A1 (Carl Zeiss, Oberkochen, Germany).

*Statistics analyses*

Height, leaf length, and new leaf formation data were analyzed in JMP Pro 12 (SAS Institute Inc., 2016–2017), using the Fit model to test both the effects of constructs and the events within constructs. To estimate and test differences between means, we used the LSMEANS protocol and applied the Tukey–Kramer's adjustment for all possible pairwise comparisons between transgenic group means.

**Results**

*FDL genes show divergent seasonal regulation*

Full-length cDNAs for the three poplar *FDL* genes were cloned from *P. deltoides* (Supplemental Table2.1 and Supplemental Figure 2.2). Both the *FDL2.1* and *FDL2.2* splice variants and *FDL1* encode proteins nearly identical to the previously reported *P. trichocarpa* cDNAs (Tylewicz et al. 2015; Parmentier-Line and Coleman 2016) except that the FDL1 reported here contains an additional 39 amino acids at its N-terminus. Sequence alignments showed that FDL3 shares the conserved C-terminal phosphorylation (T)/SAP motif and groups closely with FD in a phylogenetic tree (Supplemental Figure 2.2).

We studied *FDL* expression in different vegetative tissues of 4-month-old *P. tremula* × *P. alba* clone 717-1B4 trees grown in a LD greenhouse. Similar to previous results (Parmentier-Line and Coleman 2016), expression of both *FDL2.1* and *FDL2.2* was mostly limited to the shoot tip (Supplemental Figure 2.3). Previous study of the seasonal expression of poplar *FT* paralogs in various tissues and organs was instrumental in revealing the divergence of *FT1* and *FT2* functions (Hsu et al. 2011). Thus, we studied expression of the *FDLs* using these same samples collected from adult *P. deltoides* growing in Mississippi, USA. Consistent with its ability to induce flowering (Parmentier-Line and Coleman 2016), *FDL2.2* was strongly upregulated in newly developing reproductive buds as was the *FDL2.1* splice variant (Figure 2.1c and 2.1d). The highest seasonal expression of *FDL1* was during late autumn-winter, especially in the preformed leaves of dormant buds (Figure 2.1a), consistent with its indicated role in mediating cold acclimation (Tylewicz et al. 2015). *FDL3* was more highly expressed in shoot apices during the growing season compared to autumn-winter season and showed a transient upregulation in leaves during early autumn (Figure 2.1c). Taken together, the different seasonal expressional patterns of poplar *FDL* genes imply that functional diversification of *FDLs* is not only in regard to a role in flowering, but also suggest that they could have distinct roles in vegetative phenology.
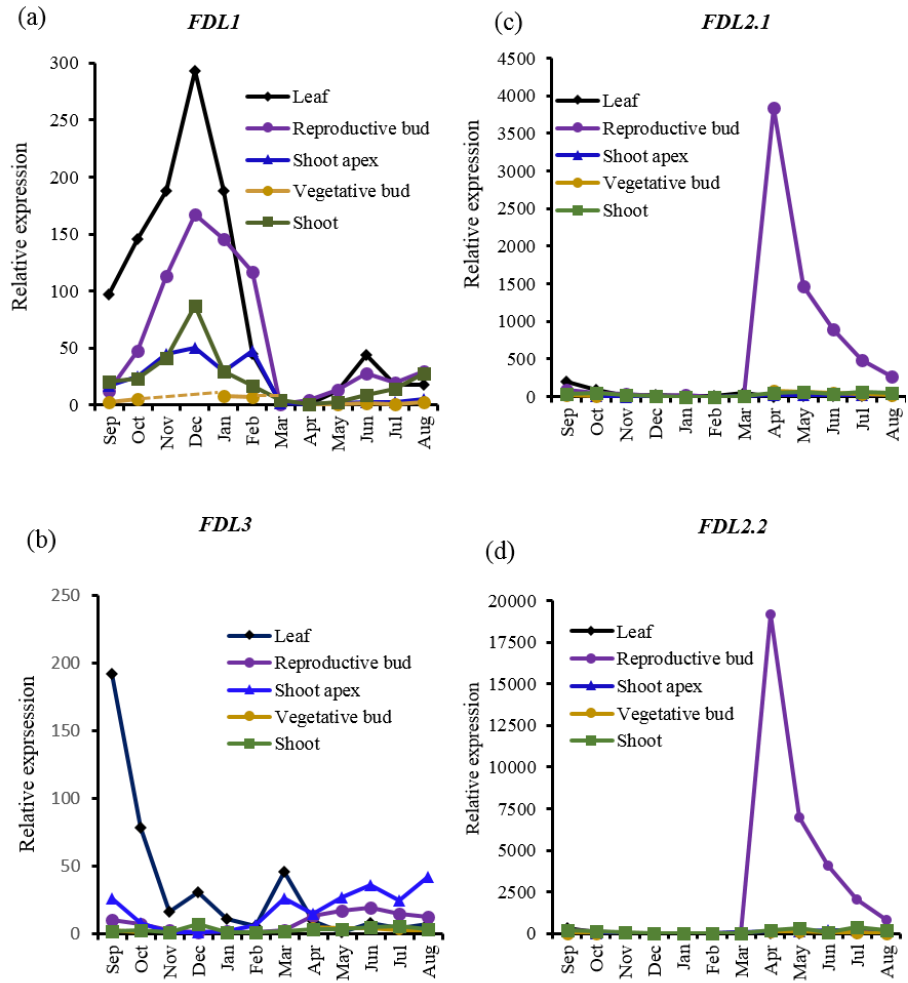
***Figure 2.1 Seasonal expression of poplar FDL genes in the same five tissues of mature P. deltoides.***

Relative expression was fold change in transcript levels of *FDL1* (a), *FDL3* (b), *FDL2.1* (c) and *FDL2.2* (d) relative to the lowest amount of expression within a tissue (n = 3). The expression was normalized against reference gene *18S rRNA*.

*Dominant repressor versions of each of the three poplar FDL reduces shoot elongation*

The 35S promoter was used to direct expression of *FDL1*, *FDL2.1*, *FDL2.2*, or *FDL3* coding regions fused to the SRDX repressor domain (transgenes designated *FDLrd*) in *P. tremula* × *P. alba* clone 717-1B4. All *FDLrd* transgenics showed varying degrees of reduced shoot elongation. The *FDL1rd* transgene imposed the most severe effect on shoot development (Figure 2.2). Many tiny transgenic shoots, confirmed positive for the *FDL1rd* transgene by PCR, initially regenerated from the explants transformed with *FDL1rd.* However, they failed to elongate when sub-cultured on shoot elongation medium and we could not regenerate any rooted plants. Six independent transgenic events of *FDL2.1rd* and eleven events of *FDL2.2rd* were regenerated (Supplemental Figure 2.4). Among them, four out of six *FDL2.1rd* events and five out of eleven *FDL2.2rd* events showed slow growth and short internodes when they were micropropagated and grown in vitro. Among all dominant repressor constructs, the *FDL2.2rd* transgene showed the weakest effect on shoot elongation and we were able to propagate and acclimate to soil several independent events. After growing in the LD greenhouse for 4 months, the *FDL2.2rd* transgenics were significantly shorter than WT plants. Compared to *FDL2.2rd*, *FDL2.1*rd transgenics showed a greater reduction in shoot growth in vitro (Supplemental Figure 2.4). *FDL2.1rd* trasngenics were not studied further, because overexpression of *FDL2.1* also reduced shoot growth (Tylewicz et al. 2015), indicating that the repressor domain might have augmented the WT protein's activity rather than repress its function.

FDL1dr on shoot elongation medium

Typical shoots on shoot elongation medium
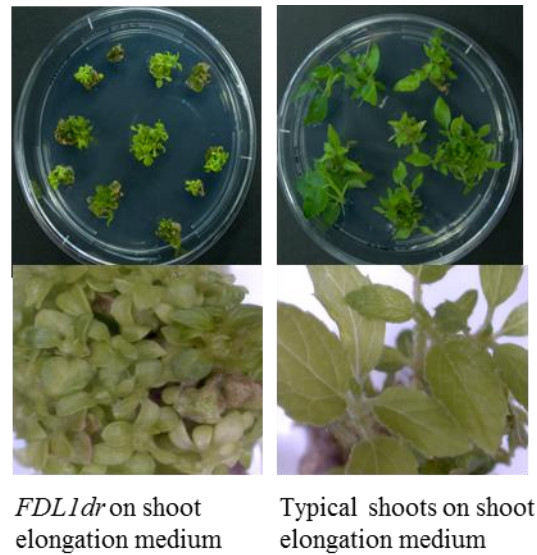
***Figure 2.2. Dominant repression of FDL1 inhibits shoot growth.***

Regeneration of 35S promoter-directed expression of dominant repressor version of *FDL1* (*FDL1dr*) transgenics are compared to an unrelated transgenic regenerated at the same time that shows typical shoot growth on shoot elongation medium.

We were able to regenerate and root in vitro five independent transgenic events of *FDL3rd*; however, internode elongation was severely affected (Figure 2.3A-a and B), and most attempts to propagate in vitro or acclimate to soil failed. However, we were able to transfer three ramets of event *FDL3rd*_56 to soil. After two months in a LD growth chamber, all three ramets grew no more than a height of 10 cm and set terminal buds, as opposed to the actively growing WT plants which reached 40 to 50 cm in height (Figure 2.3B_a). In addition, five ramets of event *FDL3rd*_52 were propagated and transferred to soil. Only two ramets survived and grew in the greenhouse for six months (Figure 2.3B-b). Besides having shorter internodes compared to WT (Supplemental Figure 2.5), the two *FDL3rd*_52 plants had significantly shorter petioles (Figure 2.3B-c), but had larger mature leaf lamina (Figure 2.3B-d). In sum, all of the dominant repressor versions of the different poplar *FDL* genes inhibited shoot elongation, but their effects varied in degree with *FDL1rd > FDL3rd > FDL2.2rd*. This suggests that the FDL proteins share incomplete functional equivalency.
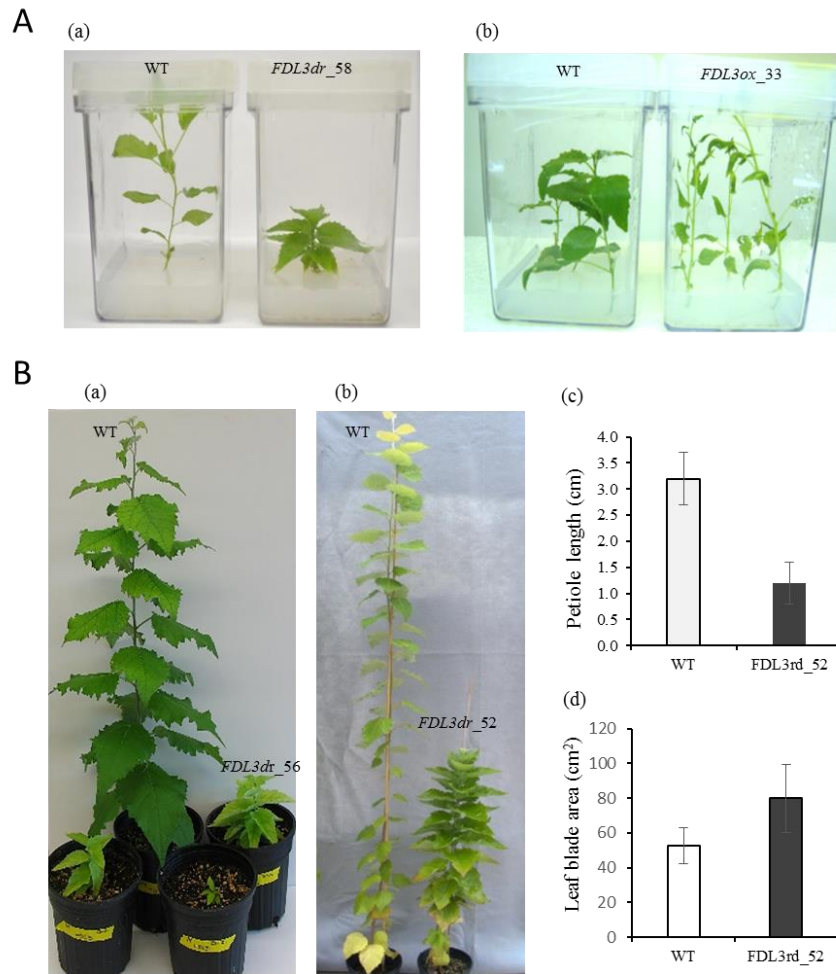
***Figure 2.3 Dominant repression of FDL3 inhibits shoot elongation, while overexpression of FDL3 promotes shoot elongation, but reduces leaf expansion under in vitro conditions, and phenotypes of FDL3dr plants in soil.***

A: Representative plants showing opposite effects on shoot elongation of (a) *FDL3* dominant repression (*FDL3dr*) versus (b) overexpression (*FDL3ox*) in *P. tremula* x *P. alba* clone 717-1B4). In (a) WT plant is 6-week-old, whereas *FDL3dr is* 10-week-old and in (b), WT and *FDL3ox* were propagated at the same time and are 4-week-old in the photo.

B: Phenotypes of FDL3dr plants in soil (a) FDL3dr_56 trasngenics showed reduced shoot growth and set terminal buds after 2 months in soil under LD conditions, whereas WT continued to grow. (b-d) FDL3dr_52 showed reduced shoot elongation (b), shorter petioles (c) but greater leaf area (d) compared to WT plants. In photo (b), plants are 6-month-old.

### *FDL2.2 but not FDL3 promotes early flowering under LDs*

As transgenics with dominant repressor versions of the FDLs were difficult to impossible to propagate in vitro and grow in soil, we produced transgenics with the 35S promoter directing expression of *FDL2.2* or *FDL3,* designated *FDL2.2ox* and *FDL3ox*, respectively. 16 independent events of each of *FDL2.2ox* and *FDL3ox* were regenerated. Micropropagated plants of *FDL2.2ox* and *FDL3ox* grown in vitro grew faster with longer internodes, but smaller leaves than WT plants propagated at the same time (Figure 2.3A-b). We observed in vitro flowering of one *FDL2.2ox* plant consistent with a previous study (Parmentier-Line and Coleman 2016). Because *FDL2.2ox* and *FDL3ox* transgenics had similar vegetative phenotypes in vitro, we directly compared the ability of the two transgenes to induce flowering under LDs. Three to five ramets of five events of *FDL2.2ox* and five events of *FDL3ox* were propagated and transferred to soil in parallel. The potted plants were grown in greenhouses with ambient light during the daytime and with extended light at the end of day to ensure a duration of 16 hr/8hr for regular LD growth conditions. All plants had small leaves as was the case in vitro, but over time, their height and diameter growth was reduced relative to WT and they also exhibited increased branching, especially the *FDL2.2ox* transgenics (Figure 2.4d and 2.4e). Given that shoot elongation was greater in vitro where sugars are supplied in the medium, the reduced growth in soil is likely a secondary effect of small transgenic leaves providing less photosynthate that WT leaves.

Within six months of growth in the greenhouse, all ramets of all *FDL2.2ox* events flowered. Trasngenics formed consecutive axillary inflorescences and terminal inflorescences were also formed on some of the plants (Figure 2.4a). However, we did not observe flowering on any of the *FDL3ox* plants, (Figure 2.4c). In addition, ramets from two of the FDL3ox events were grown for an additional 10 months (16 months in total) with no flowering. Thus, we conclude, that whereas *FDL2.2* and *FDL3* overexpression induces similar vegetative phenotypes under LDs, they are not equivalent in their ability to induce flowering, consistent with their different seasonal expression patterns and predominance of *FDL2.2* expression in reproductive buds (Figure 2.1). Because *FDL2.2* overexpression has been previously studied (Parmentier-Line and Coleman 2016), we focused on further characterization of *FDL3* vegetative effects.

**Figure 2.4 Overexpression of FDL2.2 but not FDL3 promotes precocious flowering under long days.**

All photos are of 6-month-old plants grown at the same time in a greenhouse with a 16-hour photoperiod. (a-c) shoot tips of (a) *FDL2.2ox* with consecutive axillary inflorescences, (b) WT and (c) *FDL3ox*. (d) A premature flowering *FDL2.2ox* plant with many inflorescences on the main shoot and branches. (e) A *FDL3ox* plant with small leaves and a few branches, but without any inflorescences on the plant.

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***Overexpression of FDL3 delays leaf development and the transition to secondary growth under LDs***

In LD growth chambers with a light density of 100 µmol $m^{-2}s^{-1}$, plants of both events *FDL3ox_*33 and *FDL3ox_*40 exhibited small leaves, but not curled, and the young trees had no branches (Figure 2.5b). This differed from the upwards curling leaves and multi-branches produced when the plant of both events were grown in greenhouses with ambient light during daytime (Figure2.4c and 2.4e), suggesting that differences in light quality or intensity between growth chamber and greenhouse affected phenotypes. However, phenotypes of both *FDL3ox* events were uniform and consistent within identical growth conditions. Despite potted *FDL3ox* plants being shorter compared with WT, grow rate (initiation of phytomers) was faster in *FDL3ox* transgenics, indicated by the formation of new leaves (leaf blades longer than 1 cm) over time (Figure 2.5a-d). However, the *FDL3ox* leaf expansion was much slower and, subsequently, the fully expanded leaf size was significantly reduced (Figure 2.5b and 2.5e). Taken together, it suggests that *FDL3* plays a role in promoting the rate of phytomer formation, whereas it inhibits expansion of leaves.

