ABSTRACT

Title of Thesis: CHARACTERIZATION OF PTFD1, A BZIP TRANSCRIPTION FACTOR USING TRANSGENIC POPLARS

Minggang Wu, Master of Science, 2006

Thesis Directed By: Associate Professor Gary D. Coleman
Department of Natural Resource Science and Landscape Architecture

Dormancy is an adaptive mechanism that enables plants to survive unfavorable environmental conditions and resume growth when the conditions become favorable again. Bud formation is the morphological event associated with bud dormancy. The research presented in this thesis focuses on the role of *PtFD1*, a bZIP transcription factor, in apical bud development in poplar. This research included the construction of binary *Agrobacterium* vectors for the overexpressing of *PtFD1* and for down regulation or silencing of *PtFD1* expression using RNAi technology. These vectors were used to create transgenic poplars (*Populus alba*×*Populus tremula*) with altered expression of PtFD1. The overexpression of PtFD1 prevented apical bud development while apical bud development appeared normal in PtFD1 RNAi expressing plants. Flowering was also induced in long days in poplars overexpressing PtFD1. Anatomical studies indicate that overexpression of PtFD1 impinges on bud scale development during short day induced bud formation.

CHARACTERIZATION OF PTFD1, A BZIP TRANSCRIPTION FACTOR USING TRANSGENIC POPLARS

By

Minggang Wu

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2006

Advisory Committee:
Associate Professor Gary D. Coleman, Chair
Associate Professor Harry Swartz
Associate Professor Joseph Sullivan

Acknowledgements

I would like to thank my advisor, Dr. Coleman, for his expertise, advise and assistance with this project and his patient guidance throughout my studies.

Thanks to Dr. Sullivan and Dr. Swartz for being on my committee and for their valuable suggestions to my research.

Thanks to Dr. Swartz for the generous use of his lab facilities.

Thanks to Dr. Parmentier-Line for her help in my research.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABA	Abscisic Acid
ABI	Abscisic Acid Insensitive
bp	base pair
C	Celsius
$CaCl_2$	calcium chloride
cDNA	complementary DNA
dNTP	deoxynucleotide triphosphate
EtBr	ethidium bromide
EtOH	ethanol
FAA	Formalin-acetic acid-alcohol
GA	Gibberellic Acid
kDa	kiloDalton
KoAc	potassium acetate
L	Liter
LB	Luria-Bertani
LD	long days
LT	low temperature
mg	milligram
µg	microgram
min	minute(s)
ml	Milliliter
µl	microliter

MS	Murashige and Skoog
ng	nanogram
nm	nano-meter
OE	over-expression
PCR	Polymerase Chain Reaction
RNAi	RNA interference
RPM	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SD	short days
sec	second(s)
S.O.C.	Salt-Optimized + Carbon
TBA	tert-Butanol
T-DNA	Transferred-DNA
UV	Ultraviolet
wk	week
w/v	weight/volume

Introduction

The growth of temperate woody perennial plants is characterized by growth periods (time between spring bud break and fall bud set) interrupted by a vegetative dormancy phase during the winter. Dormancy is an adaptive mechanism that enables plants to survive unfavorable environmental conditions and resume growth when conditions become favorable. Vegetative bud dormancy also influences the morphology and architecture of trees (Rohde et al., 2000).

Formation of the apical buds is the most obvious morphological change associated with the transition from vegetative growth to dormancy (Rohde et al., 2000). The physiological and morphological changes that occur during dormancy allow trees to cope with water and temperature stress (Thomas and Vince-Prue, 1997). The release from dormancy requires exposure to chilling temperature and once the chilling requirement has been fulfilled, bud breaking occurs and vegetative growth resumes in favorable conditions (Powell, 1987).

Apical bud formation is central to bud development (Juntilla and Kaurin, 1990). Although extensive research has been performed in this field, most studies focused on physiological aspects of bud dormancy. The molecular mechanisms that control apical bud formation still remain elusive. However, with the development of modern genetic technologies, research has begun to concentrate on discovering the genes regulating this process.

The research in this thesis focuses on PtFD1, a basic leucine zipper (bZIP) transcription factor expressed in poplar apical buds. The expression of PtFD1 is coincident with the process of apical bud formation and maturation. PtFD1 mRNA level peaks after several weeks of short day (SD) treatment and then decline. Exogenous abscisic acid (ABA) application can also induce the expression of PtFD1 (Gnewikow, 2001). From this, *PtFD1* appears to be a candidate gene regulating poplar apical bud formation and maturation.

To test this hypothesis, transgenic poplars that either overexpress PtFD1 or which downregulate PtFD1 expressing using RNAi were generated. The experiments presented in this thesis focused on: 1) the role of *PtFD1* in poplar apical bud formation; 2) the possible interactions of *PtFD1* with other bud formation-related genes; 3) whether *PtFD1* is involved in other physiological events.