The stems of *FDL3ox* plants were not self-supporting, indicating defects in secondary growth. As leaf maturation and the transition to secondary growth are coordinated (Larson 1971; Larson and Isebrands 1974), we first examined these relationships in WT *P. tremula x P. alba.* Anatomical features of a stem developmental gradient under LDs (Supplemental Figure 2.6) shows that as leaves are maturing (only apical part of a leaf acting as source), the internodes are transitioning to secondary growth.
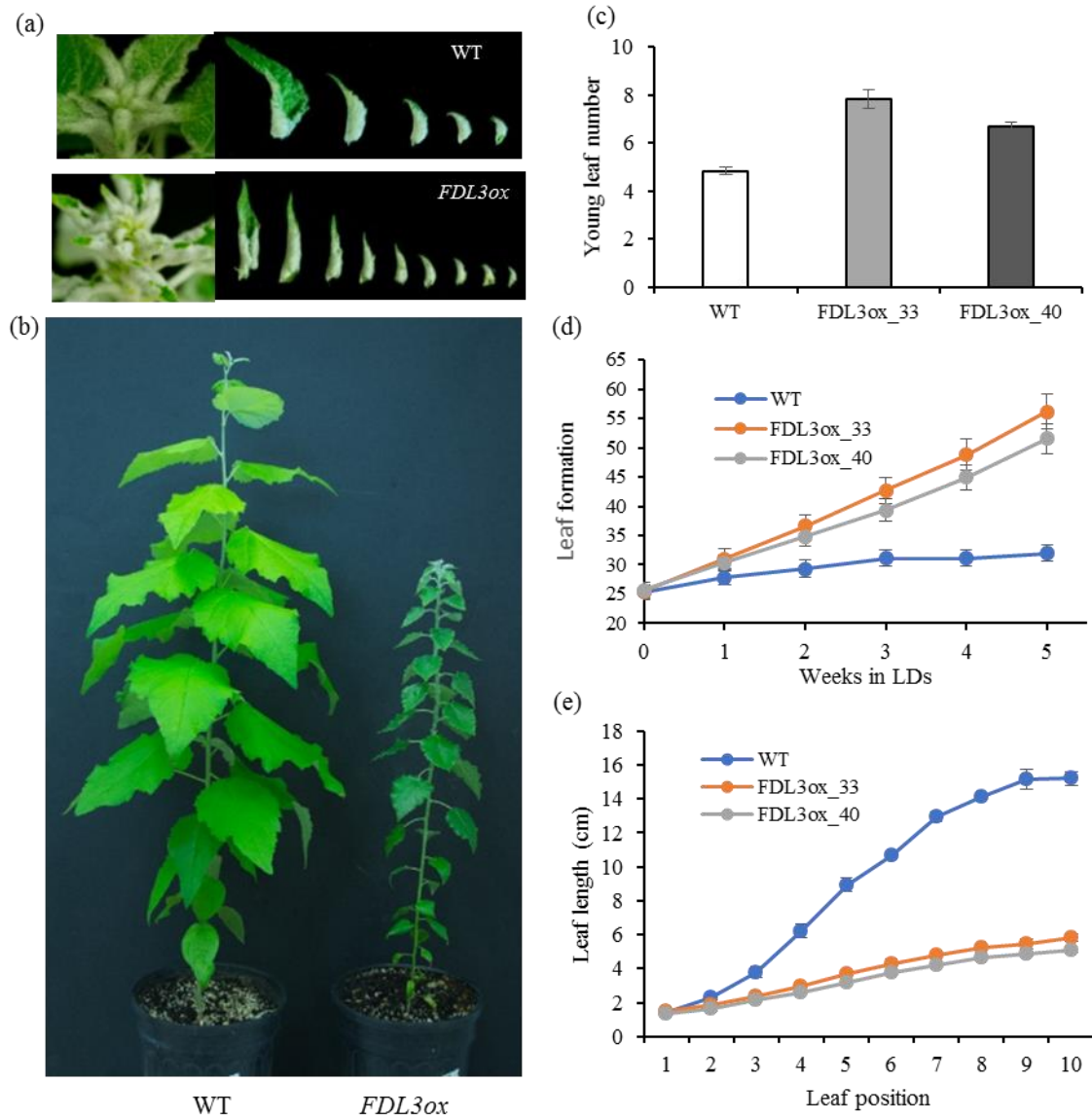
***Figure 2.5 Overexpression of FDL3 promotes leaf production, but represses leaf development when FDL3ox plants growing in long day (LD) conditions.***

Ramets of two *FDL3ox* events and WT were grown in a growth chamber under 16 hour photoperiods for 2 months. (a) Shoot tips with young rolled leaves of a *FDL3ox* plant compared with that of a WT plant. (b) A *FDL3ox* plant and a WT plant after 2 months in a LD growth chamber. (c-e) Means ± SE (n = 6) for two *FDL3ox* events (33 and 40) and WT (c) Number of young rolled leaves (d) Emergence of new leaves (leaf blades longer than 1 cm) over time. Leaf number was counted weekly, beginning 3 weeks after transplantation. (e) Progression of leaf length with position on the shoot.  Leaf position 1 is the youngest leaf, whose blade is longer than 1 cm.

Concomitant with the delayed leaf development of *FDL3ox* plants grown in LDs, secondary growth was also severely affected. Multiple events of *FDL3ox* trees show stalled leaf development and vine-like stems (Supplemental Figure 2.7a). Detailed study of leaf and stem development in two events, clearly illustrate the coordinate delay in leaf maturation and transition to secondary growth (Figure 2.6a and 2.6b).

First, transverse sections of IN2 of *FDL3ox* had nascent semi-continuous vascular cambium-like bundles composed of small and dark stained cells surrounded by ground tissue (Figure 2.6b1), in contrast to the clear primary vascular bundles presented in the elongating IN2 of WT (Figure 2.6b5). Secondly, the development of the vascular system in the IN4 and IN6 of *FDL3ox* remained nearly the same as in the IN2, with the exception that there were red-stained lignified cells appearing at the position where secondary xylem would normally develop. Additionally, no phloem fiber bundles were formed in either IN4 or IN6 (Figure 2.6b2, and 2.6b3), whereas transitional secondary growth in IN4 and secondary growth in IN6 progressively occurred in the stem of WT plants (Figure 2.6b6, and 2.6b7). Lastly, phloem fiber bundles were present even though the secondary xylem in the lower IN10 of *FDL3ox* still remained in a much less developed state (Figure 2.6b4). We cross-sectioned further down the stem of FD3ox plant, yet even IN20 lacked well-developed secondary xylem, but was eventually evident in IN30 (Supplemental Figure 2.7b, c). Thus, this suggests that *FDL3* may play a role in inhibiting the formation of a functional vascular cambium concomitant with inhibition of leaf maturation.
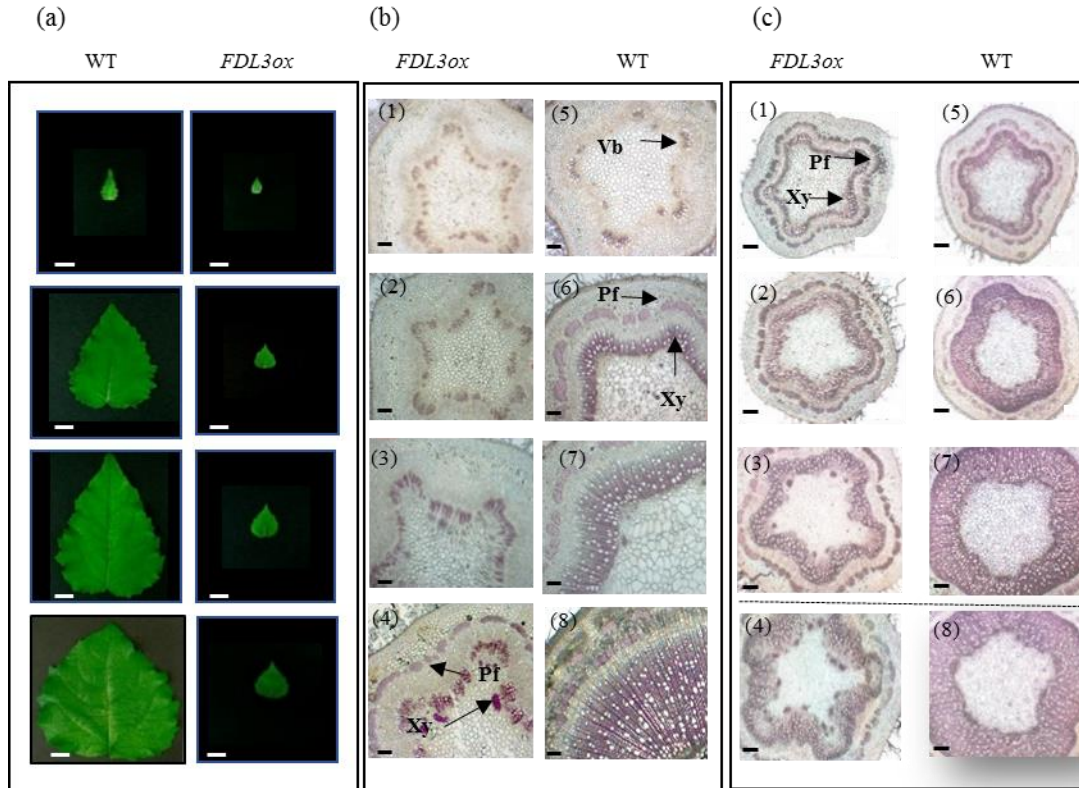
***Figure2.6 Overexpression of FDL3 reduces leaf size and inhibits stem secondary growth of FDL3ox plants grown in long days (LD), but stem secondary growth is restored in short days (SD).***

Both *FDL3ox* and WT plants were grown in a LD greenhouse for 6 months, and subsequently transferred into a SD growth chamber for 8 weeks. Leaves were counted from top to bottom according to leaf plastochron index (LPI). Internodes (INs) referred to the internodes beneath the corresponding LPI. All panels show from top to bottomLPI2, LPI4, LPI6 and LPI10 or corresponding INs. (a) Extremely slow growth of *FDL3ox* leaves compared to WT in LDs. Scale bars = 2 cm. (b) Severely inhibited secondary growth in *FDL3ox* plants (b1-4) compared to progressive transition to secondary growth in WT (b5-8) in LDs. (c) Secondary growth in IN2, IN4 and IN6 formed after exposure to SDs of *FDL3ox* plants. Note that INs 4 and 6 now resemble the same INs of WT plants grown in LDs (b5-7). In contrast, IN10 (images below the dot line) formed before SD treatment (in LDs) remained underdeveloped in *FD3ox*. After exposure to SDs, WT plants ceased elongation growth, IN2 transitioned to secondary growth (c5) and substantial secondary xylem accumulated in IN4 and IN6 of WT (c6-7). Transverse sections were 60 µm thick, Scale bars = 100 µm. Vb: vascular bundles, Pf: phloem fiber, Xy: xylem.

*Overexpression of FDL3 delays SD-induced growth cessation*

After exposure to SDs, poplars complete the processes of growth cessation and bud set within four to six weeks. In our SD experiments, height growth of WT plants stopped after three weeks, whereas *FDL3ox* plants showed no sign of growth cessation (Figure 2.7b). Meanwhile, terminal buds progressively formed in WT. After five weeks in SD, WT plants had formed reddish brown apical buds, while shoot apices of *FDL3ox* plants remained active (Figure 2.7a). Hence, *FDL3ox* plants produced more new leaves under SDs conditions (Figure 2.7c). However, *FDL3ox* plants eventually formed apical buds after 10 weeks in SDs (Figure 2.7a).
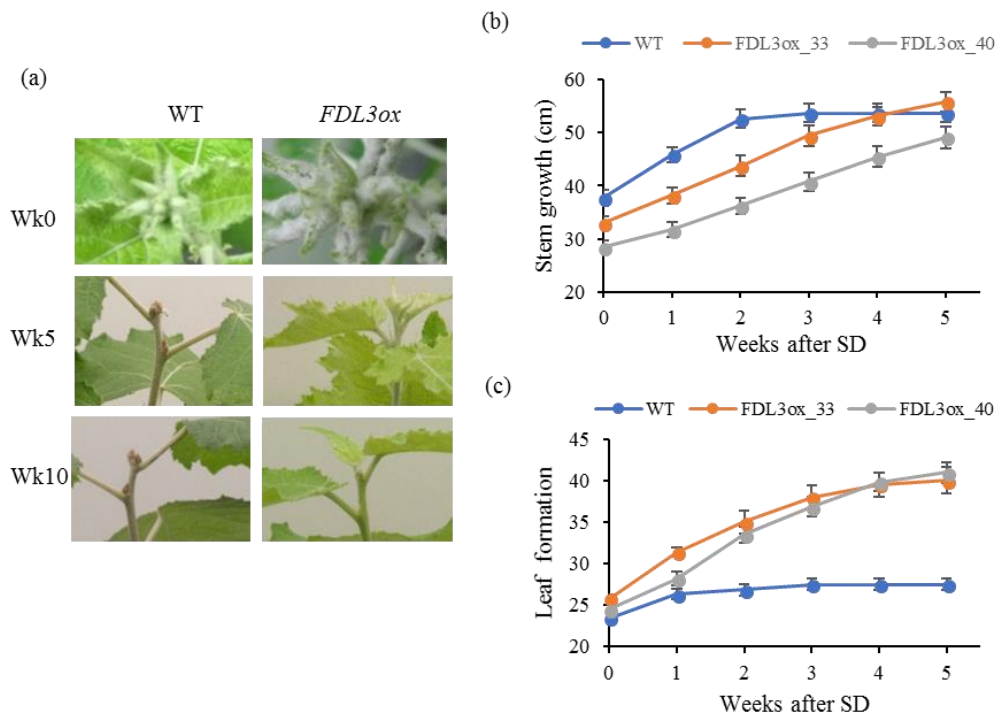


**Figure 2.7 Overexpression of FDL3 delays growth cessation and bud set under short days.**

Plants of WT and two independent *FDL3ox* events (33 and 40) were grown in long days for 2 months before exposure to short days (SDs). (a) Apical bud development of *FDL3ox* plants compared to WT after 5 and 10 weeks (Wk5 and Wk10) in SD. By Wk5, WT plants had formed buds. In contrast, *FDL3ox* plants maintained an actively growing apex. By Wk10, FDL3ox plants formed buds. (b-c) Cumulative stem growth (b) and leaf formation (c) were measured weekly during the first 5 weeks in SDs. (b) Plant height (y axis) and (c) Leaf numbers (y axis) indicate mean $\pm$ SE (n = 6). The duration of SDs is shown (in weeks) on the x axis.

*SD restores leaf development of FDL3ox plants*

Intriguingly, we observed that, in SDs, *FDL3ox* plants not only continued to grow, but leaves formed after transfer to SDs, developed similar to the leaves of WT grown in a LD greenhouse

(Figure 2.8a and 2.8b, above the red arrows of the *FDL3ox*). However, SDs did not reverse the size of small leaves of *FDL3ox* plants formed under LDs (Figure 2.8a and 2.8b, below the red arrows of the *FDL3ox* plant). In WT plants, SDs induced the leaves directly below the forming apical bud to expand (Figure 2.8 a, b). To further investigate the effects of photoperiod, we tracked the dynamics of leaf development of *FDL3ox* and WT plants. Plants were first grown under LD conditions for two months, then under SD conditions for four weeks, and finally back to LD conditions for three weeks (2nd LD). While fully expanded leaf size of WT was not affected by the photoperiodic changes, the extent of leaf expansion in *FDL3ox* plants was determined by the length of photoperiod received during leaf emergence and development (Figure 2.8). Most strikingly, leaves formed on the *FDL3ox* plants became small again as the lighting was reset back to LD conditions (Figure 2.8d, above the yellow arrow and Figure 2.8e, marked as 2nd LD).

As described above, phenotypes of *FDL3ox* plants grown under LDs in greenhouse differed somewhat from plants grown in growth chambers, suggesting that light quality or intensity differences affected FDL3ox phenotype. Thus, to specifically study the effect of photoperiod, we transferred plants from tissue culture to pots and grew them entirely in a growth chamber with only photoperiod altered. Within four weeks in SDs, leaf expansion in *FDL3ox* was the same as WT. *FDL3ox* plants were still actively growing, while apical buds had started to form in WT (Figure 2. 9b and 2.9c). In contrast, young *FDL3ox* plants grown in LD conditions at the same time were shorter with small leaves (Figure 2.9a and 2.9c). Thus, the effect of *FDL3ox* overexpression on leaf development is dependent on photoperiod.
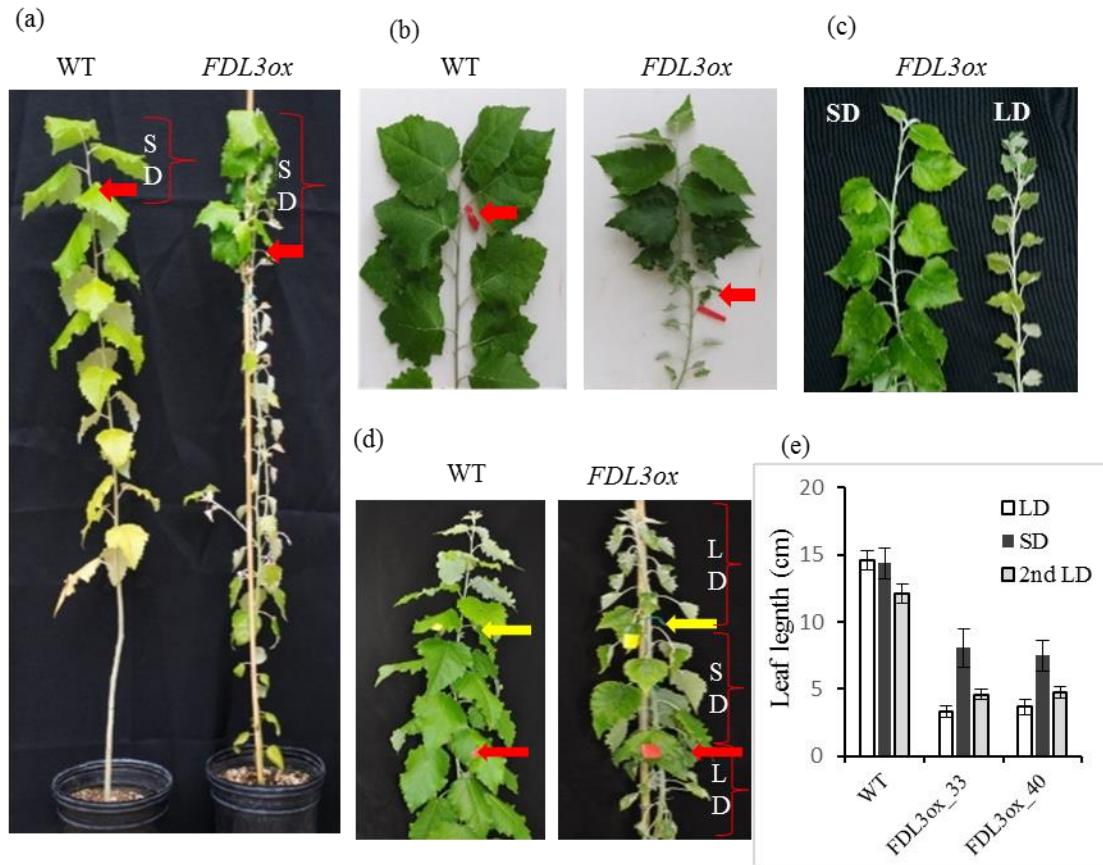
**Figure 2.8 Resumption of leaf development FDL3ox plants in short days.**

(a-b) Both *FDL3ox* and WT plants were grown in a long day (LD) greenhouse for 6 months, and then were transferred into a short day (SD) growth chamber for 8 weeks. *FDL3ox leaves formed* after transfer to SDs (above the red arrows) showed leaf development of actively growing WT plants, in contrast to underdeveloped leaves formed on *FD3ox* in LDs (below the red arrows). Plants (a) and shoots (b) were imaged after 8 weeks exposure to SDs. (c) Shoots from two ramets of the same *FDL3ox* event grown 8 weeks in SDs (left) or in LDs (right). (d-e) The changes in leaf expansion size of *FDL3ox* plants followed the changes of photoperiod duration. *FDL3ox* and WT plants were grown for 2 months in LDs (below the red arrows), followed by 4 weeks of SDs (between red arrows and yellow arrows), and then 3 weeks of LDs (above the yellow arrows). (d) Leaves formed in SDs on *FDL3ox* are larger than leaves formed in LDs on the same plants, while the expanded leaf size of WT remained the same. (e) Leaf size of fully expanded leaves of WT and *FDL3ox* plants formed in SDs and LDs. Leaves were measured for 6 fully expanded leaves of each plant. Leaf length (y axis) indicate mean ± SE (n =4).
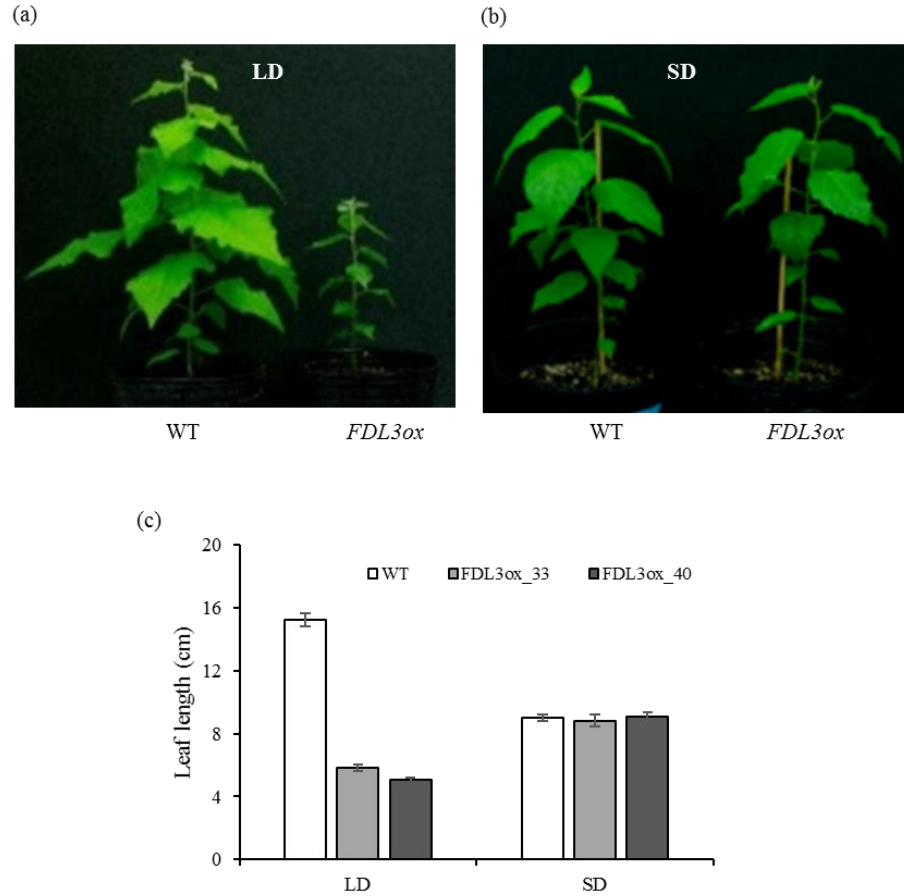
*Figure 2.9 Effects of photoperiod on young plants grown in a growth chamber.*

After 2 weeks acclimation in soil pots, very young *FDL3ox* and WT seedlings were divided into 2 groups grown in two growth chambers for 4 weeks. (a) *FDL3ox* with small leaves and reduced growth compared to WT in a long day (LD) growth chamber. (b) WT-like *FDL3ox* plants with no phenotypical alteration in a short day (SD) growth chamber. (c) Leaf length of two independent events of *FDL3ox* and WT plants in the LDs and SDs. Six fully expanded leaves of each plant were measured after 4 weeks in LDs and SDs. Leaf length (y axis) indicate mean ± SE (n =4).

### SD restores secondary growth of FDL3ox plants

As the leaf expansion in *FDL3ox* were restored once grown in SD conditions, we examined restoration of the secondary growth of the stem segments formed under SD conditions in *FDL3ox* and WT plants. In contrast to the poor secondary growth of *FDL3ox* plants under LD conditions (Figure 2.6b1-4), a closed circle of secondary xylem and phloem fiber bundles were present in all three internodes of *FDL3ox* plants formed after eight weeks in SD conditions (Figure 2.6c1-3). Normally, the transition of primary to secondary growth advances to the top internode directly under the apical bud in poplar trees after 4-6 weeks of growth in SDs (Goffinet and Larson 1981). We observed substantial secondary xylem and phloem fiber bundles in the IN2 of WT plants, which set bud before the internode was collected after eight weeks of SD treatment (Figure 2.6c5). As small leaves formed before the onset of SD described above was not reversed (Figure 2.8a and 2.8b), the secondary xylem in the lower stem segment of IN10, which formed in LDs before the start of SD treatment, remained poorly developed (Figure 2.6c4). Collectively, overexpression of *FDL3* represses leaf development and secondary growth during LD growing conditions. However, a short photoperiod can counteract this repression. Considered together, this suggests that *FDL3* has a role in coordinating leaf and stem development and that this is dependent on other factors whose activity is regulated by photoperiod.

### Gene expression changes associated with FDL3ox phenotypic effects

To look for gene expression alterations induced by *FD3ox* that might contribute to the leaf and stem phenotypes, we took a candidate gene approach based on work in both poplar and Arabidopsis. *FT2* is rapidly downregulated in response to SD signals (Bohlenius et al. 2006; Hsu et al. 2011) and studies suggest that *LIKE-AP1a (LAP1a)* acts downstream of an FT2-FD complex in the shoot apex to promote growth under LDs (Azeez et al. 2014; Tylewicz et al. 2015).  In Arabidopsis, the small, curled leaf phenotype induced by overexpression of *FT* under SDs was dependent on *FD* and linked to high expression of *FUL* and *SEP3* in leaves (Teper-Bamnolker and Samach 2005). Moreover, studies have shown roles for *FUL* homologs in leaf development of diverse species, including tomato and *Aquilegia* (Bar and Ori 2014). Thus, we hypothesized that repression of leaf development in *FDL3ox* plants might involve FT2-dependent ectopic or elevated activation of *AP1/FUL* family genes in the leaves under LD conditions. When in SDs, downregulation of *FT2* might prevent promotion of *AP1/FUL* family

gene expression. The poplar genome contains five members of the *AP1/FUL* subfamily (De Bodt et al. 2006) and (Supplemental Figure 2.8b), including two *AP1* co-orthologs (*LAP1a, LAP1b*), one *FUL* ortholog, and two members that do not have a clear Arabidopsis ortholog. To characterize leaf expression, we first studied *FDL3* and *FT*2 expression in a WT leaf developmental series under LDs. Whereas *FT2* was dramatically upregulated in the first mature leaf (LPI6), leaf developmental stage had little effect on *FDL3* expression (Supplemental Figure 2.9). Compared to WT plants, *FT2* expression in LPI6 leaf was 4 to 6-fold higher in *FD3ox* transgenics (Figure 2.10a). Consistent with its sensitivity to SD signals, expression levels of *FT2* were significantly reduced in WT leaf after three weeks in SD conditions. Moreover, the expression of *FT2* in the leaves of *FDL3ox* plants were downregulated in a similar manner in SD conditions. In *FDL3ox* transgenics, only three *AP1/FUL* members, *LAP1a, LAP1b* and *FUL* were upregulated under LDs and their expression level was correlated with expression level of the *FDL3ox* transgene (Supplemental Figure 2.8c-e). Thus, we studied expression of these three genes in LPI6 leaf and for comparison to previous studies (Azeez et al. 2014; Tylewicz et al. 2015; Parmentier-Line and Coleman 2016) also in the shoot apex. In WT plants under LDs, *LAP1a, b* were low to barely detectable in shoot apices and mature leaf, whereas *FUL* was relatively highly expressed (Supplemental Figure 2.10). However, all three genes were upregulated in LPI6 leaf of FDL3ox trees under LDs (Figure 2.10b-d). Similar to *FT2* expression, the three *AP1/FUL* homologs were markedly downregulated in the leaves of both WT and *FDL3ox* transgenics under SDs (Figure 2.10). In shoot apices, the *AP1/FUL* homologs showed similar expression changes, except that their downregulation in FD3ox plants under SDs was weaker than in leaves (Supplemental Figure 2.11). Taken as a whole, these results suggest that under LDs, *FDL3ox* transgenics could have elevated levels of a FT2-FDL3 complex in leaves that activates *AP1/FUL* homologs but that under SDs, reduced *FT2* levels limit complex formation and hence, *AP1/FUL* expression.
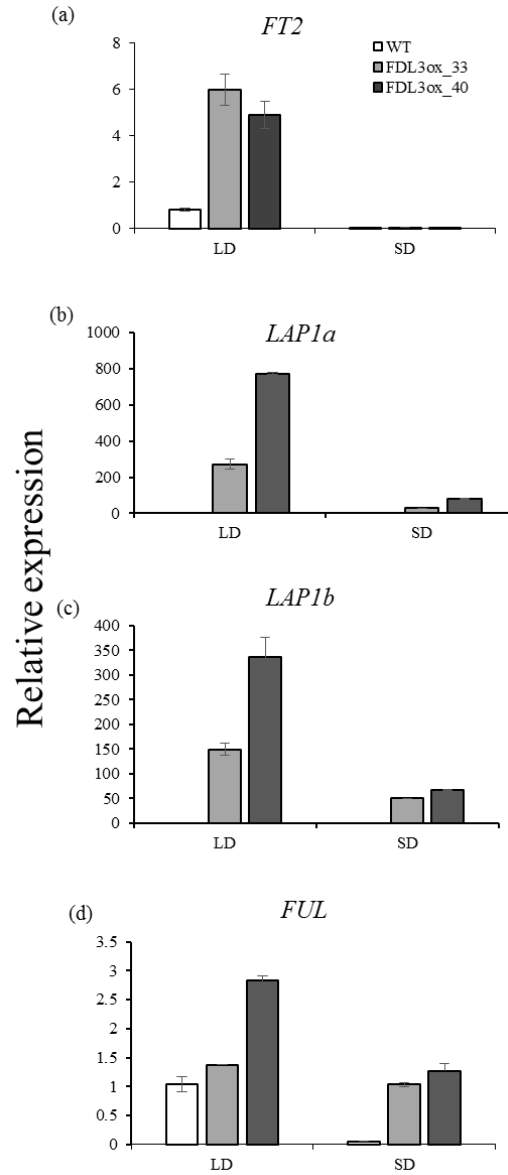
***Figure 2.10 Downregulated expression of FT2 and downstream targets AP1/FUL members in the leaf of FDL3ox and WT after 3 weeks in short days.***

Fully expanded leaves were collected from WT and two independent events of *FDL3ox* (33 and 40) plants grown for 2 months in long days (LDs), followed by 3 weeks in short days (SDs). Relative fold changes in transcript levels of *FT2* (a), *LAP1a* (b), *LAP1b* (c), and *FUL* (d) were relative to that of WT. Expression of two *AP1/FUL* members, *MADS14* and *MADS28*, was not detectable in either LDs or SDs. The expression was normalized against reference gene *UBQ*. LD:16 hr light/8 hr dark, SD: 8 hr light/16 hr dark.

**Discussion**

*Seasonal expression patterns of poplar FDL family genes reveal regulatory diversification*

*FD* was originally identified as a flowering time gene acting at the shoot apex (Abe et al. 2005; Wigge et al. 2005; Azeez et al. 2014) and homologs have similar roles in rice (Taoka et al. 2011). However, studies in different species also indicate that homologs are expressed in leaves and other tissues and have functions in vegetative development (Teper-Bamnolker and Samach 2005; Tsuji et al. 2013; Tylewicz et al. 2015). For example, the rice *FD* homolog *OsFD2* is expressed in the leaf blade and leaf sheaf, and overexpression did not alter flowering time but did affect shoot branching and panicle architecture (Tsuji et al. 2013). Moreover, the flowering promoter OsFD1 forms a floral activation complex (FAC) in the leaf as well as at the shoot apex (Brambilla et al. 2017). Similarly, poplar *FDL1* was reportedly to play a role in cold adaption and bud maturation. In agreement with its function, expression of *FDL1* was upregulated by SD conditions in the apical bud during the final stages of bud maturation (Tylewicz et al. 2015). We studied all poplar *FDL*s over an annual cycle in multiple tissues. *FDL2.1* and its splice variant *FDL2.2* are distinct in that they are the only *FDL* showing predominately floral expression (Figure 2.1c and Figure2.1d). Specifically, they are primarily expressed in initiating reproductive buds that are first distinguishable shortly after vegetative bud flush (Brunner et al. 2014) consistent with a canonical function in the control of the floral transition. Interestingly, expression of *FT1* peaks in preformed leaves and shoot approximately two months before the peak in *FDL2* expression (Hsu et al. 2011) and (Figure 2.1c and Figure2.1d) suggesting that the FT1 protein persists until *FDL2* upregulation allows the formation of FAC.

Previous study of *FDL1* suggested it functions with *FT2* to control shoot growth and has an *FT*2-independent function in bud maturation and acclimation to freezing temperatures (Tylewicz et al. 2015). FDL1 was shown to interact with the transcription factor ABSCISIC ACID-INSENSITIVE3 (ABI3), a gene previously shown to have a role in bud formation (Rohde 2002; Ruttink et al. 2007; Tylewicz et al. 2015). Moreover, transgenics misexpressing either gene show altered expression of genes involved in adaptation to cold and dehydration. *FDL1* was upregulated in late fall and winter, particularly in preformed leaves within the bud (Figure 2.1a), consistent with a role in promoting bud maturation and cold acclimation. Upregulation of *FT1* expression occurs in late winter (Hsu et al. 2011), after *FDL1* expression has declined. In

addition to promoting flowering, various lines of evidence suggested that *FT1* also promotes dormancy release (Rinne et al. 2011; Brunner et al. 2014; Evans et al. 2014). In controlled experiments with juvenile trees, *FT1* upregulation in a dormant vegetative bud occurs during chilling and correlates with the opening of plasmodesmata, which is necessary for SAM reactivation (Rinne et al. 2011). Single nucleotide polymorphisms (SNPs) in the *FT1* locus were also associated with bud flush time in a genome wide association study (Evans et al. 2014). Finally, the previously demonstrated role of conserved *FT* antagonist, poplar *CEN1*, in repressing dormancy release suggested that *FT1* promotes dormancy release (Mohamed et al. 2010; Brunner et al. 2014). Dormancy and adaptation to freezing temperatures prevent growth. Thus, downregulation of *FDL1* might be necessary for subsequent *FT1* upregulation or putative activity in promoting dormancy release.

In contrast to *FDL1*, *FDL3* is expressed in shoot apices during the growing season, and is transiently upregulated in leaves in late summer/early fall (Figure 2.1 b). Taken together, differential seasonal expression patterns in different tissues of poplar *FDL* genes indicate that *FDL*s have multifaceted roles in poplar tree development, including roles in both reproductive development and different phases of vegetative phenology.

*Partial diversification of FDL protein functions*

That the dominant repressor version of each of the FDL genes reduced shoot elongation in LDs to different degrees (Figure 2.2, Figure 2.3Aa and B, Supplemental Figure 2.5) indicates that the proteins share partial functional equivalency. Interestingly, *FDL3ox* plants developed new leaves at a faster rate than WT under LDs (Figure 2.5). Combined with its expression in shoot apices during the growing season, *FDL3* might be the *FDL* paralog that has a primary endogenous role in promoting growth. Gibberellin (GA) has a key role in promoting stem elongation and a number of studies have implicated GA metabolism and signaling in SD-mediated growth cessation (Busov et al. 2003; Zawaski et al. 2011; Zawaski and Busov 2014). Thus, exploring the participation of *FDLs* in GA signaling pathways is a promising direction for further study of the multifunctionality of *FDL* family genes. We have started to work on comparing expression levels of the GA biosynthesis gene to find candidate *FDL3* downstream targets as well as comparing effects of GA treatments on *FDL3dr* plants and of uniconazole-P (inhibitor of GA biosynthesis)

treatment on *FDL3ox* plants. Previous research and this study have also shown that overexpression of *FDL1*, *FDL2.2* or *FDL3* delays SD-induced growth cessation and bud set (Tylewicz et al. 2015; Parmentier-Line and Coleman 2016) and (Figure 2.7), indicating that the proteins share some degree of functional equivalency.

Only overexpression of *FDL2.2* induced precocious flowering under LDs (Parmentier-Line and Coleman 2016). Although the *FDL2* splice variants differ in their ability to induce flowering, both show a similar seasonal expression pattern (Figure 2.1c-d). However, *FDL2.2.* is more highly upregulated than *FDL2.1* in initiating floral buds, suggesting that the ratio of the two splice variants might have a role in determining whether an axillary vegetative meristem transitions to a reproductive meristem. Overexpression of *FD1* did not induce any obvious changes in growth and development under LDs (Tylewicz et al. 2015), whereas both *FDL2.2ox* and *FDL3ox* plants had smaller leaves in LDs (Parmentier-Line and Coleman 2016) and (Figures 2.4d-e). However, these transgenics differed in that SDs did not alter leaf size in transgenics overexpressing *FDL2.2*. Although considerable evidence supports that a combination of regulatory divergence and partial diversification of encoded proteins has resulted in functional diversification of the *FDL* paralogs, definitive evidence of the endogenous roles of the *FDLs* is still lacking. Gene editing can potentially reveal gene-specific functions; however, given the difficulties in regenerating dominant repressor transgenics, knock outs could be equally challenging, especially for *FDL1* and *FDL3*.

***FDL3 coordinately affects leaf maturation and the transition to secondary growth***
Study of *FDL3ox* plants revealed a joint effect on leaf and stem development that was dependent on daylength (Figure2.6). *FDL3dr* plants were difficult to impossible to propagate and study in depth; however, two ramets of one event grew for six months in soil under LDs and in contrast to the *FDL3ox* plants, they had larger mature leaf lamina than WT (Figure 2.3d). Although the coordination among leaf development and the transition to secondary growth has long been recognized (Larson 1971; Larson and Isebrands 1974), the signaling pathways regulating this synchronization are unknown. From a physiological standpoint, the formation of a functional vascular cambium introduces another strong sink; thus, that this transition occurs some distance from an active SAM and below a leaf that has at least partially matured could reflect sink-source

relationships. $^{14}$C tracer studies in *Populus* showed that the direction of carbon transport changes as leaves develop (Dickson and Larson 1981; Isebrands and Nelson 1983). In general, transitional leaves (i.e., only an apical portion is mature) mostly transport carbon upward to younger leaves and the SAM, recently mature leaves transport in both directions, and older mature leaves primarily transport carbon to the lower stem and roots. The greatly delayed transition to secondary growth of *FDL3ox* plants under LDs might be a direct consequence of the stalled leaf maturation preventing transport of sufficient sugar to initiate and support secondary growth. It is also possible that a more active SAM (faster leaf initiation) increases sink strength and limits sugar import into leaves for expansion and correspondingly, less carbon is available for cambium formation and secondary growth. Whereas sugar transport and signaling could have a key regulatory role in coordinating the developmental transitions of a woody shoot, other long-distance signaling pathways are also likely. In tobacco, GA-mediated signaling from maturing leaves was necessary for both shoot elongation and radial growth (Dayan et al. 2012). Although the FT protein is a long-distance signal that moves to the SAM where it forms a complex with FD to promote the transition to flowering, there is increasing evidence that FACs also form in leaves (described above) and have other roles (Tsuji et al. 2013; Tylewicz et al. 2015). In Arabidopsis, *FT* has a role in leaf development under certain environmental conditions, including SDs and blue light LDs, that is dependent on *FD* as well as *FUL* (Teper-Bamnolker and Samach 2005). These studies combined with that *FT2* expression is predominately limited to mature leaves Supplemental Figure 2.9b) and *AP1/FUL* family members have been implicated in photoperiodic control of growth (Teper-Bamnolker and Samach 2005; Azeez et al. 2014), prompted us to study expression of these genes in WT and *FDL3ox* transgenics under different photoperiods.