Literature Review

I. Tree Growth and Dormancy

Woody perennial plants of the temperate zone synchronize their growth cycle with changing seasons, fluctuating temperature, different photoperiod (Villiers, 1975). Typically, tree growth involves two phases: a period of active growth and a dormant period. The growing period begins with spring bud break during which the shoot elongates. The shortening of daylength induces growth cessation and bud formation in late summer and autumn. The formation of an apical bud usually signals the beginning of dormancy and is an adaptive response to harsh environments. The breaking or release from dormancy requires exposure to chilling temperatures. After the chilling requirement has been fulfilled, the apical bud is released from dormancy and shoot elongation can resume (Powell, 1987). In *Populus*, a perennial woody plant, growth cessation, bud formation and dormancy are phytochrome-mediated SD photoperiod responses (Howe et al, 1996; Zhu & Coleman, 2001).

Although dormancy is not completely understood, researchers have defined dormancy based on practical terms. Dormancy is defined as "the temporary suspension of visible growth of any plant structure containing a meristem" (Lang, 1987). Based on this definition, dormancy can occur in any organ that contain meristems, including seeds, buds, tubers, roots and vascular cambium.

Dormancy is an adaptive mechanism that enables meristems to survive

unfavorable environmental conditions such as cold temperature or desiccation, and resume growth when the conditions become favorable again (Rohde et al., 2000). For trees, dormancy also has a morphogenetic effect in influencing growth habit and tree form. Because of dormancy, woody plants can adapt to a wide range of circumstances.

Dormancy has been classified into three categories: ecodormancy, paradormancy and endodormancy (Lang, 1987). In ecodormancy, growth cessation is imposed by one or more unsuitable environmental factors, such as nutrient or water deficiencies or unfavorable temperature. Growth can resume when the conditions become favorable. In paradormancy, growth control is imposed by plant structures other than the affected organ. When the control of dormancy occurs within the dormant organ, it is defined as endodormancy. In endodormancy, growth cannot resume even under favorable environmental conditions (Lang, 1987). Once the chilling requirement has been fulfilled, apical buds can break (Amling, 1980). However, low temperature may still prevent the buds from breaking. Only when temperatures become warm can apical buds be released from dormancy (Martin, 1991).

Trees can undergo each of the three types of dormancy in their growth cycle (Critchfield, 1960). During shoot elongation, the growth of lateral buds is inhibited by auxin from the shoot apex, which is the effect of paradormancy. Endodormancy occurs when the dormancy enters into a stage when growth will not resume even under favorable conditions. Chilling requirements must be fulfilled before the buds

are released from dormancy. Also the buds may be held in a dormant status until a favorable temperature is reached, which can be defined as ecodormancy. The type of dormancy that this research focused on is endodormancy.

Seed and bud dormancy have some common features such as chilling requirements, the regulatory role of GA and ABA, accumulation of reserve proteins and acquisition of desiccation tolerance (Powell, 1987; Dennis 1996). Although little is known about the genetic regulatory factors involved in bud dormancy, it has been shown that *Abscisic Acid Insensitive 3* (*ABI3*) plays an important role in late seed development (Giraudat et al., 1992; Parcy et al., 1994). The poplar *ABI3* homologue *PtABI3* is essential for correct embryonic leaf differentiation during bud set in poplar (Rohde et al., 2002). Because of the similarities between seed and bud dormancy, it is possible that similar regulatory mechanisms exist between them.

II. Poplar as a Model Plant to Study Bud Dormancy

Poplar can serve as a model system for the study of bud dormancy in woody plants. First, as a woody perennial plant, poplar undergoes dormancy in response to short day (SD) photoperiod and forms apical buds (Howe et al, 1996; Zhu & Coleman, 2001). These features can be manipulated in growth chambers using different photoperiods. Second, it is one of the fastest growing temperature trees and can be easily propagated. Third, many genetic techniques have been practiced successfully in poplar, such as *Agrobacterium*-mediated transformation. Poplar also has a relatively small genome size (450-550 Mbp) that has been sequenced. Besides,

poplar also has commercial values as a tree for timber, plywood, pulp and paper (Taylor, 2002).

III. The Apical Bud

A. Bud Structure

Two types of buds are found in woody plants, apical and lateral buds. Apical buds consist of the apical meristem that was formed during embryogenesis while lateral buds are found in the axis of leaf petioles and contain meristems for branch shoots. Lateral buds often do not elongate during the season in which they were formed. In angiosperms, the meristem consists of several zones including a central zone with three layers of cells, which acts to maintain the population of indeterminate cells. Beneath the central zone is the peripheral zone, which is the major source of new cells in the apical meristem and of organ primordia. The rib meristem in the very center is where cells begin to elongate (Kerstetter and Hake, 1997).

The apical bud is located at the apex of a stem. It is a short axis consisting of a densely packed series of leaf primordial that is produced by the shoot apical meristem (Rohde et al, 2000). In buds that are actively growing, the axis elongates with progressive formation of the primordia. During bud set, internode elongation ceases above the primordial that will develop into bud scales. Bud scales are formed by overlapping, modified stipules of unexpanded leaf primordia. An apical bud of *Populus trichocarpa* usually consists of apical meristem at the center and 6-9 leaf

primordia, which are enveloped by bud scales (Critchfield, 1960).