Under SDs, cessation of elongation growth, which reduces SAM sink strength, is coordinated with internodes immediately below the apex transitioning to secondary growth (Goffinet and Larson 1981) and (Figure 2.6c). Expression of *FT2* in a mature leaf was elevated under LDs in *FDL3ox* plants, but similar to WT, *FT2* expression was downregulated under SDs. Three *AP1/FUL* family members showed a similar elevation in the shoot apex as well as mature leaf in *FDL3ox* plants, but while SD-mediated downregulation occurred in both organs, it was greater in leaf.  Thus, these results suggest the possibility that *FDL3* overexpression increases the level of a

*FDL3-FT2* complex in leaf as well as SAM under LDs that elevates the expression of putative downstream targets to promote SAM activity, but limit leaf maturation and the transition to secondary growth. Under SDs perhaps reduced *FT2* expression is sufficient to maintain growth, but not to affect leaf development and the transition to secondary growth. 35S promoter-directed expression of *FT2* induces precocious formation of flowers and reduces vegetative growth (Hsu et al. 2011), thus, its direct effect on leaf development is unclear. Although not specifically studied, *LAP1a* overexpression did not appear to alter leaf development under LDs (Azeez et al. 2014). Moreover, *LAP1a* promotes the expression of *AINTEGUMENTA-like (AIL1)* which activates core cell cycle genes such as D type cyclins (Karlberg et al. 2011; Azeez et al. 2014). Leaf development involves spatial and temporal coordination of cell cycle activity and cell expansion. A developing poplar leaf stops cell division when it reaches one third of its full size: thus, cell expansion is predominantly responsible for leaf enlargement (Dickmann 2001). Leaves are initiated at a faster rate in *FDL3ox* plants (Figure 2.5d) and the first couple leaves below the apex are about the same size as the corresponding WT leaves (Figure2.5e). Therefore, reduced cell expansion might be the primary cause of stalled leaf growth in *FDL3ox* plants. To further characterize the differences in leaf development, we are investigating expression of genes related to cell division and expansion in FDL3ox and WT leaves. Various FT and FD homologs have been shown to interact with other proteins and to have functions independent of one another (Tsuji et al. 2013; Tylewicz et al. 2015; Brambilla et al. 2017); thus, it is possible that the effect of *FDL3* on leaf development and secondary growth is independent of *FT2*. Alternatively, *FT2* could have an indirect role, in that a more active SAM (faster leaf initiation) increases sink strength and limits sugar import into leaves for expansion and for cambium formation in the stem.

***Conclusion***

Similar to the two poplar *FT* paralogs (Hsu et al. 2011), the three *FDL* paralogs have diverged in regulation and their encoded proteins show a degree of functional divergence. Among them, only *FDL2.2* promoted precocious flowering in very young trees, whereas *FDL1* and *FDL3* appear to have distinct roles in vegetative growth and phenology. Overexpression of either *FDL1* (Tylewicz et al. 2015) or *FDL3* delayed SD-induced growth cessation and bud set, but only *FDL3* coordinately altered leaf development and the transitioned to secondary growth. Thus,

further study of *FDL3*, including identification of targets, could provide the first inroad into understanding the genetic pathways that link these two developmental aspects of a woody shoot.

**Supplemental Figures and Tables**

(a)



(b)
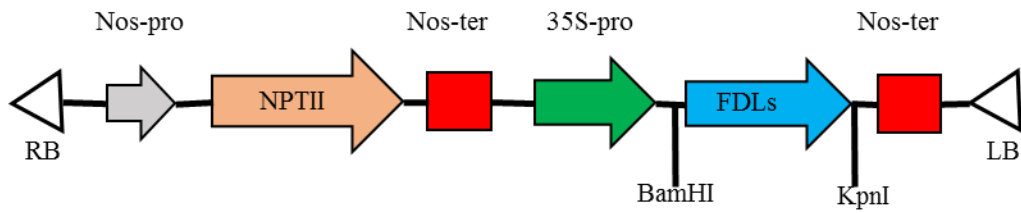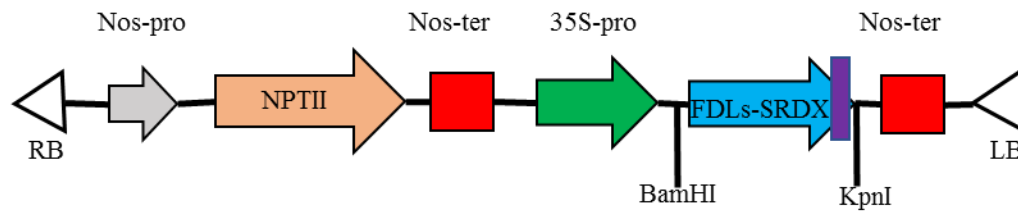


*Supplemental Figure 2.1 Diagram of the overexpression of FDL (FDLox) and dominant repression of FDL (FDLrd) constructs used for poplar transformation.*

(a) Coding regions of *FDL2.2* and *FDL3* in *Populus deltoids*, and (b) coding regions of *FDL1*, *FDL2.1*, *FDL2.2* and *FDL3* in *P. deltoids* directly fused with a dominant repressor, SRDX, were clone into PBI121 vector between constitutive 35S promotor (35S-pro) and *nos* terminator (Nos-ter).

**Supplemental Figure 2.2 Poplar FD-like (FDL) family members contain a conserved T/SAP motif at the C-terminus.**

(a) Alignment of amino acid sequences of FD family members from *Arabidopsis thaliana* (At), and FDL1, FDL2.1, FDL2.2 and FDL3 in *Populus deltoides* generated using Clustal Omega, and then visualized using BoxShade server (ExPASy). (b)Phylogenetic tree of poplar FDLs and FD proteins from other plant species. FDs from Arabidopsis are in red, FDLs from poplar are in blue.

***Supplemental Figure 2. 3 Diverse expressions of poplar FDL genes in various organs of young poplar trees.***

Expressions of poplar *FDL* genes in 4-month-old trees of WT *P. tremula x P. alba* clone 717-1B4) grown in greenhouse under LDs. ST, shoot tips; AB, axillary buds; YL, young leaf (leaf plastochron index 2, LPI2 (LPI1 was the first leaf below the apex, whose lamina length larger or equal of 1 cm); ML, mature leaf (fully expanded leaf, LPI6); IN2, internode beneath LPI2 (primary growth zone); IN6, internode beneath LPI6 (transitional zone from primary to secondary growth; Ph, phloem; Xy, xylem; Rt, root. The expression was normalized against UBQ and expressed as fold upregulation to the sample showing the lowest expression. Error bars represent SD for three replicate reactions performed on a pooled sample from three trees.
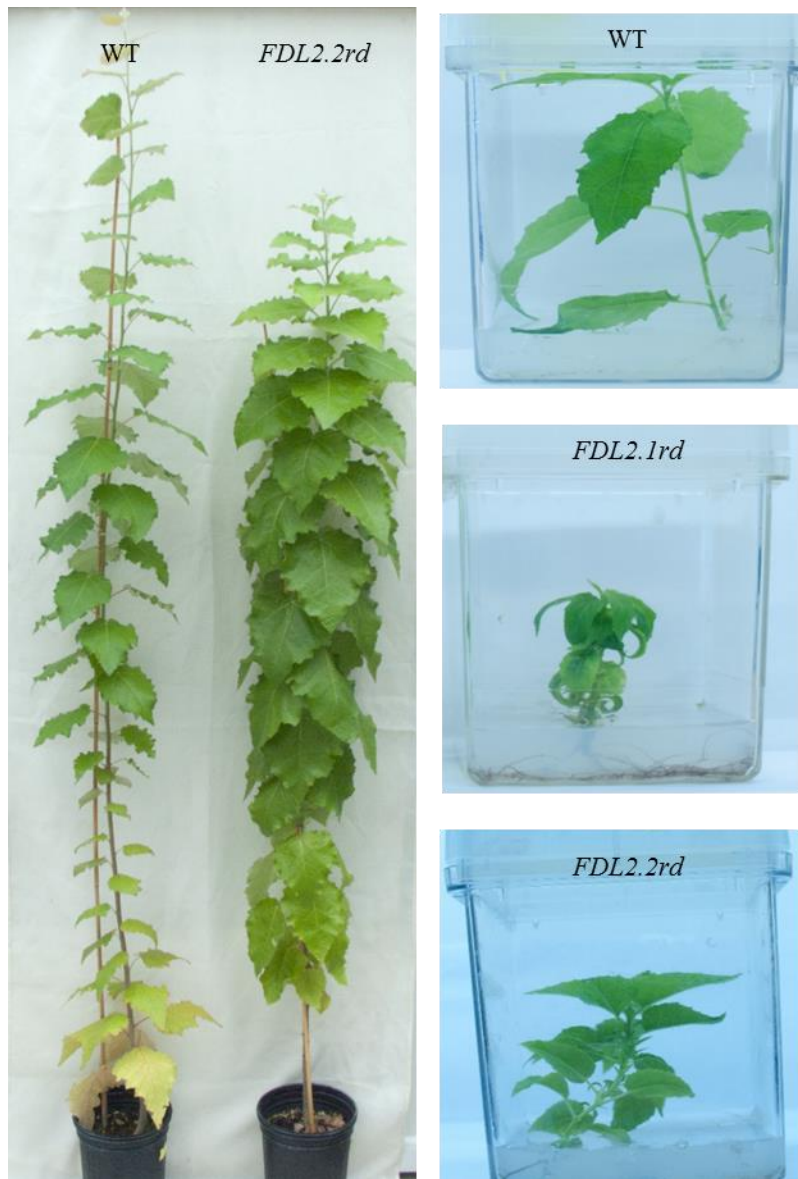
***Supplemental Figure 2.4 Dominant repression of FDL2.1 and FDL2.2 inhibits shoot growth.***
Overexpression each of two splice variants of *FDL2.1* and *FDL2.2* fused to a dominant SRDX repressor under the constitutive CaMV *35S* promoter (*FDL2.2rd FDL2.1rd*) in WT *Populus* (*P. tremula* x *P. alba* clone 717-1B4) inhibited shoot development. Plants were in greenhouse for 4 months and in vitro for 2 months.
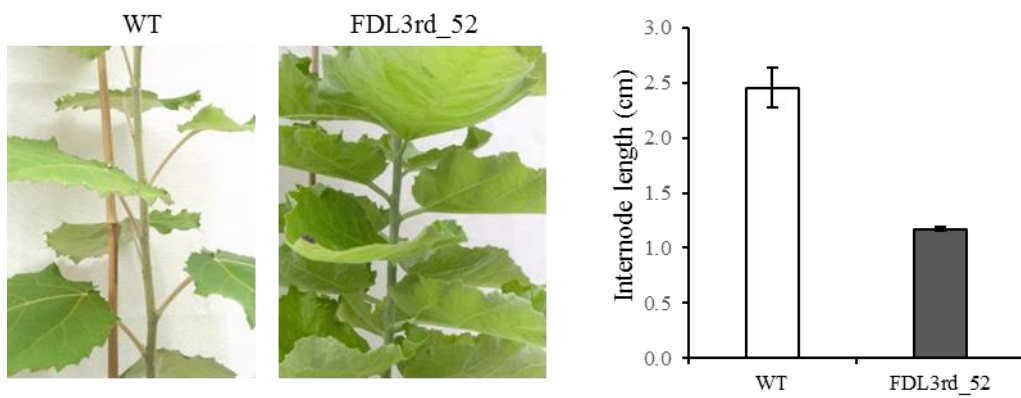
***Supplemental Figure 2.5 Dominant repression of FDL3 inhibits internode elongation.***

Overexpression of *FDL3* fused to a dominant SRDX repressor under the constitutive CaMV *35S* promoter (*FDL3rd*) in WT *Populus* (*P. tremula* x *P. alba* clone 717-1B4) showed shorter internodes
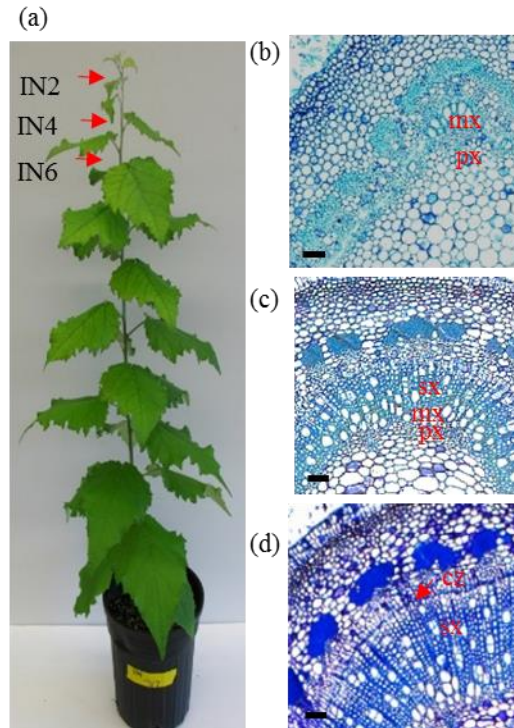
***Supplemental Figure 2.6 Secondary growth of poplar trees is closely associated with leaf development.***

Anatomical features of cross-sections along the leaf development zone of the stem of a 2-month-old *P. tremula* × *P. alba* clone 717-1B4 tree under LDs. including primary, transitional and secondary growth. (a) Sample positions along stem are indicated. Internode 2 (IN2), IN4 and IN6 are the internodes beneath the leaves of leaf plastochron index 2, 4 and 6 (LPI2, LPI4 and LPI6). LPI1 was defined as the first leaf below the apex, whose lamina length larger or equal of 1 cm. (b-d) Transverse sections (2 µm thick) of IN2, IN4 and IN6 respectively. (b) INT2 contains primary xylem (protoxylem and metaxylem) in vascular bundles, which represent the primary growth stage. (c) IN4, which is beneath a transitional leaf is transitioning to secondary growth. Xylem fibers have begun to differentiate adjacent and centrifugal to the last formed metaxylem vessels and secondary vessels have begun to expand. In IN6, which is beneath the first mature leaf, displays secondary growth. A well-structured secondary xylem (a matrix of xylem fibers, secondary vessel elements, and xylem rays) and a continuous circle of cambium are evident. cz, cambial zone; mx, metaxylem; px, protoxylem; sx, secondary xylem. Scale bars = 50 µm.
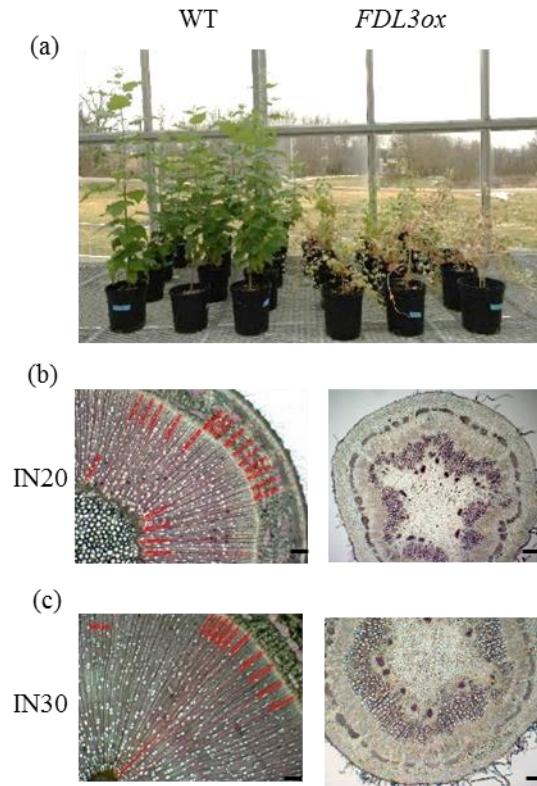
***Supplemental Figure 2.7 Dominant repression of FDL3 inhibits stem secondary growth.***

Underdeveloped stem secondary growth in *FDL3ox* plants. (a) Plants were grown under LD conditions in a greenhouse for 6 months, WT (left) compared with *FDL3ox* (right). (b-c) Low sections to base of the stems of 6-month-old *FDL3ox* and WT plants were cut to examine secondary xylem development. Transverse sections (60 µm thick) were from the 20th and 30th internode slices (IN20, b and IN30, c). Scale bars = 200 µm.
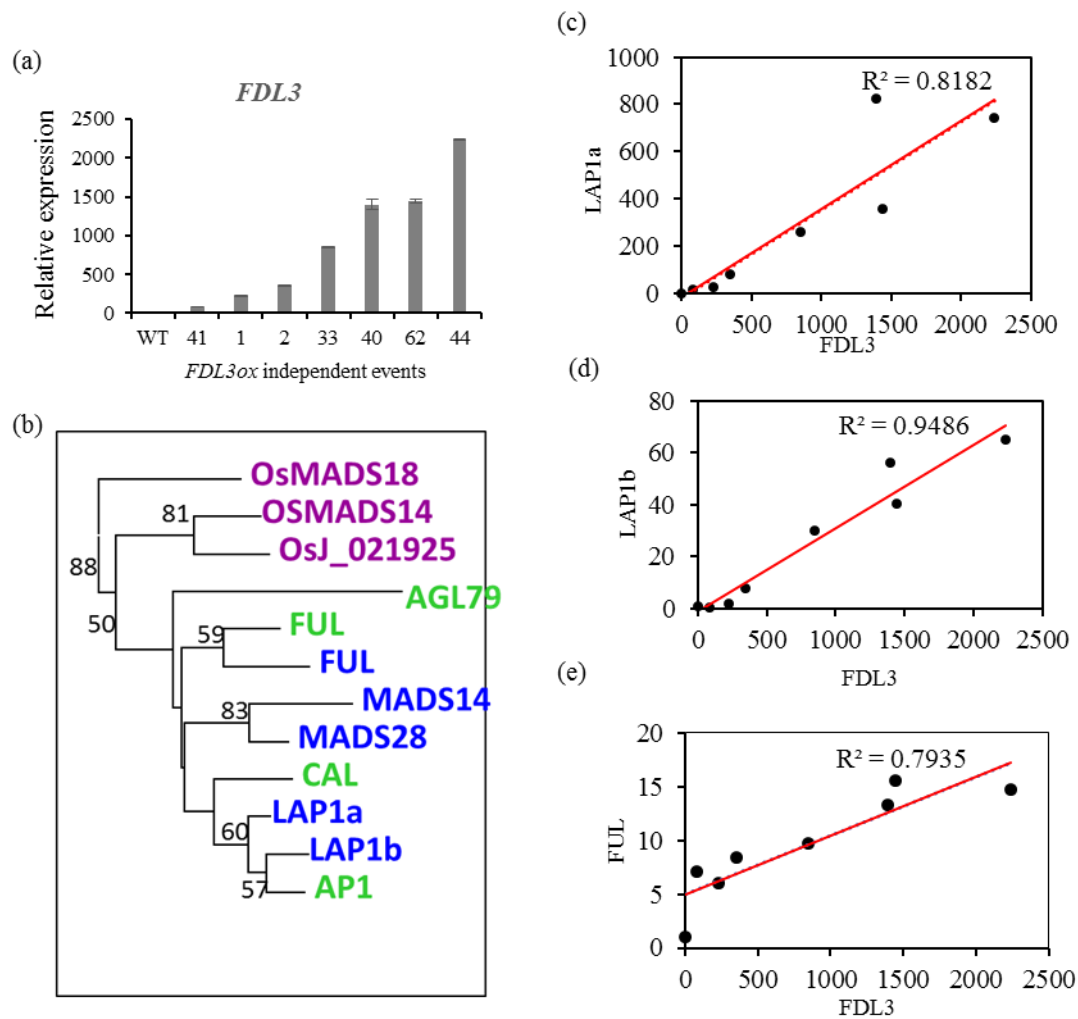
**Supplemental Figure 2.8 Overexpression of FDL3 induces expressions of three AP1/FUL members in the shoot apex of FDL3ox plants.**

Shoot apex of one-month-old *FDL3ox* plants in vitro grown in magenta boxes under LD conditions. (a) Transgene *FDL3* expression in the shoot tips of WT and seven independent *FDL3ox* events. (b) Phylogenetic tree of AP1/FUL members from poplar (in blue), Arabidopsis (in green), and rice (in magenta). (c-e) Expression levels of *LAP1a* (c), *LAP1b* (d) and *FUL* (e) were highly correlated with that of *FDL3*.

(a)



(b)
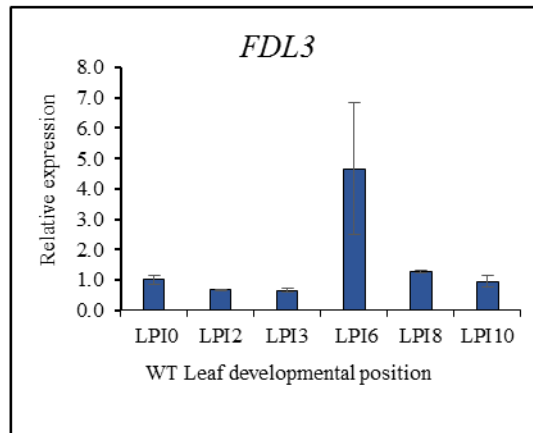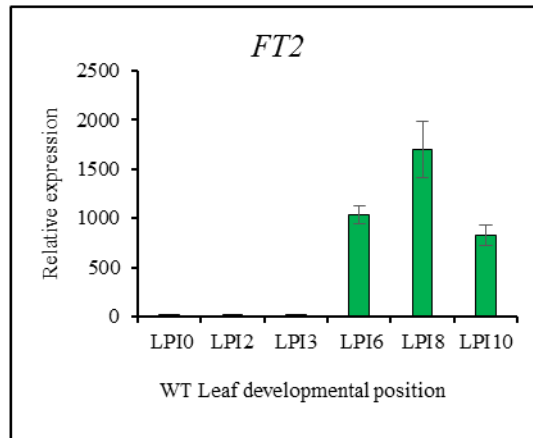


**Supplemental Figure 2.9 Expression of FDL3 and FT2 in development leaves of WT in long days.**

Developmental leaves were collected from WT plants grown for 2 months in long days s (16 hr light/8 hr dark). Leaves were counted from top to bottom according to leaf plastochron index (LPI). LPI1 was the first leaf below the apex, whose lamina length larger or equal of 1 cm. The expression was normalized against *UBQ* and expressed as fold upregulation to the sample showing the lowest expression.
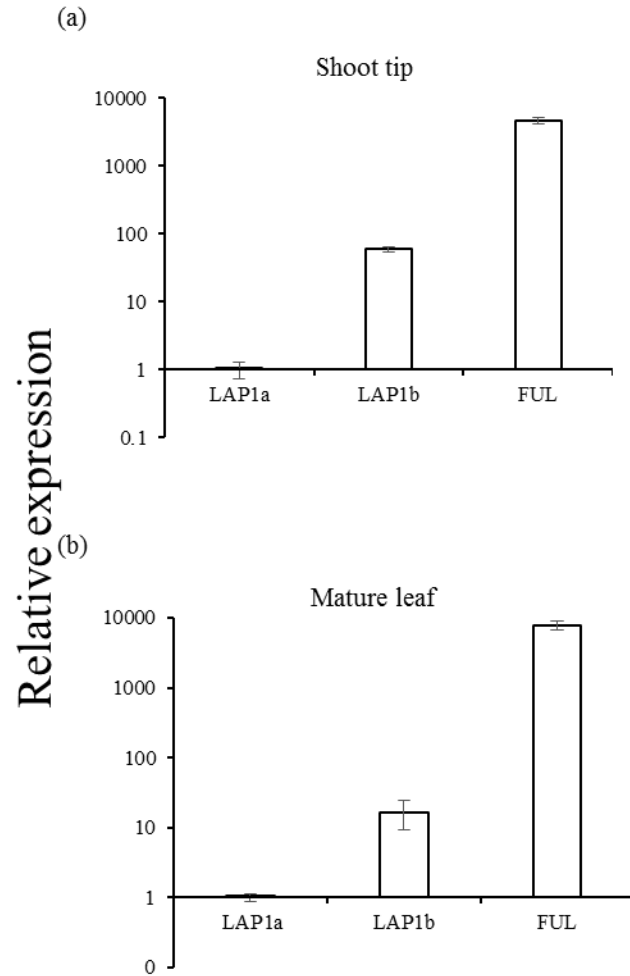
**Supplemental Figure 2.10 Expression of three AP1/FUL members in shoot apex and mature leaf of WT in long days.**

Shoot apiece and fully expanded leaves were collected from WT plants grown for 2 months in long days s (16 hr light/8 hr dark). The expression was normalized against *UBQ* and expressed as fold upregulation to the gene showing the lowest expression.

**(a)** *LAP1a*

**(b)** *LAP1b*

**(c)** *FUL*

Relative expression
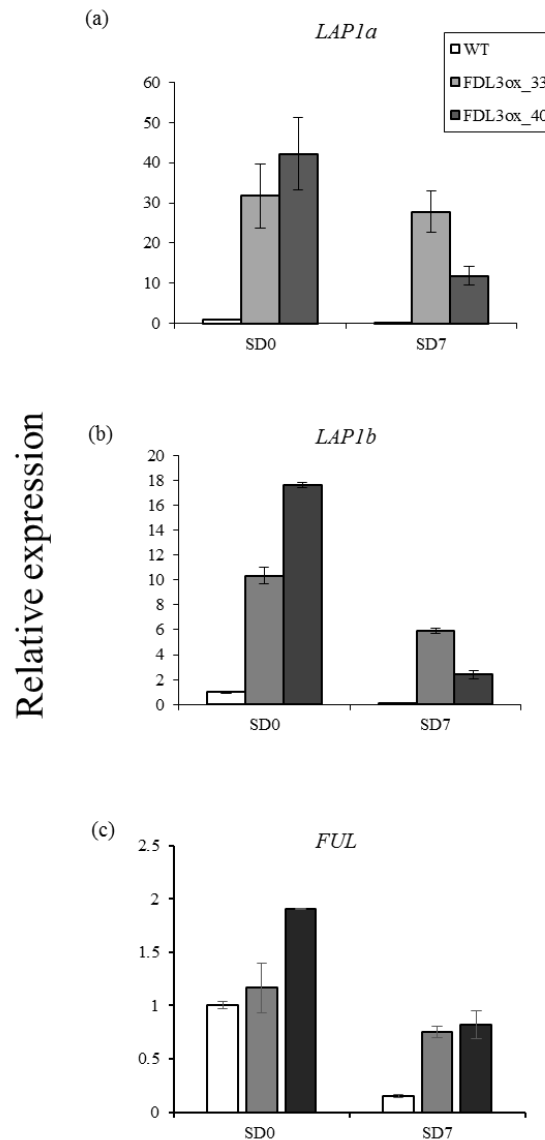
*Supplemental Figure 2.11 Downregulated expression of three AP1/FUL members in shoot apex of FDL3ox and WT after 7 days in short days (SDs).*

WT and two independent events of *FDL3ox* (33 and 40) plants grown for 2 months in long days (LDs). Shoot apiece were collected on the day started to SD treatment (SD0) and after 7 days in SDs (SD7). Relative fold changes in transcript levels *LAP1a* (a), *LAP1b* (b), and *FUL* (c) were relative to that of WT. The expression was normalized against *UBQ*. SD0:16 hr light/8 hr dark, SD: 8 hr light/16 hr dark.

*Supplemental Table 2.1 Populus FDL gene family members*

| Sheng et al. (this paper) | Tylewicz et al. 2015 | Parmentier-Line & Coleman 2016 | Potri ID | Differences with Potri gene models and relationship to previous studies |
|---|---|---|---|---|
| *FDL1* | *FDL1* | *FD2* | Potri.002G018400.1 | *P. deltoides* cDNA is spliced differently from predicted Potri gene model. *P. trichocarpa* EST DT479848.1 encodes a protein of identical length that shares 99.5% identity with *P. deltoides* FDL1. Compared to these proteins, FDL1 reported by Tylewicz et al. 2016 lacks 39 amino acids from the N-terminus. |
| *FDL2.1* | *FDL2* | | Potri.005G243400.1 | Splice variant studied by Tylewicz et al. 2016 |
| *FDL2.2* | | *FD1* | Potri.005G243400 | Splice variant studied by Parmentier-Line & Coleman 2016; no corresponding transcript predicted in Phytozome. |
| *FDL3* | | *FD3* | Potri.005G109500.1 | *P. deltoides* cDNA differs from Potri gene model; model lacks 35 amino acids of the bZIP domain. A *P. trichocarpa* cDNA encoding a protein that includes these 35 amino acids was also isolated (data not shown). |

*Supplemental Table 2.2 Populus AP1/FUL subfamily members*

| Name | Potri ID | Previous studies |
|---|---|---|
| *FUL* | Potri.012G062300.1 | |
| *LAP1b* | Potri.008G098500.1 | |
| *LAP1a* | Potri.010G154100.1 | Azeez et al. 2014 |
| MADS14 | Potri.017G099800.1 | |
| MADS28 | Potri.004G115400.1 | |

*Supplemental Table 2.3. Nucleotide sequence of oligonucleotide primers used in this study*

| Primer | Sequence (5'-3') | Orientation | Purpose |
|---|---|---|---|
| FDL2.2F | ATGTGGTCATCGCCAGGAGC | Forward | FDL2.2 |
| FDL2.2R | TCAAAATGGAGCTGTTGAGG | Reverse | cloning |
| FDL3F | ATGTTGTCGCCAACAGATTG | Forward | FDL3 |
| FDL3R | TCAAAATGGAGCTGTTGATG | Reverse | cloning |
| FDL1F | ATGAGCACCAATAAAGTCTC | Forward | FDL1 |
| FDL1R(SRDX) | TCAGGCGAATCCGAGTCTCAGTTCGAGATCCAGATCGAG AAATGGAGCCGTTGAGG | Reverse | With SRDX |
| FDL2.1F | ATGTGGTCATCGCCAGGAGC | Forward | FDL2.1 |
| FDL2.1R(SRDX) | TCAGGCGAATCCGAGTCTCAGTTCGAGATCCAGATCGAG AAATGGAGCTGTTGAGG | Reverse | With SRDX |
| FDL2.2F | ATGTGGTCATCGCCAGGAGC | Forward | FDL2.2 |
| FDL2.2R(SRDX) | TCAGGCGAATCCGAGTCTCAGTTCGAGATCCAGATCGAG AAATGGAGCTGTTGAGG | Reverse | With SRDX |
| FDL3F | ATGTTGTCGCCAACAGATTG | Forward | FDL3 |
| FDL3R(SRDX) | TCAGGCGAATCCGAGTCTCAGTTCGAGATCCAGATCGAG AAATGGAGCTGTTGATG | Reverse | With SRDX |
| PdFDL1qF | CAGCAACAACAACACCGACGACCATG | Forward | FDL1 |
| PdFDL1qR | CAACTCGGTCGTGTAAGCCTGCTTCC | Reverse | RT-PCR |
| PdFDL2.1qF | CTAATTTTGCAGGCTTACACAGTTG | Forward | FDL2.1 |
| PdFDL2.1qR | GAGCAAGTGGGATTAATGTATTCTC | Reverse | RT-PCR |
| PdFDL2.2qF | GGAAAGCAGTGATACTGTCCCTGTG | Forward | FDL2.2 |
| PdFDL2.2qR | CTCAACTGTGTAAGCCTGCTTTCTAGC | Reverse | RT-PCR |
| PdFDL3qF | CCGATCAAGAACTTTCCATGACCCCA | Forward | FDL3 |
| PdFDL3qR | CAGGCCTCTGAGGATGATCAGAGTTC | Reverse | RT-PCR |
| Pd18SrRNAqF | GGAATTGACGGAAGGGCACCACCAGGC | Forward | 18SrRNA |
| Pd18SrRNAqR | GGACATCTAAGGGCATCACAGACCTG | Reverse | RT-PCR |
| 717FDL1qF | GAGAAGTAAGAAATCTATGCTTAGCCCAT | Forward | FDL1 |
| 717FDL1qR | CCTTTCTTTTCCCCTCCTTTCTTTTACAT | Reverse | RT-PCR |
| 717FDL2.1qF | CAGGAATCTGGCTCTCCTTTTGAAAATT | Forward | FDL2.1 |
| 717FDL2.1qR | CAGCTTCACGCTCCAACTCAACT | Reverse | RT-PCR |
| 717FDL2.2qF | GCTAGAAAGCAGGCTTACACAGTTGA | Forward | FDL2.2 |
| 717FDL2.2qR | GTGGGATTAATGTATTCTCAAAATGGAGC | Reverse | RT-PCR |
| 717FDL3qF | TGGCCCTGGCTTTGATTTTCTTGAG | Forward | FDL3 |
| 717FDL3qR | AGATGAATCTAAACCCTCAAAAGGACTAG | Reverse | RT-PCR |
| 717FT2qF | CTACCGGGGCGAACTTTGGGCAAGAGG | Forward | FT2 |
| 717FT2qR | TCATGGTCTCCTTCCACCGGAGCCAC | Reverse | RT-PCR |
| 717UBQ2qF | TGTACTCTTTTGAAGTTGGTGT | Forward | UBQ |
| 717UBQ2qR | TCCAATGGAACGGCCATTAA | Reverse | RT-PCR |
| 717LAP1aqF | GAGAAGGAGAAGAAGGATAAAGCTG | Forward | LAP1a |
| 717LAP1aqR | CCAAATATTCATGCTCCGTAACC | Reverse | RT-PCR |
| 717LAP1bqF | AGATCAAGGAGAAGGAGAAAGCAC | Forward | LAP1b |
| 717LAP1bqR | ACCCAAATATTCATGTTCCAAAGC | Reverse | RT-PCR |
| 717FULqF | CCACTCCAATCCAACATCGAG | Forward | FUL |
| 717FULqR | CAACATTTTCGCTTCATCAGACAG | Reverse | RT-PCR |
| 717MADS28qF | GATGCTTCGCCATGTCAACG | Forward | MADS28 |
| 717MADS28qR | GAAAGTTACGAAATACATTCTTGTGCTC | Reverse | RT-PCR |
| 717MADS14qF | GGATGCTTCGCCATGTCACTG | Forward | MADS14 |
| 717MADS14qR | TTATTGTGACTTAGCACTTGAAG | Reverse | RT-PCR |

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# 3. DIFFERENTIATING THE FUNCTIONS OF THE *POPULUS* PARALOGS *FT1* AND *FT2* IN THE ANNUAL GROWTH CYCLE USING CRISPR/CAS9 GENE EDITING APPROACH

**Sheng X,** Horner L and Brunner AM.

**Abstract**

The phenology of growth and dormancy transitions is a key adaptive trait in trees from temperate latitudes and the phenology of plantation genotypes must be closely matched to local climates for optimal biomass production. Studies indicate that *Populus FLOWERING LOCUS* (*FT*) paralogs, *FT1* and *FT2*, have distinct functions in different aspects of vegetative phenology as well as flowering. *FT1* is upregulated by low temperatures in winter and proposed to promote flowering and dormancy release, whereas *FT2* is upregulated by warm temperatures and long daylengths (LDs) and is proposed to promote vegetative growth in LDs. However, their endogenous functions have yet to be definitively shown. The two paralogs exhibit functional redundancy when either of them is overexpressed. Furthermore, gene-specific downregulation was not achieved via RNA-mediated methods due to high sequence conservation. To reveal the distinct functions of *FT1* and *FT2*, we used the CRISPR/CAS9 system to generate *ft1ft2* double mutants and *ft1*-specific mutants. The *ft1* mutant plants were WT-like under LDs and in short daylengths (SDs), they ceased growth and set terminal buds at the same time as WT. However, after subsequent exposure to chilling temperatures and transfer to LDs and warm temperature conditions, bud flush was delayed in *ft1* mutants compared to WT. In contrast, double *ft1ft2* mutants showed extremely reduced shoot elongation and set terminal buds in tissue culture under 16 hours daylengths. Our results show that *FT1* promotes dormancy release, and that *FT2* is necessary to sustain vegetative growth.

**Introduction**

In boreal and temperate ecosystems, the annual alterations of growth and dormancy are vital to the adaptation of forest trees. Natural populations must be matched to local climates for optimal growth under favorable conditions and survival from dehydration and freezing stress in winter months (Evans et al. 2014). Reduction of daylength in fall induces growth cessation, formation of a bud with bud scales enclosing preformed leaves and the shoot apical meristem (SAM) and bud dormancy (Rohde 2002). At the same time, cellular changes result in acclimation to

freezing temperatures and dormancy prevents resumption of growth during fall and winter. Meanwhile, exposure to an extended period of chilling throughout the winter promotes release from dormancy, thereby enabling reinitiation of growth when warm temperatures return in the spring (Brunner et al. 2014; Yordanov et al. 2014).

First identified in Arabidopsis, *FLOWERING LOCUS T* (*FT*), is a highly conserved protein that integrates environmental and endogenous signals to promote flowering at an appropriate seasonal time (Corbesier et al. 2007; Andres and Coupland 2012). The *Populus* genome contains two *FT* orthologs, *FT1* and *FT2*, whose encoded proteins share some functional redundancy, while they have markedly different seasonal expression patterns (Hsu et al. 2011). *FT2* is upregulated in leaves in warm temperatures and LDs during late spring through summer, while it is rapidly downregulated in response to SDs in the fall. Therefore, *FT2* has been proposed to promote shoot growth under LD conditions, and conversely must be downregulated to induce growth cessation and entry into dormancy under SD conditions (Hsu et al. 2011). In contrast, *FT1* is upregulated in the shoot apex, preformed leaves, and preformed stem within a winter bud by cold temperatures. Thus, *FT1* has been suggested to participate primarily in promoting reproductive bud onset in early spring (Hsu et al. 2011). In addition, under controlled conditions, *FT1* was upregulated by cold temperature regardless of photoperiods (Hsu et al. 2011), and also upregulated in buds of juvenile hybrid aspen trees after 8 weeks of chilling temperatures (Rinne et al. 2011). Therefore, the transcript accumulations of *FT1* suggests that it could also have a role in dormancy release.

Winter dormancy, also known as endodormancy, is a state of meristems defined as the inability to initiate growth under favorable growing conditions such as warm temperatures, suitable photoperiods, and moisture. The growth arrest is maintained by the SD-induced plasmodesmata (PDs) closure, which prevents endogenous growth-promoting signals from accessing the meristem (Rinne et al. 2011; Tylewicz et al. 2018). Consequently, release from dormancy by prolonged chilling temperatures is required to restore a meristem's competency to respond to growth promoting warm temperatures and long daylengths (Cooke et al. 2012). In dormant hybrid aspen (*Populus tremula* × *Populus tremuloides*), chilling treatment induced production of gibberellic acid (GA) and thereby increased GA-inducible 1,3-β-glucanase genes, glucan

hydrolase family 17 (GH17s) (Rinne et al. 2011). Studies indicate that PD-localized GH17s hydrolyze 1,3-β-glucan (callose) (Levy et al. 2007). As callose is deposited at the sieve plate pores and PD in the shoot apical meristem (SAM) during establishment of dormancy, induction of GH17s could mediate PD opening. The reopening of PDs was hypothesized to allow movement of *FT1* to the SAM to reinitiate growth as soon as favorable conditions returned (Rinne et al. 2011). However, the inability to produce gene-specific downregulation via RNAi-mediated methods has limited *FT1* functional characterization and its interaction with GA synthesis and signaling. In addition, *FT2* is downregulated not only by SDs but also by other conditions that inhibit growth such as drought and cold temperatures (Hsu et al. 2011). This suggests that *FT2* integrates multiple signals to maintain growth under appropriate conditions. We used the CRISPR/CAS9 system to generate gene-specific mutants and address these hypotheses: 1) *FT1* promotes dormancy release and 2) *FT2* is necessary to sustain vegetative growth.