B. Apical Bud Formation and Dormancy in Poplar

1. Endodormancy Establishment is a Complex Process

For most temperate trees, formation of an apical bud is prerequisite to the development of dormancy (Junttila and Kaurin, 1990). In poplar, like many woody plants, apical bud formation is a phytochrome-mediated SD response (Howe et al., 1996; Zhu and Coleman, 2001). The process starts once the critical photoperiod for cessation of active vegetative growth occurs and before leaf abscission, allowing the plant enough time to prepare for bud dormancy prior to freezing temperatures (Perry, 1971; Vegis, 1964; Rohde et al., 2000). Typically there is a time lag between growth cessation and the establishment of endodormancy (Rinne et al., 1994; Heide, 1974; Junttila, 1976). After the cessation of stem elongation, the growth in diameter still continues until leaf abscission (Perry, 1971). Root growth will also continue as long as the soil temperature is favorable (Barney, 1951).

2. Stages of Apical Bud Development

Poplar apical bud development can be defined by three stages. During the first stage, bud morphogenesis, bud scales are initiated and enclose the shoot tips (include meristem, embryonic leaves, leaf primodia and stipules) and in controlled environment condition occurs with the first 3 weeks of SD (8 hours light and 16 hours dark) exposure.

During the second stage, bud maturation, apical buds enlarge and elongate with continued SD treatment. Their colors turn from green to reddish-brown. This

stage is also characterized by the decrease of bud water content and accumulation of storage reserves. During bud maturation, the meristem is not dormant and when plants are transferred to long day (LD, 16 hours light and 8 hours dark) or SD with night break, shoot growth will resume and leaves will emerge from the bud.

The third stage is bud dormancy. In controlled environment conditions, this stage occurs after more than 6 weeks of SD exposure. During this stage, bud growth eventually ceases, bud water content continues to decline and the meristem becomes dormant. At this stage, bud burst and regrowth will be delayed by at least 4 weeks when treated with LD.

C. Significance of Bud Formation

The actual growth period of temperate woody perennials occurs between spring bud break and fall bud set and determines tree productivity and wood quality (Rohde et al., 2000). In tree breeding programs, an appropriate bud flushing date, an indeterminate shoot growth and the timing of bud set are of great importance (Dickmann and Keathley, 1996). The timing of bud set in temperate climates is crucial for the trees to avoid frost damage. Failure of bud break when chilling requirements cannot be fulfilled in warm climates can significantly impact productivity.

Apical bud formation is prerequisite to the development of bud dormancy. Since bud scales enclose leaf primordia, they may act as possible regulators controlling growth and dormancy (Perry, 1971). In some species, it seems that the bud scales produce some unknown compounds that inhibit growth. It has been

shown that the presence of bud scale inhibits the growth of primordia enclosed within the bud and this effect correlate with the stage of quiescence the bud has reached (Iwasaki and Weaver, 1977; Tinklin and Schwabe, 1970; Swartz et al., 1984).

Since bud formation functions in both adaptive and morphological responses, it is essential in the understanding of tree biology. Such knowledge will assist in tree breeding, maintenance and improvement.

IV. Physiological and Biochemical Changes during Dormancy

The physiology of bud dormancy has been extensively studied for decades. In addition to the visible morphological changes, many physiological and biochemical changes occur within the plant during the process of bud formation and dormancy. These include changes in enzyme activities, membrane lipid composition, bud water status, amino acids, carbohydrates, proteins and respiration.

SD treatments result in the accumulation of starch, markedly thickened cell walls and much denser cytoplasm, suggesting that plants synthesize and accumulate starch, cellulose, lipids, proteins and other bio-polymers during dormancy induction (Perry, 1971; Bonicel et al., 1987; Coleman and Chen, 1996).

Dormant buds usually have reduced water content and water molecules appear to be associated with macromolecules (Faust et al., 1997). More than half of the bound water molecules disasscociate from macromolecules after the chilling requirement has been fulfilled (Faust et al., 1995).

SD leads to the closure of plasmodesmata that connect the cells in the apical meristem. The altered cell-to-cell communication may initiate growth cessation and dormancy development (Jian et al., 1997; Rinne and van der Schoot, 1998). Release from bud dormancy by chilling involves restoration of the cell-to-cell connection through plasmodesmata.

Growth cessation leads to the accumulation of photosynthesis assimilates and proteins in the plant (Sauter et al., 1996). Cellulose synthesis is reduced prior to dormancy while synthesis of lignin is accelerated (Perry, 1971). During the induction of dormancy, carbon and nitrogen are stored as starch and protein respectively (Coleman and Chen, 1996). SD also induces the expression of bark storage protein (BSP) genes (Coleman et al., 1992).

V. Regulators of Bud Formation

Regulators of bud formation and dormancy include light (photoperiod), temperature, water and hormones (abscisic acid, gibberellins) (Rohde et al., 2000)

A. Environmental Factors

1. Photoperiod

Photoperiod is a key regulator of bud formation and dormancy in poplar and many other trees with indeterminate growth patterns (Howe et al., 1996). It affects both the vegetative growth period and the onset of bud set.