**Materials and methods**
*Cas9 target site selection and guide RNA constructions*
The coding sequences (CDSs) of *FT1* and *FT2* share 89% identity (Supplemental Figure 3.1). As online tools were not available for poplar, I manually designed specific guide RNA (gRNA) target sites as described in the workflow supplemental figure 3.2a (Li et al. 2013). Firstly, I aligned the two *FT* CDSs and identified 23-base pair (bp) sequences ending with NGG, the protospacer-adjacent motif (PAM). Secondly, I checked if there is an intron at N21GG sites using predicted gene models in *Populus trichocarpa* v3.0 in Phytozome database and AspenDB, a database site for poplar INRA 717-1B4 (*P. tremula* x *P. alba*), the clone used for transformation. Thirdly, I only used N21GG candidates located in exons for further blast search of the 12-bp core sequence (perfect match) plus 3' NGG and selected the N21GG with perfect match in the 12-bp core sequence with no hit in other exon as candidates for Cas9 targets. Finally, three target sites were chosen to make gRNA constructs.

Each of the three targeting sequences was introduced into a new gRNA construct through PCR primers using a plasmid with Arabidopsis U6 promoter preceded gRNA, guide RNA backbone, and a poly T terminator as templates (Supplemental Figure 3.2c; kindly provided by Dr. Zack

Nimchuk). The primer sequences used for gRNA constructs are presented in the supporting information (Supplemental Table 3.1). Each gRNA construct was inserted between the Cas9 cassette and a kanamycin selection marker for plant transformation in the binary vector the pMOA33 (Supplemental Figure 3.2b; kindly provided by Dr. Zack Nimchuk). The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 using the freeze-thaw method.

### Plant transformation and transgenic mutant selection

Transformation of *P. tremula* x *P. alba* clone INRA 717-1B4 , hereafter referred to as WT or 717, was carried out as previously described (Meilan and Ma 2006). CRISPR/Cas9-induced mutagenesis usually involves the introduction of small insertions or deletions (indels) into the target sequences. Genomic DNA was extracted from leaves of individual rooted transgenic and WT plants and PCR amplicons of the short (~300 bp) target regions were sequenced. The primers for a specific locus are listed in Supplemental Table 3.1.

### Short day and chilly induction of dormancy release

WT and *ft1* #1 and *ft1* #3 mutant plants were propagated in vitro. Rooted plantlets were transferred from tissue culture to soil (Promix B, Canada), grown in a growth chamber under LD conditions (16 hr light/8 hr dark) at 22/20 °C. When the plants reached heights of 65 to70 cm-tall, we changed the setting of the growth chamber to SD conditions (8 hr light/16 hr dark) with the same temperatures (22°C day/20 °C night) to induce growth cessation and dormancy. After 6 weeks in SD, we lowered temperatures to 15/12 °C for 2 weeks, a transitional phase for acclimation to chilling temperatures, followed by 10/8 °C temperatures for 6 weeks to release dormancy. Finally, the plants were transferred to a LD and warm temperature (22/20 °C) greenhouse to assess dormancy release by scoring time of bud flush (Figure 3.5a).

### Bud-internode unit assay for bud flush

A fast bud-internode unit assay for bud flush was modified from the culture system of bud-internode units previously described (Rinne et al. 2011) . Before the experiment, mutant and WT plants were exposed to 8 weeks of SD followed by 6 weeks of chilling temperatures as described in the preceding paragraph. The stem of each chill-treated plant was cut into segments, with an

axillary bud and 1- to 2-cm-long internodal segment at both ends. The apical bud-internode unit along with each of the next 23 axillary bud-internode units was placed in a well with 1 ml of water as treatment control or 1 µM GA$_3$ (Sigma-Aldrich) solution.  To uncover any effects of bud position on the shoot, bud-internode units were placed in order from left to right, in each row of a 24-well cell culture plate. The plates were incubated in forcing conditions of continuous light at room temperature to monitor the time of visible bud flush.

## Results

### *CRISPR/Cas9-induced double ft1ft2 mutants reduced shoot elongation and set terminal buds*

We designed three single-guide RNA constructs to target three different sites. One site is at the end of their first exons, simultaneously targeted both *FT1* and *FT2*. The FT1ex4 site specifically target *FT1* at the beginning of its fourth exon. The FT2ex1 site is specific to *FT2,* located at the beginning of the coding region (Figure3.1). All three gRNA constructs were introduced into wild type poplar clone 717. We generated a series of double *ft1ft2 and* single *ft1* mutants, but we could not regenerate any transgenics with the *FT2*-specific construct. This could be due to off-target effects or that a mutation at the FT2-specfic site had a stronger effect on regeneration than a mutation at the shared *FT1/FT2* target site.

Consistent with *FT2* being essential for sustained growth, most transformants with the gRNA targeting both *FT1* and *FT2* failed to regenerate shoots. Only a few short shoots were regenerated, and some of these set buds while still on shoots elongation media plates under LD growing conditions (Figure 3.2a). We were able to root three independent events harboring different combinations of indels (Table 3.1). All 3 independent events were double *ft1ft2* loss-of-function mutants. For example, in the *ft1ft2* #4 mutant*,* an *ft1* A insertion introduced a premature stop codon that if translated would encode a protein of 71 amino acids (aa), while *FT1* encodes a protein of 174 aa; Similarly, the *ft2* A deletion would encode a protein of 88 aa compared to *FT2* encoding a protein of 174 aa. The mutants had extremely short internodes and set terminal bud when grown in magenta boxes for 6 weeks under LDs (Figure 3.2b).
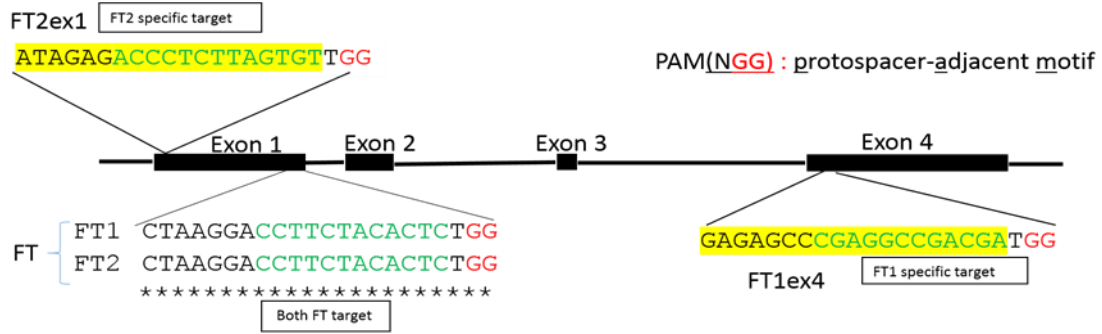
*Figure 3.1 Diagrams of guide RNA (gRNA) target designs.*

Selected gRNA sites targeting both *FT1* and *FT2* are located near the 3' end of the first exon, the *FT1*-specific site is near the 5 end of the fourth exon and the *FT2*-specific site is at the start of the first exon. The gRNA constructs were introduced into clone INRA 717-1B4 (*P. tremula x P. alba*) to regenerate mutants.
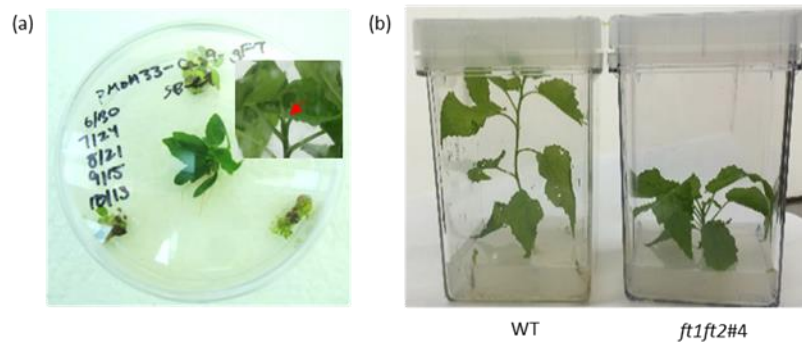


*Figure 3.2 CRISPR/Cas9-induced double ft1ft2 mutant showed reduced shoot elongation and set terminal buds in tissue culture under 16 hr daylengths*

(a) Overview of a shoots elongation media tissue culture plate with regenerated new shoots from explants transformed with the gRNA targeting both *FT1* and *FT2*. Many new shoots failed to elongate. Only a few short shoots formed and some even set terminal buds on the plates. The red arrow indicates a terminal bud. (b) A rooted double mutant (ft1ft2#4) showing dramatically reduced shoot elongation. Plants were grown in LDs for 6 weeks.

# Table 3.1 Analysis of double ft1ft2 CRISPR mutants

| | Genomic sequences at FT1 and FT2 loci around the sgRNA sites | | Indels | |
| --- | --- | --- | --- | --- |
| | *FT1* | *FT2* | *FT1* | *FT2* |
| **WT** | GATCTAAGGACCTTCTACACTCTGGTAC | GATCTAAGGACCTTCTACACTCTGGTAA | wt | wt |
| **#4** | GATCTAAGGACCTTCTACAACTCTGGTAC | GATCTAAGGACCTTCTAC--CTCTGGTAA | A ins | A del |
| **#13** | GATCTAAGGACCTTCTACATCTCTGGTAC | GATCTAAGGACCTTCTAC--CTCTGGTAA | T ins | A del |
| **#20** | GATCTAAGGACCTTCTAC--CTCTGGTAC | GATCTAAGGACCTTCTAC--CTCTGGTAA | A del | A del |

## CRISPR/Cas9-induced ft1-specific mutants appear wild-type under LDs

To differentiate the functions of *FT1* and *FT2*, we designed gRNA specifically targeting each of them. We were unable to regenerate any plants following transformation with the *FT2*-specific gRNA. However, we regenerated multiple events and studied two independent events harboring different combinations of heterozygous or homozygous indels in the *FT1* locus (Table 3.2 and Supplemental Figure 3.3). Similar to the double mutants, *ft1* mutants had 1-2 bp indels. In the *ft1* #3 mutant, an A insertion introduced amino acid changes and a premature stop codon to encode a protein of 147 aa compared to *FT1* of 174 aa. In the mutant of *ft1* #1, either a T insertion or CG deletion caused similar changes as that of the *ft1*#3, except for with CG deletion resulted in one aa shorter than the T insertion (Figure 3.3c). However, no phenotypic alterations were noted in both *ft1* mutants compared to WT when grown under long-day conditions (Figure 3.3a-b).

# Table 3.2 Analysis of *ft1*-specific CRISPR mutants

| | Genomic sequences at FT1 locus around the sgRNA sites | Indels |
| --- | --- | --- |
| | *FT1* | *FT1* |
| **WT** | ACTGTGTGCTATGAGAGCCCGAGGCCGA-CGATGGGGATTCATCG | wt |
| **#1** | ACTGTGTGCTATGAGAGCCCGAGGCCGA---ATGGGGATTCATCG<br>ACTGTGTGCTATGAGAGCCCGAGGCCGATCGATGGGGATTCATCG | CG del/T ins Heterozygote |
| **#3** | ACTGTGTGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCG | A ins Homozygote |

(a) WT    ft1 #3

(b) WT    ft1 #3

(c)

```
FT1         MSRDRDPLSVGRVIGDVLDPFTKSISLRVTYSSREVNNGCELKPSQVANQ
ft1#1CGdel  MSRDRDPLSVGRVIGDVLDPFTKSISLRVTYSSREVNNGCELKPSQVANQ
ft1#1Tins   MSRDRDPLSVGRVIGDVLDPFTKSISLRVTYSSREVNNGCELKPSQVANQ
ft1#3Ains   MSRDRDPLSVGRVIGDVLDPFTKSISLRVTYSSREVNNGCELKPSQVANQ

FT1         PRVDIGGEDLRTFYTLVMVDPDAPSPSDPSLREYLHWLVTDIPATTGASF
ft1#1CGdel  PRVDIGGEDLRTFYTLVMVDPDAPSPSDPSLREYLHWLVTDIPATTGASF
ft1#1Tins   PRVDIGGEDLRTFYTLVMVDPDAPSPSDPSLREYLHWLVTDIPATTGASF
ft1#3Ains   PRVDIGGEDLRTFYTLVMVDPDAPSPSDPSLREYLHWLVTDIPATTGASF

FT1         GHETVCYESPRETMGIHRFVGVLFRQLGRQTVYAEGWRQNFNTRDFAEVY
ft1#1CGdel  GHETVCYESPRP-NGNSSVCFRLVSATGQANCVCPWVAPELQHQRLG---
ft1#1Tins   GHETVCYESPREIDGNSSVCFRLVSATGQANCVCPWVAPELQHQRLG---
ft1#3Ains   GHETVCYESPRPNDGNSSVCFRLVSATGQANCVCPWVAPELQHQRLG---

FT1         NLGSPVAAVYFNCQRESGSGGRRR
ft1#1CGdel  ------------------------
ft1#1Tins   ------------------------
ft1#3Ains   ------------------------
```

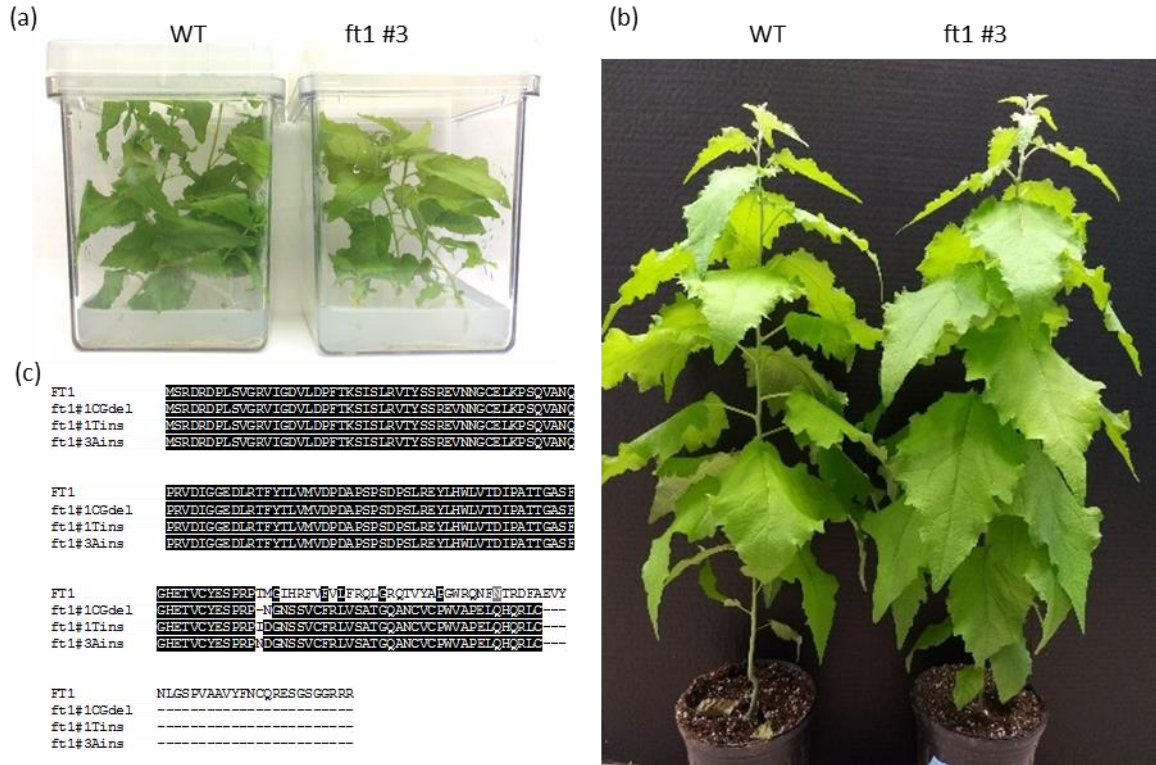***Figure3.3 CRISPR/Cas9-induced indels at the FT1 target locus resulted frame shifts, but no phenotypic changes under LDs compared to WT.***

*FT1* mutant, *ft1* #3, plants appeared WT during growth in (a) magenta boxes for 2 months; and (b) soil for 2 months. (c) Alignment of predicted amino acid sequences of *FT1* and *ft1* mutants generated using Clustal Omega, and then visualized using BoxShade server (ExPASy).

***CRISPR/Cas9-induced ft1-specific mutants showed growth cessation and bud set under SDs***

Previous studies demonstrated that *FT2* downregulation is an early marker for induction of growth cessation and bud set in response to SDs (Bohlenius et al. 2006; Hsu et al. 2011) and the double mutant phenotype (Figure 3.3) supports that is essential for sustained growth. However, it is unclear whether *FT1* has any role in dormancy induction under SD conditions. In this study, we investigated the growth response of *ft1* mutants during the first 4 weeks grown in SDs. As expected, both *ft1*#1 and *ft1*#3 mutants ceased growth at the same time as WT after they were transferred to SD conditions (Figure 3.4a). Meanwhile, they showed no differences in progression of terminal bud formation from WT, and they completed bud set after exposure to 4 weeks of SDs as did WT (Figure 3.4b). Thus, our results show that *FT1* does not have a role in SD-induced growth cessation and bud formation and set.



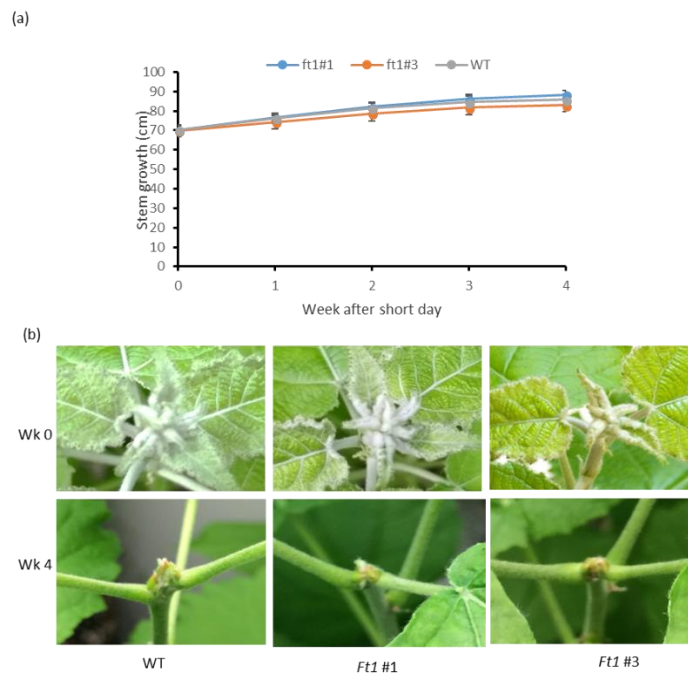***Figure 3.4 Both CRISPR/Cas9-induced ft1 mutants ceased growth and set terminal buds under short days (SDs) the same as WT trees.***

WT and *ft1* #1 and *ft1* #3 mutant plants were grown in LDs for 3 months before the SD treatment. (a) Stem growth of plants during the first 4 weeks in SDs. Plant height (y axis) indicate mean ± SE. (b) Apices at the start of the SD treatment (Wk0) and after 4 weeks in SDs (Wk4).

***CRISPR/Cas9-induced ft1-specific mutants delayed bud flush***

Prolonged chilling in winter is required to release dormancy and thus enable reinitiation of SAM activity and bud flush when favorable conditions return in spring (Rinne et al. 2011; Cooke et al. 2012). *FT1* is expressed in dormant buds in winter and induced by low temperatures under control environments, suggesting that is associated with dormancy release and reinitiation of growth (Hsu et al. 2011; Rinne et al. 2011). To determine *FT1's* function in dormancy release and bud flush, ramets of WT and two mutants, *ft1* #1 and *ft1* #3, were exposed to 8 weeks SDs and warm temperatures followed by 6 week of chilling temperatures, which was previously shown to be effective for inducing and releasing endodormancy in WT (Figure 3.5a; (Rohde and Bhalerao 2007; Ruttink et al. 2007; Mohamed et al. 2010; Rinne et al. 2011). Plants were then transferred to a warm temperature and LD greenhouse (Figure 3.5b top panel, Wk14). Terminal and axillary buds on all WT plants started to swell after 7-10 days in the greenhouse, and internode elongation began after preformed leaves emerged and unfolded within 11-15 days. By contrast, all plants of *ft1* #1 and *ft1* #3 remained dormant (Figure 3.5b; middle panel, Wk16). However, terminal buds and a few axillary buds on the four *ft1* #1 mutant plants flushed after 4 weeks in the greenhouse, while buds of *ft1* #3 plants flushed after 6 weeks in the greenhouse (Figure 3.5b; bottom panel, Wk18). Despite the variation in time of bud flush, the two loss-of-function *ft1*-specific mutants demonstrated delayed dormancy release/bud flush. Thus, *FT1* has a critical role in promoting in dormancy release and subsequent bud flush in juvenile poplar trees.

*Figure 3.5 Chilling-induced dormancy release of ft1 mutants was delayed.*

(a) Light and temperature (day/night) conditions for dormancy induction, establishment, release, and regrowth. (b) WT, *ft1* #1 and *ft1* #3 mutant plants were grown in a warm temperatures and LD growth chamber for 3 months to a height of 50- 70-cm before SD treatment. After exposure to 8 weeks of SDs followed by 6 weeks of chilling temperatures, trees were transferred to a LD and warm temperature greenhouse to assess dormancy release and bud flush. Photos were taken at week 14 (the day of transfer to warm temperatures and LDs); week 16 (two weeks in warm temperatures and LDs); and week 18 (4 weeks in warm temperatures and LDs).

***Position and GA3 effects on bud flush of CRISPR/Cas9-induced ft1-specific mutants***

To further understand the role of *FT1* in dormancy release, we adopted a culture system of bud-internode units (Rinne et al. 2011), to assess the impact of chilling temperatures and GA-feeding on dormancy release. Before the experiment, plants of two mutants *ft1* #1 and *ft1* #3, and WT were exposed to 8 weeks of SD followed by 6 weeks of chilling temperatures as described above. As soon as plants were moved out of the low-temperature growth chamber (Figure 3.5d), the stem of each plant was cut into segments. Each segment with an axillary bud and 1- to 2-cm-long stem at both ends was placed in a well with either water (control treatment) or a GA3 (1 µM) solution.

In the control treatment, except for the terminal bud, the top 10 axillary buds of WT flushed within 7-14 days (Figure 3.6a and 3.6b). Despite the delayed axillary bud flush on both *ft1* #1 and *ft1* #3 mutants, most of them flushed after 14 days incubation in water. In contrast, none of the buds on intact plants of both *ft1* mutants flushed, after 2 weeks in LD and warm greenhouse (Figure 3.5b; middle panel, Wk16), suggesting that the responses of detached buds are not completely equivalent to bud responses on intact plants. Moreover, all the axillary buds from the lower region of the stem, (buds at postions $11^{th}$ to $23^{rd}$) of the two *ft1* mutants and WT flushed after 7 days in water (Supplemental figure 3.4). This suggests that the impact of *FT1* on dormancy release of axillary buds might reduce with increasing distance from the apical bud. In many plants, application of GA can substitute for a prolonged exposure to chilling temperatures (Saure 1985). In hybrid aspen (*P. tremula* × *P. tremuloides*), chilling induced the upregulation of GA biosynthesis genes and promoted the reopening of PD and response to growth promoting signals(Rinne et al. 2011). Thus, we tested whether GA application can compensate for *ft1* mutation in dormancy release.  We placed bud-internode units collected from *ft1* mutants and WT plants in wells filled with either a low concentration solution of $GA_3$ (1 µM) or water. All tested apical and the axillary buds from both mutants *ft1* #1 and *ft1* #3 flushed as rapidly as WT plants, within 5-7 days incubation in $GA_3$ solutions (Figure 3.6c), in contrast to the delayed bud flush of *ft1* mutants in water (Figure 3.6a). Moreover, canonical bud flush with a new shoot growing out was induced by $GA_3$ Thus, bud flush time of *ft1*mutants was restored to that of WT by $GA_3$ treatments, consistent with *FT1* promoting dormancy release by activating GA synthesis, transport or signaling.
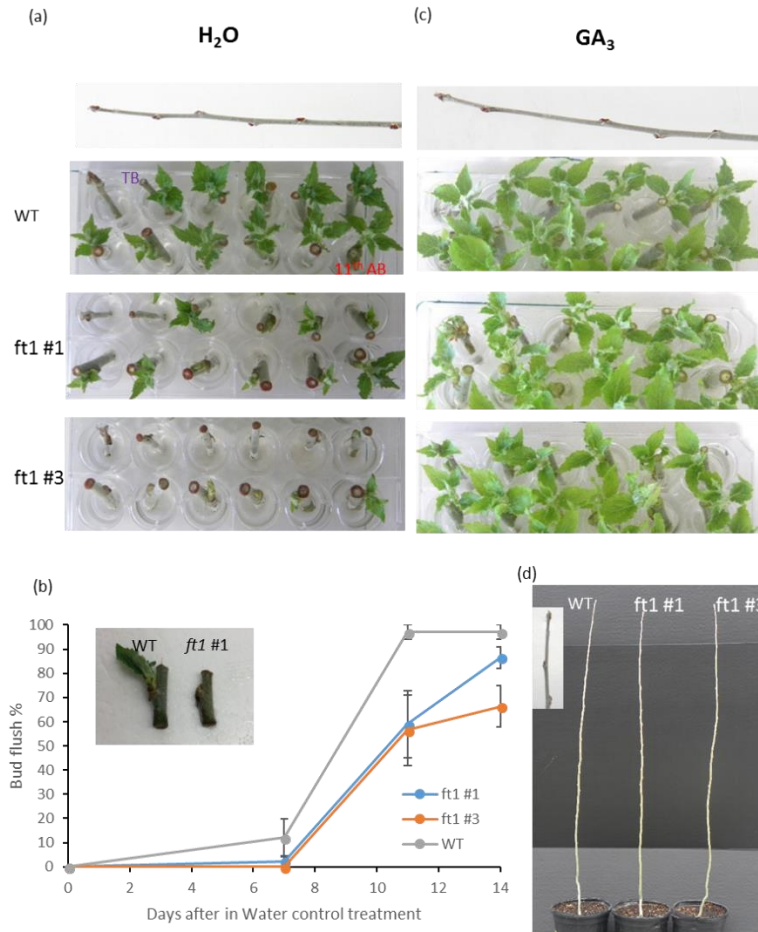
***Figure 3.6 Axillary buds below the apical buds of both ft1 #1 and ft1 #3 showed delayed bud flush after 6 weeks in chilling temperatures, while GA3 advanced bud flush time.***

Before the experiments, 8 plants each of the two mutants, *ft1* #1 and *ft1* #3, and WT were exposed to 8 weeks of SDs, and warm temperatures followed by 6 weeks of chilling temperatures. The apical bud-internode unit along with each of the next 11 axillary bud-internode units was placed in a well with 1 ml of water as treatment control or $GA_3$ (1 µM) solution in the order from left to right of a 24-well cell culture plate. The plates were incubated in forcing conditions (constant light and room temperature). (a) Axillary buds between the terminal bud (TB) and 11th axillary bud (AB) of both *ft1* #1 and *ft1* #3 showed delayed flush compared to WT, when the bud-internode units were incubated in water. Photos were taken after 2 weeks incubation in water. (b) Axillary bud flush percentage over time. Y axil: Values are means (±SE) of 4 plants; X axil: days after incubation in the control treatment of water. The insert showed bud-internode units of the 5th axillary buds of *ft1* #1 compared to WT after 10 days incaution in water. (c) Photos of axillary buds from *ft1* mutants and WT after 2weeks incubation in 1 µM $GA_3$. (d) WT and *ft1* mutant plants following 8 weeks of SDs followed by 6 weeks of chilling temperatures (source of bud-internodes used for plate assay).

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**Discussion**

We generated *ft1ft2* double mutants and *ft1*-specific mutants using the CRISPR/CAS9 system to differentiate the functions of *FT1* and *FT2* in *Populus*. The strikingly different phenotypes of *ft1ft2* double mutants and *ft1*-specific mutants strongly suggest that *FT2* is necessary to sustain vegetative growth, while *FT1* promotes dormancy release. Three main lines of experimental evidence indicate that *FT1* plays an important role in the dormancy release, but is not required for vegetative growth. First, no phenotypic changes were noted in *ft1*-specific plants when grown under LD conditions. Second, *ft1*-specific mutants showed the same growth cessation and bud set phenology as the WT in response to SD signals. Third, *ft1*-specific mutants showed significantly delayed bud flush after an extended chilling period, when subsequently moved to warm temperatures and LD conditions.

In contrast to the *ft1* mutants, double *ft1ft2* mutants showed severely reduced shoot elongation and set terminal buds in tissue culture under LD conditions. These results support that *FT2* is necessary to sustain vegetative growth under growth promoting conditions. We were not able to regenerate *ft2*-specific mutants, possibly because of off-target effects or that mutations near the start codon generated a stronger loss-of-function allele compared to the mutation in the double mutant thereby preventing regeneration. New Cas9 variants and novel RNA-guided nucleases are needed to expand the possible target sites. However, given that *ft1* mutants showed WT-like growth under LDs and responded to SDs the same as WT, our results strongly support that *FT2* is necessary to maintain vegetative growth during spring and summer when conditions are favorable for growth as previously indicated (Hsu et al. 2011).

A role for *FT1* in promoting dormancy release was proposed based on 1) Conserved *FT* antagonist poplar *CENTRORADIALIS1* (*CEN1)* represses dormancy release (Mohamed et al. 2010); 2) its upregulation during chilling treatment in juvenile poplars was correlated with increases in GA biosynthesis genes, GA-inducible GH17s, opening of PDs and dormancy release (Rinne et al. 2011); and 3) Single nucleotide polymorphisms in *FT1* were associate with time of bud flush in *P. trichocarpa* (Evans et al. 2014). Thus, it was proposed that induction of *FT1* expression in winter coupled with low levels of *CEN1* promote dormancy release (Brunner et al. 2014). In Arabidopsis a PD-localized GH17 was linked to callose degradation (Levy et al. 2007).

Moreover, heat-inducible induction of *FT1* expression upregulated a *GH17* (Hsu et al. 2011), suggesting the possibility, that *FT1* promotes the opening of PDs. Application of $GA_3$ and especially GA4 was able to substantially substitute for chilling, based on detached bud-internode unit assay (Rinne et al. 2011). Thus, we tested whether GA3 application could compensate for *ft1* mutation in dormancy release. After 6 weeks at chilling temperatures, GA3 application advanced bud flush time to that of WT buds (Figure 3.6c), suggesting that GA synthesis and signaling acts downstream or in parallel to *FT1*.

However, it is still unclear how *FT1*, GA and PD opening are connected during dormancy release. Therefore, examinations of sphincters in axillary buds of *ft1* mutants at different time points during chilling using transmission electron microscopy; and whether dormancy release similar to WT via introduction of a *FT1pro::GH17* transgene in the *ft1* mutant background will inform on the role of *FT1* on symplastic inter-cellular communication. Furthermore, a comparison of transcriptomic responses to chilling treatment in WT and *ft1* buds could identify putative downstream targets.

Most frost damages in trees occur around the time of bud flush in late spring or during growth cessation and bud formation in early fall (Timmis et al. 1994). However, late spring frost injury after bud-break cause more severe damage than early fall frost injury before dormancy (Ningre and Colin 2007). Given that most research has focused on of dormancy induction and establishment, further analysis of *FT1's* in dormancy release using *ft1* mutants should provide additional insights into the regulation of this important phenology transition.

**Supplemental Figures and Table**



***Supplemental Figure 3.1 Alignment of coding sequences of the two FT paralogs, FT1 and FT2, in WT Populus (P. tremula x P. alba clone 717-1B4).***

The alignment was generated using Clustal Omega, and then visualized using BoxShade server (ExPASy).
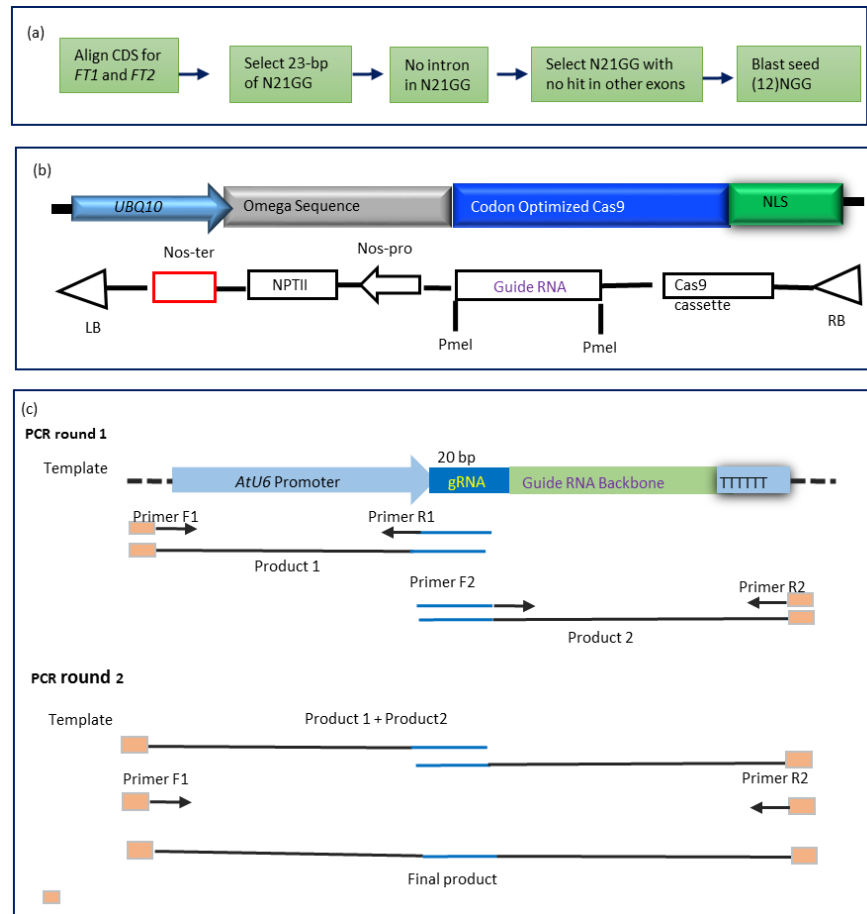
***Supplemental Figure 3.2 Diagrams of guide RNA (gRNA) target design and the two components for the CRISPR system.***

(a) Manual design for specific gRNA targeting sites in the two *FT* paralogs. First, align the two coding sequences (CDSs); Second, identify 23-bp sequences ending with GG; Third, Select N21GG candidates with no intron; Fourth, Blast the 12-nt core sequence (perfect match) plus 3' NGG, the protospacer-adjacent motif (PAM); Last, Select the N21GG with perfect math in the 12-core sequence and no hit in other exon as a target site. (b) Cassette of Cas9 (codon optimized for plants) driven by UBQ10 promoter, and a nuclear localization signal (NLS) fused at the end was inserted into the binary vector pMOA33. The gRNA cassette is inserted into the pMOA33 between the Cas9 cassette and a kanamycin selection marker for plant transformation. (c) The gRNA cassette includes an Arabidopsis U6 promoter a 20-base pair (bp) gRNA, a guide RNA backbone and a poly T terminator. A new gRNA construct is assembled by two steps of overlapping PCR. The specific target sequence is introduced into a new gRNA through PCR primers, primer R1 and primer F2, and plasmids with the gRNA cassette as templates in the first round of PCR. A complete new gRNA cassette can be synthesized using primer F1 and primer R2, and the two products of the first round PCR as templates in the second round PCR.

(a)

FT1 specific target                                          two alleles

```
WT_FT1ex4_c7    TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCGG
WT_FT1ex4_c2    TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCGG
WT_FT1ex4_c3    TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCGG
WT_FT1ex4_c6    TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCGG
WT_FT1ex4_c8    TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCGG
WT_FT1ex4_c1    TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCGG
WT_FT1ex4_c4    TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCGG
WT_FT1ex4_c5    TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCGG
WT_FT1ex4_c9    TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCGG
WT_FT1ex4_c10   TGCTATGAGAGCCCGAGGCCGACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCGG
                ************************************************************ ***
```

(b)

T insertion/CG deletion

```
ft1-1_c2        TGCTATGAGAGCCCGAGGCCGA--ATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-1_c3        TGCTATGAGAGCCCGAGGCCGA--ATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-1_c5        TGCTATGAGAGCCCGAGGCCGA--ATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-1_c8        TGCTATGAGAGCCCGAGGCCGA--ATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-1_c9        TGCTATGAGAGCCCGAGGCCGA--ATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
WT_FT1ex4_c7    TGCTATGAGAGCCCGAGGCCGA-CGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCG
ft1-1_c4        TGCTATGAGAGCCCGAGGCCGATCGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCG
ft1-1_c1        TGCTATGAGAGCCCGAGGCCGATCGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-1_c6        TGCTATGAGAGCCCGAGGCCGATCGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCG
ft1-1_c7        TGCTATGAGAGCCCGAGGCCGATCGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCG
                *******************     ***********************************  **
```

(c)

A insertion

```
ft1-3_c9        TGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCG
ft1-3_c2        TGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCG
ft1-3_c8        TGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-3_c3        TGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-3_c6        TGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-3_c1        TGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-3_c7        TGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
ft1-3_c5        TGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
WT_FT1ex4_c7    TGCTATGAGAGCCCGAGGCCG-ACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTCCG
ft1-3_c4        TGCTATGAGAGCCCGAGGCCGAACGATGGGGATTCATCGGTTTGTTTTCGTCTTGTTTCG
                *********************  ************************************  **
```

***Supplemental Figure 3.3 Sequences of single colonies from PCR of the FT1 specific target region cloned in Teasy vector.***

(a) *FT1* specific target region of WT with a SNP at 29 bp downstream of the target site. (b) ft1 #1 with T insertion and CG deletion. (c) ft1 #3 with A insertion.