Growth cessation is one of the initial events in the dormancy process and is

mainly induced by perception of critical photoperiod, which is the longest photoperiod that can induce growth cessation. The critical photoperiod shows ecotypic variation among species and is inherited as a quantitative trait (Hummel et al., 1982; Junttila, 1982; Li and Adams, 1993). Generally, northern ecotypes have longer critical photoperiods than southern ecotypes so that trees in higher latitudes can enter growth cessation and bud formation earlier to cope with the frost that will come sooner (Junttila, 1980).

Phytochrome is the primary photoreceptor in photoperiodism. They are dimeric chromoproteins with monomers of 120-130kD, which exist in two forms that are interchangeable. They convert to an active far-red light (FR) absorbing form (Pfr) after absorbing red light (R); after FR treatment, they convert to an inactive R absorbing form (Pr). In darkness, phytochromes are converted from Pfr to Pr. The ratio of R to FR declines during sunset and is employed by the plants to measure the length of the day (Tai and Zeiger, 2002).

In *Arabidopsis*, five distinct phytochrome genes were identified, named phyA to phyE. Both phytochrome A and phytochrome B may be involved in photoperiodic responses. PhyA is a type I phytochrome that is much more abundant in dark-grown seedlings. PHYA protein accumulates in the dark and breaks down rapidly after converting to its Pfr form in light. Experiments with transgenic hybrid aspen suggest that responses to photoperiod could be affected by the amount of phytochromes present in plants. Olsen et al. 1997 showed that poplars (*Populus tremula* × *P. tremuloides*) that overexpress oat *PHYA* did not stop shoot elongation

under SD treatment, and were unable to shed leaves and cold acclimate (Olsen et al., 1997)

2. Temperature

Lavender et al. 1973 suggested a scenario in which air and soil temperatures and photoperiod all interact to permit the earliest possible bud activity compatible with the risk of frost for any given year (Lavender et al., 1973). Dormancy intensity is promoted in woody plants exposed to a few weeks of chilling temperature after bud formation (Walser et al., 1981). Junttila et al. 2003 showed that induction and depth of bud dormancy in birch are significantly affected by temperature. In six ecotypes of *Betula pubescens* Ehrh and two ecotypes of *Betula pendula* Roth that were tested by raising temperature during dormancy induction, bud dormancy developed most rapidly at 15-18°C and was delayed by both 9-12°C and 21°C temperatures (Junttila et al., 2003). These results are consistent with what was found in Norway spruce and *Acer rubrum* (Heide, 1974; Downs and Borthwick, 1956). In some species that are insensitive to photoperiod, such as apple and pear, growth cessation, formation of bud scales and winter buds, leaf senescence and abscission, dormancy induction and release occur in response to low temperature (Heide and Prestrud, 2005). Plant receptors for low temperatures have not been found. Örvar et al. 2000 showed that changes in membrane fluidity in response to a decrease in temperature trigger calcium influx from vacuoles or extracellular storage and induces a signaling cascade leading to changes in the expression of genes that are responsible for increased tolerance of freezing (Örvar et al., 2000).

Temperature also has a role in release of dormancy, bud break and resumption of shoot elongation. Buds need to be exposed to chilling temperature for a certain time to be released from dormancy. After the chilling requirements have been fulfilled, shoot elongation resumes in response to rising temperature (Perry, 1971).

3. Water and Nutrition

Both water supply and mineral nutrition interact with dormancy induction. Dehydration has been shown to be an integral part of bud dormancy development (Rohde et al., 2000). Water stress will deepen dormancy and if severe enough will result in a resting bud and leaf abscission in some species. Mineral nutrition, in particular nitrogen status influence dormancy induction. High levels of nitrogen will delay the onset of dormancy, induce bud break and growth resumption if applied to plants in late summer or early fall.

B. Hormonal Control of Bud Dormancy

1. Abscisic Acid (ABA)

Abscisic acid (ABA) is known to be a “stress hormone” involved in plant responses to abiotic stress, such as dehydration, low temperature and salinity. ABA also plays a role in the regulation of plant development, including embryogenesis, shoot growth, seed dormancy and leaf transpiration. ABA triggers rapid stomata closure by ion effluxes from guard cells to inhibit water loss through transpiration (Taiz and Zeiger, 2002). In seeds, ABA acts as an efficient inhibitor of germination and occurs in high concentrations in dormant seeds (Bewley, 1997). The involvement of ABA in potato microtuber dormancy has been demonstrated (Suttle

& Hulstrand, 1994).

ABA is assumed to be associated with vegetative growth cessation. Seasonal changes in ABA levels have been observed in leaves, buds and xylem saps in various species of woody plants. The level is the highest during mid-summer or autumn and declines during winter (Rohde et al., 2000). Rinne et al. 1994 showed that in apical and lateral buds of *Betula pubescens*, ABA levels are 5-8 fold higher under SD conditions than under LD. Under water stress, the ABA levels in lateral buds doubled (Rinne et al., 1994).