**(a)** WT      ft1 #1      ft1 #3

H$_2$O
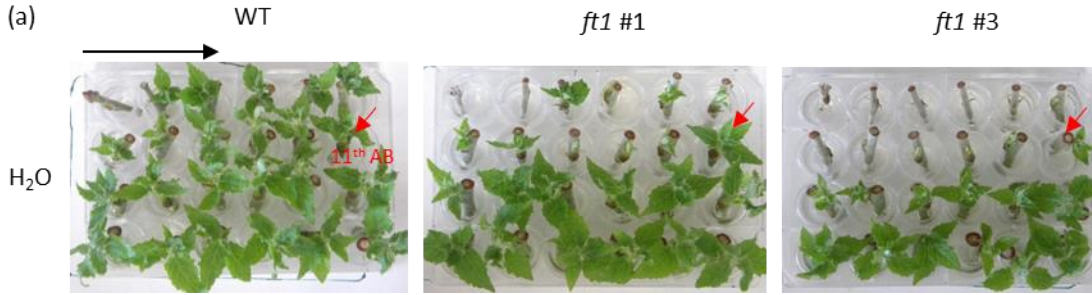
11$^{th}$ AB

***Supplemental Figure 3.4 Axillary buds below the 11$^{th}$ axillary buds of both ft1 #1 and ft1 #3 flushed the same as WT flush after 6 weeks of chill.***

Before the experiment, 4 plants each of the two mutants *ft1* #1 and *ft1* #3, and WT were exposed to 8 weeks of SD followed by 6 weeks of chill. Stem of each chilly treated plant was cut into segments, with an axillary bud and 1- to 2-cm-long internodal segment at both ends. The apical bud-internode unit along with each of the next 23 axillary bud-internode units was placed in a well with 1 ml of water in the order from left to right in each row of a 24-well cell culture plate. The plates were incubated in forcing conditions with constant lights and at room temperature. The axillary buds below the 11$^{th}$ axillary buds (red arrow) of both mutants flushed the same as WT.

*Supplemental Table Table 3.1 Nucleotide sequence of oligonucleotide primers used in this study*

| Primer | Sequence (5'-3') | Orientation | Purpose |
|---|---|---|---|
| AtU6P_F | GTTTAAACAAGCTTTCGTTGAACAACG | Forward (F) | F primer for guide RNA (Primer F1) |
| gRNA_R | GTTTAAACAAAAAAAGCACCGACTCGG | Reverse (R) | R primer for guide RNA (Primer R2) |
| 717FTgRNA_F1 | CTAAGGACCTTCTACACTCgttttagagctagaaatagc | Forward (F) | F for targeting both loci of *FT1* and *FT2* |
| 717FTgRNA_R1 | GAGTGTAGAAGGTCCTTAGcaatcactacttcgactctag | Reverse (R) | R for targeting both loci of *FT1* and *FT2* |
| 717FT2ex1_F | ATAGAGACCCTCTTAGTGTgttttagagctagaaatagc | Forward (F) | F for targeting specific to *FT2* locus |
| 717FT2ex1_R | ACACTAAGAGGGTCTCTATcaatcactacttcgactctag | Reverse (R) | R for targeting specific to *FT2* locus |
| 717FT1ex4_F | GAGAGCCCGAGGCCGACGAgttttagagctagaaatagc | Forward (F) | F for targeting specific to *FT1* locus |
| 717FT1ex4_R | TCGTCGGCCTCGGGCTCTCcaatcactacttcgactctag | Reverse (R) | R for targeting specific to *FT1* locus |
| CRISPR_FT_F | GTTGGCCGTGTTATAGGGGAC | Forward (F) | F for PCR to validate for double mutations at both loci of *FT1* and *FT2* |
| 717FTFT1R_a | GGAGGAGGCTGCAGAGACGTA | Reverse (R) | R for PCR to validate double mutations specific to locus of *FT1* |
| 717FTFT2R_a | GAGGAGGTCATGGTTGGGACTTA | Reverse (R) | R for PCR to validate double mutations specific to locus of *FT2* |
| FT1ex4_seqF | CGACATTTTGCGAAGGCCATGAAAC | Forward (F) | F for PCR to validate single mutation specific to locus of *FT1* |
| FT1ex4_seqR | TATTTAAAGGGATATCTTCCTGTTATCGC | Reverse (R) | R for PCR to validate single mutation specific to locus of *FT1* |

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# 4. FINAL DISCUSSION AND FUTURE PERSPECTIVE

In temperate zones, forest trees adapt their growth-dormancy rhythm with seasonal changes to increase their chances of survival. To avoid frost damage in the winter, poplar trees rely on short photoperiods to induce dormancy and low temperatures to break dormancy, which allows resumption of growth when favorable conditions return in the spring (Rohde and Bhalerao 2007; Cooke et al. 2012; Brunner et al. 2014; Maurya and Bhalerao 2017). Short day (SD) conditions that induce growth cessation and bud formation in poplar trees have been studied in the past (Rohde 2002; Bohlenius et al. 2006; Ruttink et al. 2007; Mohamed et al. 2010; Hsu et al. 2011; Azeez et al. 2014; Tylewicz et al. 2015; Tylewicz et al. 2018), but our knowledge of the underlying molecular mechanisms is still limited. In this research, I have focused on understanding poplar flowering time gene homologs, *FLOWERING LOCUS T* (*FT*) and *FLOWERING LOCUS D- like* (*FDL*), in the regulation of development and growth when responding to SDs and prolonged chilling temperatures. The discovery of the novel role of *FDL3*, one of the three poplar *FDL* family genes, in photoperiod mediated controlling of leaf development and secondary growth, as presented in Chapter II, raises new questions regarding the vital functions of *FDL*s vegetative growth in poplar trees. In Chapter III, I discussed how I regenerated specific-*ft1* mutants using the CRISPR/Cas9 system and showed for the first time that *FT1* has a dual role in promoting dormancy release in juvenile poplar trees. In this chapter, I will highlight the significance of my discoveries in a broader context and present possible future research directions.

**Distinct life cycle of annual and perennial plants**
Annual herbaceous plants, such as *Arabidopsis thaliana*, and the perennial tree poplar (*Populus* species) differ significantly in their life cycles. *Arabidopsis* completes its life cycle in eight weeks consisting of a short vegetative phase followed by a single irreversible transition to a reproductive phase for flowering and seed production leading to the end of the life cycle (Somerville and Koornneef 2002). In contrast, long-lived poplar trees need six to ten years of vegetative growth before being competent to flower (Dickmann 2001; Yuceer et al. 2003; Hsu et al. 2006; Mohamed et al. 2010). Moreover, adult poplars contain branches with both axillary vegetative and reproductive buds, reflecting that the transition of axillary meristems to

inflorescence meristems is limited to a short seasonal time frame and vegetative meristems are maintained to enable indeterminate growth (Brunner et al. 2014).

In addition to age maturation growth, poplar trees undergo seasonal cycles between growth and winter dormancy in temperate and boreal zones. Decreases in day length and temperature during fall induce growth cessation, bud set, cold acclimation, and endodormancy (Rohde and Bhalerao 2007). Following an extended chilling period in winter to release dormancy, the increasing temperatures and photoperiod in spring stimulate the reinitiation of growth (Rinne et al. 2011; Yordanov et al. 2014). Photoperiod and a prolonged chilling period (vernalization) also regulate flowering time in *Arabidopsis* and many plants (Amasino and Michaels 2010). Thus, that homologous genes regulate vegetative phenology in poplar and flowering phenology in Arabidopsis likely reflects their common environmental signals. The functions of some regulatory modules may be context dependent and gene duplications may have enabled the evolution of paralogous modules that act in different signaling pathways.

**Photoreceptors, the circadian clock and the CO/ FT regulatory module**
Photoperiodism occurs in all plants. Many flowering plants sense changes of photoperiod, especially night length, as a signal to determine their flowering time. According to their photoperiod requirements, plants can be categorized as long day plants, short day plants, and day neutral plants. The model plant *Arabidopsis* is a long day plant. Under a long inductive day, the key flowering time gene, *FT,* is expressed in the leaf that leads to flowering (Andres and Coupland 2012; Song et al. 2013). Similar to *Arabidopsis*, vegetative growth is dependent on daylenths being above a certain threshold. Under long day (LD) conditions, the expression of *FT2* sustains active growth in poplars (Hsu et al. 2011). In contrast, rapid downregulation of *FT2* by SD conditions is essential and sufficient to induce growth cessation (Bohlenius et al. 2006; Azeez et al. 2014). In addition to the *FT* gene, plants depend on phytochromes to perceive light signals and circadian genes to regulate *CONSTANS* (*CO*), which is the direct upstream regulator of *FT* (Putterill et al. 1995; Shim et al. 2017*)*. For example, poplar plants overexpressing oat *PHYTOCHROME A* (*oatPHYA*) showed growth promotion and delayed bud set in response to SD conditions (Olsen et al. 2002) . In contrast, poplar trees with reduced expression of

endogenous *PHYA* by RNAi showed earlier growth cessation and bud formation under SD conditions (Kozarewa et al. 2010).

**FT-FD complex participates in photoperiodic regulation of flowering and vegetative phenology**

In *Arabidopsis,* when plants grow to a stage with 14 rosette leaves under a daylength of 12 to 16-hours, *FT* is expressed in rosette leaves (Kobayashi et al. 1999). Then, the FT protein is transported to the shoot apical meristem (SAM) where it interacts with FD to form a FT-FD complex (Abe et al. 2005; Wigge et al. 2005; Corbesier et al. 2007). The FT-FD complex activates downstream flowering genes**,** *APETALA1* (*AP1*), and the closely related genes, *FRUITFULL* (*FUL*) and *CAULIFLOWER* (*CAL*), which leads to flowering (Andres and Coupland 2012).

Our results in this study and previous reports demonstrated that several poplar flowering gene homologs have diversified following duplication to have roles in flowering or vegetative phenology or have dual roles in both flowering and seasonal growth and dormancy transitions (Table 4.1). Overexpression of poplar *FT1* induced precocious flowering in poplar (Bohlenius et al. 2006; Hsu et al. 2011), and our study of CRISPR-induced *ft1* mutants showed that FT1 promotes dormancy release and hence, bud flush. Overexpression of poplar *FT2* promoted early flowering and delayed bud set in SD conditions. RNAi suppression of both *FT1* and *FT2* paralogs were more sensitive to SD signals and accelerated growth cessation in SD conditions (Bohlenius et al. 2006; Hsu et al. 2011). Our CRISPR-induced double *ft1ft2* mutants displayed reduced shoot growth and even set bud under LD conditions. Taken together, *FT1* has an important role in promoting bud flush and *FT2* in sustaining vegetative growth. While they both promote flowering when overexpressed, *FT1* is a more potent inducer of flowering, including the production of WT-like inflorescences.

Similarly, the three poplar *FDL* genes have distinct functions in flowering and vegetative growth. Overexpression of *FDL2.2* (Parmentier-Line and Coleman 2016) promoted flowering. In contrast, overexpression of either *FDL1* (Tylewicz et al. 2015) or *FDL3* (shown in this study) did not result in early flowering. Instead, overexpression of *FDL1* enhanced bud maturation and cold adaptation (Tylewicz et al. 2015) in response to SD conditions. Our study of *FDL3*

indicated a unique function and interaction with photoperiod. *FDL3ox* trasngenics showed a faster rate of leaf initiation in LDs, but leaf development and the transition to secondary growth were dramatically delayed. However, leaf maturation and secondary growth were restored in SDs and an activity SAM was maintained.


**A Model for flowering gene homologs regulate seasonal growth changes in poplar**
Based on our results and previous studies (Hsu et al. 2011; Rinne et al. 2011), we propose the following model for flowering homologous genes regulating seasonal growth transitions in poplar in response to photoperiod and temperature changes (Figure 4.1). During winter cold temperatures, *FT1* expression is hyper-induced in winter buds, leading to upregulation of a number of gibberellic acid (GA) biosynthesis genes. GAs upregulate glucan hydrolase family 17 genes (*GH17*s), which encode enzymes to hydrolysis callose deposited at plasmodesmata (PDs) before dormancy. The upregulations of GH17s result in re-opening of PDs, hence, dormancy release. After buds become competent for growth reinitiation, as warm temperatures return in spring, vegetative buds (VBs) flush rapidly.  Meanwhile, within certain axillary buds, FT1 interacting with FDL2.2 promotes their transition to flowering in early spring. Conversely, warm temperatures and long daylengths (LDs) in later spring and summer induces *FT2* expression in mature leaves (LM). FT2 is transported to the shoot apex and may interact with FDL3 forming a FT2/FDL3 complex. The complex upregulates LAP1/FUL MADS box genes expression promoting leaf formation and primary growth. By contrast, under LDs, overexpressed FDL3 induces FT2 expression in young leaves (YL), which triggers ectopic expression of two close paralogs, *LAP1a* and *LAP1b*, that may result in delayed leaf development and transition to secondary growth. Thus, according to this model, *FT1* promotes dormancy release by activating GA synthesis, transport or signaling, and flowering by the encoded FT1 interacting with FDL2.2. Photoperiod mediated-FDL3 promotes primary growth, but delayed leaf development and the transitioned to secondary growth possibly depending on FT2 in response to the changes of photoperiods.


**Future perspectives**
Genetic and molecular understanding of genes regulating flowering and vegetative growth opens the door for accelerating breeding of woody perennial plants. The discovery of *FDL3*

coordinately affects leaf maturation and primary to secondary growth transition depending on photoperiod could ultimately lead to strategies for increasing secondary growth.

A long juvenile phase of forest trees is a major bottleneck which limits breeding progress. Overexpression of *FT1*, *FT2* (Bohlenius et al. 2006; Hsu et al. 2006; Hsu et al. 2011) or *FDL2.2* (Parmentier-Line and Coleman 2016) and this study) drastically shortened the flowering time from 6-10 years to 3-4 months. Constitutive 35S promoter has been used for gene functional studies in promoting early flowering, but is often accompanied by pleiotropic effects that impair vegetative growth or yield precocious, but infertile flowers. New approaches such as using inducible promoters or developing effective grafting techniques are needed for this knowledge of floral promoters to be translated to application.

Meanwhile, the knowledge of dual functions in vegetative growth of floral regulatory genes in poplar trees provides important insights for potential applications. Our results show that *FT1* promotes dormancy release, therefore suggesting *FT1* is not a suitable target for genetic containment (biosafety regulation).

More importantly, we showed the value of CRISPR/CAS9 system in enabling homozygous or biallelec mutations that can differentiate the functions of close paralogs to advance understanding of the functional evolution of gene duplicates.
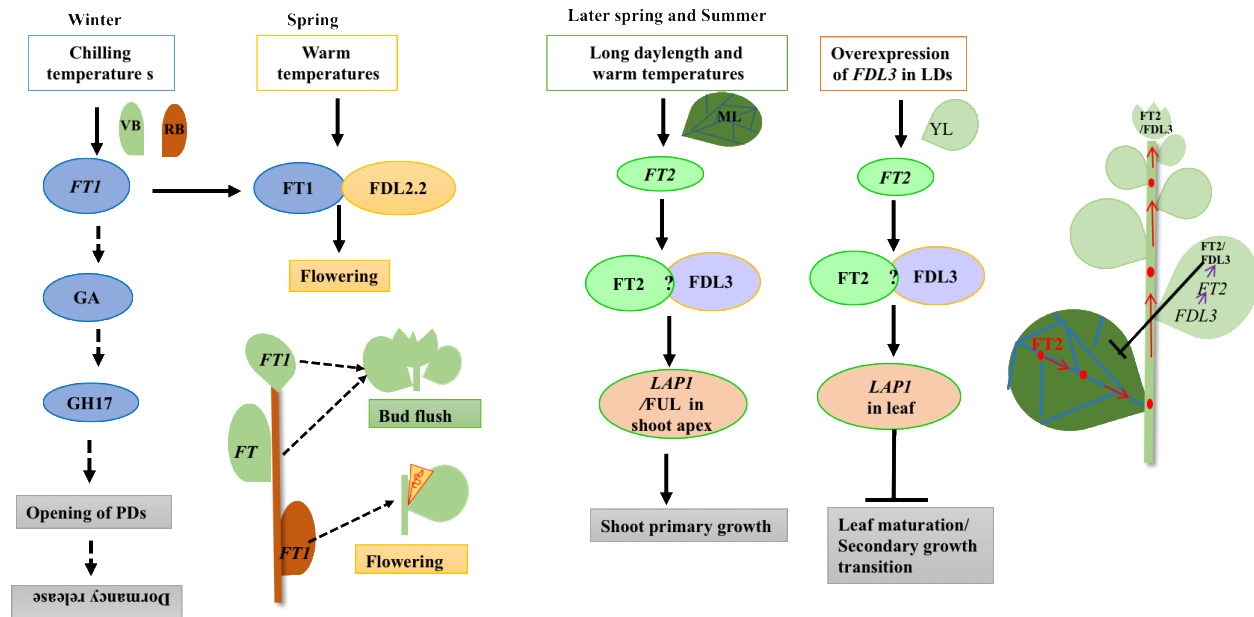
*Figure 4.1 Flowering gene homologs regulate seasonal growth changes in poplar.*

In winter, prolonged chilling temperatures induce expression of *FT1* in winter buds, which upregulates gibberellic acid (GA) biosynthesis genes. GAs upregulate glucan hydrolase family 17 genes (*GH17*s). GH17s hydrolysis callose deposited at plasmodesmata (PDs) during dormancy induction to re-open PDs and thereby release dormancy. When warm temperatures return in spring, vegetative buds (VBs) flush. Within a subset of the axillary meristems, FT1 interacting with FDL2.2 promotes flowering. In later spring and summer, warm temperatures and long daylengths (LDs) induce *FT2* in mature leaves (LM). FT2 is transported to the shoot apex, and there may interact with FDL3 to form an FT2/FDL3 complex. The complex promotes expression of *LAP1/FUL* MADS box genes leading to leaf formation and primary growth. Under LDs, overexpressed *FDL3* induces *FT2* expression in young leaves (YL), which triggers ectopic expression of two close paralogs, *LAP1a* and *LAP1b*, that may result in delayed leaf development and transition to secondary growth.

*Table 4.1 List of flowering gene homologs in flowering and seasonal growth changes in Poplar compared to Arabidopsis*

| Genes | Type of construct | Bud phenology | Vegetative growth | Flowering | Refs |
|---|---|---|---|---|---|
| ft1 | CRISPR induced null mutation | Delayed bud flush after prolonged chilly | | | This study |
| ft1ft2 | CRISPR induced null mutation | Bud set in LDs | Shoots growth reduced in LDs | | This study |
| FDL2.2 | Overexpression | | | Early flowering | This study, 17 |
| FDL3 | Overexpression | Delayed bud set in SDs | Leaf size and secondary growth inhibited in LDs | | This study |
| FDL1 | Dominant negative fused SRDX | | Unable to regenerate shoots | | This study |
| FDL2.1 | Dominant negative fused SRDX | | Shoots elongation reduced | | This study |
| FDL2.2 | Dominant negative fused SRDX | | Shoots elongation reduced | | This study |
| FDL3 | Dominant negative fused SRDX | | Shoots elongation reduced | | This study |
| | | | | | |
| FT1 | Overexpression | Delayed bud set in SDs | | Early flowering | 5, 10 |
| FT2 | Overexpression | Delayed bud set in SDs | | Early flowering | 5, 10, 11 |
| FT1FT2 | Downregulation (RNAi) | Accelerated bud set in SDs | | | 5 |
| FDL1 | Overexpression | Delayed bud set in SDs | | | 27 |
| CEN1 (TFL1) | Overexpression | Delayed bud flush | | Delayed flowering | 15 |
| CEN1/CEN2 | Downregulation (RNAi) | Accelerated bud flush | | Early flowering | 15 |
| LAP1 | Overexpression | Delayed bud set in SDs | | | 4 |
| | | | | | |
| At_FT | Overexpression | | | Early flowering | 12 |
| At_ft | Null mutation | | | Delayed flowering | 12 |
| At_FD | Overexpression | | | Early flowering | 1, 28 |
| At_fd | Null mutation | | | Delayed flowering | 1, 28 |
| At_TFL | Overexpression | | | Delayed flowering | 12 |
| At_tfl | Null mutation | | | Early flowering | 12 |

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