In poplar, it has been found that ABA contents are much higher in apices exposed to LD than to SD and the contents increase when temperature is lower (Welling et al., 2002). ABA levels increase transiently in developing poplar apical buds after 24-27 days of SD treatment and decline with continued SD treatment (Rhode et al., 2002). Exogenous ABA treatment has also been observed to cause growth cessation and bud dormancy in some species under LD conditions and can also prevent release of bud in ecodormancy (El-Antably et al., 1967; Rinne et al., 1994). Maintenance of endodormancy requires continuous endogenous ABA biosynthesis (Le Bris et al., 1999).

All evidence indicates that ABA plays a role in maintaining dormancy in apical bud. Whether it is an inducer of dormancy remains elusive, but it appears that ABA alone is not enough to induce apical bud dormancy.

2. Gibberellin (GA)

In addition to ABA, the role of GAs in bud dormancy has been extensively

studied. GAs was suggested to act as an antagonist of ABA and functions in accelerating growth (Taiz and Zeiger, 2002). Among the GAs, GA1 appears to be the active GA involved in stem elongation and growth cessation in woody plants (Junttila et al., 1991; Olsen et al., 1995).

The levels of GAs are lower under SD than under LD in many species, this suggests that photoperiodic regulation of shoot elongation may be mediated by the regulation of GA biosynthesis (Jackson and Thomas, 1997). SD may block some steps in GA biosynthesis and is mediated by PHYA (Olsen et al., 1997). Reduction in GA1 levels affect cell divisions in the subapical meristem and result in growth cessation (Hansen et al., 1999). Olsen et al. 2004 showed that in deciduous plants, GA1 can completely substitute for a long photoperiod, and SD induced growth cessation is preceded by a significant reduction of GA1 levels, particularly in the elongation zone. Apart from the phytochrome pathway, cessation of growth and initiation of hardening in trees can also be controlled through the GA mediated pathways (Olsen et al., 2004).

Due to current experimental results, it is reasonable to hypothesize that GAs are involved in shoot growth cessation induced by SD, further evidence are needed to confirm this hypothesis.

VI. Genes Involved in the ABA Signal Transduction Pathway

A. Abscisic Acid-Insensitive (*ABI*) Genes

The ABA signal transduction pathway involves hormone binding to its receptor, amplification of the primary signal and initiation of gene expression that are responsible for the physiological effects (Taiz and Zeiger, 2002). In *Arabidopsis*, a series of mutants that have normal ABA biosynthesis but display alternative sensitivity to ABA have been isolated to identify the components of the pathway. These mutants consist of five loci, *abi1*, *abi2*, *abi3*, *abi4* and *abi5* that were selected by the ability of the seeds to germinate in the presence of inhibitory concentrations of ABA. Mutant *abi1*, *abi2* and *abi3* display significant reduction in seed dormancy while *abi3*, *abi4* and *abi5* show defects in various aspects of seed maturation. Also, *abi1* and *abi2* affect ABA responses in vegetative tissues (Koornneef et al., 1984; Finkelstein, 1994; Nambara et al., 2000). The *ABI1* and *ABI2* genes encode homologous type 2C serine/threonine protein phosphatases (Leung and Giraudat 1998). The other three, *ABI3*, *ABI4*, and *ABI5*, encode putative transcription factors (Giraudat et al. 1992; Finkelstein and Lynch 2000). *ABI3* is the ortholog of the maize *vp1* gene, encoding a B3-domain transcription factor. ABI4 contains an APETALA2-like DNA binding domain. *ABI5* encodes a bZIP transcription factor (Finkelstein et al., 2002).

The ABA response effects of ABI3, ABI4 and ABI5 during seed germination are well known. Research in poplar demonstrated that ABI3 (PtABI3) is an essential factor of bud set and a precondition for dormancy establishment (Rohde et

al., 2002). The three transcription factors may form a regulatory complex to mediate gene expression (Brocard et al., 2002).

B. Genes Regulated by *ABI5*

ABI5 is a member of the basic leucine zipper transcription factor family, grouped as AtbZIP39 in Group A. In *Arabidopsis*, it is expressed in both seeds and vegetative tissues and is required for ABA-regulated gene expression (Finkelstein and Lynch, 2000). Mutations in *abi5* will cause reduced ABA sensitivity during seed germination. Furthermore, *abi5* mutation has decreased expression of some LEA (Late Embryogenesis Abundant) genes that are ubiquitous in most of the higher plants. LEA proteins accumulate during late stages of embryo development and are thought to be involved in desiccation tolerance (Bensmihen et al., 2002). In *Arabidopsis*, among the ABREs (ABA-responsive elements) of Em (encodes a class I LEA protein) promoter, a G-box type element shows strong binding with ABI5, suggesting ABI5's role in regulating these genes (Carles et al., 2002). In sunflower, genes encoding DPBFs (Dc3 promoter-binding factors) that can bind to the promoter of Dc3 (a carrot lea class gene) have been isolated. An *Arabidopsis* homolog of DPBF, AtDPBF-1 is identical to ABI5 (Kim et al., 2002).

VII. Basic Leucine Zipper (bZIP) Transcription Factors

Transcription factors are proteins that bind DNA at a specific promoter or enhancer region and facilitate its transcription. They play critical roles in almost all

biological processes. Despite the difference in the structures, the transcription factors share two functional domains: a DNA-binding domain that recognizes and binds to the specific DNA sequence of the promoter or enhancer, a transcription activation domain that interacts with other proteins and increasing the efficiency of transcription. Transcription factors are categorized according to the structure of their DNA binding domains, including zinc finger proteins, helix-turn-helix proteins, and leucine zipper proteins. Transcription factors can be activated or deactivated by other proteins.

One group of transcription factors is defined as basic leucine zipper (bZIP) motif. Plant bZIP proteins play a role in gene control of many processes, such as seed storage, photomorphogenesis and organ establishment (Schmidt et al., 1990; Oyama et al., 1997; Waltch et al., 1998). They also exhibit functions of gene control in response to stimuli including ABA, light and developmental signals (Menkens et al., 1995).

Basic leucine zipper transcription factors function as dimers. Each of the mononers contains a basic DNA binding domain at the carboxyl terminus (C-terminus), adjacent to a leucine zipper helix that is characterized by several leucine residues regularly spaced at seven-amino acid intervals. These leucine residues or other bulky hydrophobic amino acids locate exact nine amino acids towards the C-terminus to generate an amphipathic helix. To form a dimer, two subunits adhere via interactions between the hydrophobic sides of their helix, creating a superimposing coiled-coil structure (so called “leucine zipper”). The

monomers can be either identical or not and form homodimers or heterodimers. The DNA binding domain consists of a basic region, a highly conserved region enriched in basic amino acids that is approximately 25 residues in length, that contacts directly with DNA to stimulate or repress transcription. To bind DNA, the two basic regions are inserted into the major groove of the DNA, each helix finding an identical DNA sequence, results in a scissors look (Landschulz et al., 1988; Pathak and Sigler, 1992; Meshi and Iwabuchi, 1995).

Plant bZIP proteins exhibit a DNA-binding specificity for DNA sequence motifs containing an ACGT core (Foster et al., 1994). Experiments demonstrated that nucleotides flanking the ACGT core also affected binding specificity. Three different types of ACGT motifs were identified: G-box, CACGTG; C-box, GACGTC and A-box, TACGTA. Correspondingly, bZIP transcription factors could also be categorized into three groups according to their qualitative and quantitative specificity for G-box and C-box elements: Group 1 have a stronger binding affinity for G-box; Group 2 show a comparable binding affinity to both G-box and C-box (Group 2); Group 3 display a stronger binding affinity for C-box (Izawa et al., 1993).

Transcription activation domains interact with basal machinery to activate transcription. The structure of activation domain has not yet been clarified. They exhibit common amino acid sequence features in some cases and thus are classified in several categories including acidic activation domains, Glutamine-rich domains and Proline-rich domains. In addition, three serine residues that are highly

conservative in bZIP transcription factors have been identified. Phosphorylation of the serine residues affects the binding preference and ability of the bZIP proteins (Meshi et al., 1998).

A. bZIP Transcription Factors in *Arabidopsis*

Transcription factors with bZIP domains are present in almost all eukaryotes. In plants, bZIP proteins regulate processes including light and stress signaling, seed maturation, flower development. In the *Arabidopsis* genome, 73 distinct members of the bZIP families were found as potential *bZIP* genes. The AtbZIP family members were further classified into ten groups, named from Group A to Group I, and Group S, according to similarities of their basic regions. A number of Group A bZIP are associated with ABA signal transduction in both seeds and vegetative tissues (Jakoby et al., 2002).

B. PtFD1

1. *PtFD1* Encodes a bZIP Transcription Factor

First termed as *PTBF1* (Poplar Terminal Bud Factor 1;GenBank Access Number: AF288616), *PtFD1* encodes a bZIP protein of 29.5kD that shares significant sequence similarity in the basic and leucine zipper region with AtbZIP14, a members of the *Arabidopsis* Group A bZIP transcription factors, which was later identified as FD. FD is a bZIP protein that is required for FT to promote flowering through protein interaction in the shoot apex in *Arabidopsis* (Abe et al., 2005).

2. Expression of PtFD1 in Poplar

PtFD1 is expressed during bud formation in response to SD photoperiod. The expression of PtFD1 occurs after bud morphogenesis and correlates with the process of apical bud formation and maturation. The abundance of PtFD1 mRNA reaches its peak after 4-6 weeks of SD treatment and then declines with prolonged SD treatment.

PtFD1 expression is limited in the shoot meristem and young leaf primordial. It was also detected in lateral buds at a lower level than in apical buds, but not expressed in other tissues including leaves, stipules, bud scales, bark.

Under LD conditions, the expression of PtFD1 in shoot apex can also be induced by ABA treatment and high water stress (Gnewikow et al., 2001).

Materials and Methods

I. Materials

A. Plant Material and Growth Conditions

The hybrid poplar (*Populus alba* × *Populus tremula*) clone 717-1B4 was used for all experiments. Plants were maintained and propagated using shoot cultures. For all growth chamber experiments, rooted plants derived from tissue culture were potted in small plastic containers, one in each pot. The plantlets were first grown in the growth chamber under LD photoperiod (16hour light/8hour dark, 18 ºC) for approximately 4 weeks or until the plantlets have reached 25-30cm in height. After this, the photoperiod of the growth chamber was switched to SD photoperiod (8hour light/16hour dark, 18 ºC). The plants were treated in SD for 8 weeks. After that, the growth chamber was set to SD plus low temperature (LT) (8hour light/16hour dark, 10 ºC/4 ºC) for another 4 weeks. After the SD and SD+LT treatments, the plants were returned to LD (16hour light/8hour dark, 18 ºC).

Table 1. Treatments for the plant materials in growth chamber

	LD	SD	SD+LT	LD
Light	16 h / 18 ºC	8 h / 18 ºC	8 h / 10 ºC	16 h / 18 ºC
Dark	8 h / 18 ºC	16 h / 18 ºC	16 h / 4 ºC	8 h / 18 ºC
Duration	5~6 weeks	8 weeks	4 weeks	∞

During the period, the plants were watered every other day supplemented with 0.5X Hoagland's solution.

B. T-DNA Binary Vectors

The T-DNA binary vectors used to construct the chimeric genes are pB7GWIWG2(II) for RNAi and pB7WG2 for overexpression (Karimi et al., 2002). Their maps were shown in the Appendix.

II. Methods

A. Sample Collection and RNA Extraction

Tissues (shoots tips, buds, leaves) were collected at specific time intervals (in LD or after 3, 6, 8,12 weeks of SD treatment) and immediately frozen in liquid nitrogen.

Following the modifications to Qiagen's RNeasy mini kit modified by O. Shevchenko and A. Brunner, the tissues were grounded to a fine powder in liquid nitrogen using a mortal and pestle. Ground tissue (~ 200mg) was added to RNA extraction buffer (1ml Qiagen RLT buffer, 0.01g soluble polyvinylpyrrolidone (PVP-40, Sigma), 10µl β-mercaptoethanol), and vortex for 1 min to homogenize. 0.4 Volume of 5M potassium acetate (KoAC) (pH6.5) was added and mixed by inversion. The mixture was incubated on ice for 15 min, divided into two 1.5ml eppendorf tubes and centrifuged at 12,000 RPM for 15 min at 4 ºC. The supernatant were transferred to 2 new 1.5 ml eppendorf tubes and 0.5 Volume of 100% ethanol (EtOH) was added, mixed by pipetting up and down. The homogenate was transferred to two RNeasy Spin Columns following Qiagen's instructions for RNA

isolation from plant tissues. The column was washed twice with 50μl RNase-free water to elute and collect RNA (Qiagen). The RNA (4×50μl) was precipitated overnight with 0.1 Volume of 3M sodium acetate (NaoAC) (pH5.2) and 2 Volumes of 100% EtOH at –20 ºC. The RNA was pelleted by centrifuging at 14,000RPM for 30 min at 4 ºC, washed twice with 70% EtOH (cold), dried under vacuum and resuspend with 30μl of RNase-free water. RNA concentration was determined by measuring absorption at 260 nm wavelength. The RNA samples were stored at –80 ºC.

B. Plasmid DNA Extraction

Plasmid DNA was extracted using Concert™ Rapid Plasmid Miniprep System kit (Gibco BRL). Bacteria were cultured at 37 ºC overnight in 5 ml LB broth supplemented with appropriate antibiotics. The cells were pelletted by centrifuging at 12,000 RPM for 10 min. Following the manufacturer's instruction, plasmid DNA was eluted from the silica-based spin columns with TE or water. The plasmid DNA was then digested with restriction enzymes and checked by agarose gel electrophoresis.

C. PCR Amplification

All PCR reactions used the GeneAmp PCR System 9700 thermocycler (PE Biosystems). A single 10μl PCR reaction consisted of 1μl 10X ExTaq Buffer, 2mM $MgCl_2$, 200 μM dNTPs, 0.2μM forward primer, 0.2μM reverse primer, 0.25 unit Takara *ExTaq* DNA polymerase (Takara Biomedicals, Japan) and 2-20ng of DNA

template. Unless otherwise indicated, the conditions for the PCR reaction are listed below:

Table 2. PCR reaction conditions

	Denature	Anneal	Extend
Temperature	94 ºC	62 ºC	72 ºC
Duration	30 sec	1 min	1.5 min

D. Gel Purification of PCR Products

Extraction of DNA PCR products from agarose gels was accomplished using the Concert™ Rapid Gel Extraction System kit (Gibco BRL). DNA bands in the gel were stained with ethidium bromide (EtBr) and visualized under UV light. The appropriate bands were cut from the gel and weighed. The agarose was dissolved in gel extraction buffer at 50 ºC for 15 min. Following the manufacturer's protocol, the DNA/PCR products were purified and eluted from the silica-based spin columns with appropriate amount of TE or water.

E. TOPO Cloning and Transformation with PCR Products

An appropriate amount of purified PCR product was mixed with 1µl salt solution and 1µl TOPO vector and sterile water (optional) to a total volume of 6µl (Invitrogen). The tube was incubated at room temperature for 5 min, then placed on ice. 2µl of the cloning reaction was added to 100µl competent *E. coli* (TOP 10) cells in an eppendorf test tube, and gently mixed. The tube was incubated on ice for 30 min, heat treated at 42ºC for 30 sec (heat-shock), and transferred immediately to ice. Room temperature S. O. C. medium (250µl) was then added to the tube. The tube

was agitated horizontally at 37ºC for 1 hour (200RPM), then centrifuged at 4,000RPM for 10 min. The supernatant was discarded and 100µl fresh S.O.C. medium was added to re-suspend the pellet. The cell suspension was spread on 2-3 pre-warmed LB agar plates supplemented with appropriate antibiotics. The plates were incubated overnight at 37ºC.

F. Gateway™ LR Recombination Reaction

Reaction ingredients were added to a 1.5ml microcentrifuge tube at room temperature, including 300ng of entry clone, 300ng of binary vectors, 1µl of Topoisomerase I and 4µl of 5X LR Clonase™ reaction buffer. Then TE Buffer (pH8.0) was added to a final volume of 16µl. The LR Clonase™ Enzyme Mix was removed from -80ºC, thawed on ice, and mixed by vortexing briefly twice (2 sec/each). 4µl of the enzyme mix was added to the 16µl reaction mix (Invitrogen). The recombination reaction was incubated at 25ºC for 1 hour. After incubation, 2µl of 2µg/µl Proteinase K solution was added to the reaction and incubated at 37ºC for 10 min to stop the reaction. The expression resulting from the Invitrogen cloning reaction was transformed to competent cells and selected on LB agar plates with antibiotics.

G. Make *Agrobacterium* Competent Cells

Agrobacterium strain C58/pMP90 were grown overnight at 28 ºC in 5ml LB broth with 20mg/L gentamicin. The next day, 2ml of the overnight culture was added to 50ml of LB broth with 20mg/L gentamicin in a 250-ml flask and was

shaken vigorously (250RPM) at 28 ºC until the culture grew to an OD_{600} of 0.5-1.0. The cell suspension was chilled on ice and centrifuged at 3,000g for 5 min at 4 ºC. The supernatant solution was discarded. The cells were re-suspended in 1ml of 20mM ice-cold $CaCl_2$, aliquoted into pre-chilled eppendorf tubes (100µl/each tube), froze in liquid nitrogen and stored in –80 ºC for future use.

H. *Agrobacterium* Transformation (Freeze-Thaw Method)

Approximately 1µg plamid DNA is added to thawed competent cells at 4ºC. The cells were then frozen in liquid nitrogen and thawed by incubating the test tube in 37ºC water bath for 5 min (Freeze-Thaw Method). After incubation at 37ºC, 1ml of LB broth was added to the tube and incubated at 28ºC for 2 –4 hours with gentle shaking (~150RPM). The cells are then centrifuged for 30 sec and the supernatant discarded. The cells were re-suspended in 100µl LB broth and spreaded on 2-3 LB agar plates containing appropriate antibiotics. The plates were incubated at room temperature in the dark. Transformed colonies appeared in 2-3 days.

I. *Agrobacterium*-Mediated Poplar Transformation

Table 3. Media for poplar transformation

<table>
<tr><td>M</td><td rowspan="4">4.57g/L basal medium MS[a]
1mg/L L-cysteine
200mg/L L-glutamine
30g/L Sucrose</td><td></td><td></td><td></td><td></td><td rowspan="5">4g/L phytagar</td></tr>
<tr><td>M1</td><td rowspan="2">10µM NAA[b]
5µM 2iP[c]</td><td></td><td></td><td></td></tr>
<tr><td>M2</td><td rowspan="2">500mg/L Carb[d]

250mg/L Cefo[e]</td><td></td><td></td></tr>
<tr><td>M3</td><td></td><td>0.1µM TDZ[f]</td><td>BASTA</td></tr>
<tr><td>M1/2</td><td>1/2 dose</td><td></td><td></td><td></td><td>BASTA</td></tr>
</table>

[a]Murashige and Skoog [b]NAA= naphthaleneacetic acid
[c]2iP=6-(y,y,dimethylally-amino)-purine [d]Carb= carbenicillin [e]Cefo= cefotaxime
[f]TDZ= thidiazuron

1. Pre-Conditioning

Explants (stems and petioles) from the plants were pre-incubated on solidified M1 medium that included 4.57g/L basal MS medium, 1mg/L L-cysteine, 200mg/L L-glutamine, 30g /L sucrose and 4g/L phytagar for 48 hours at 24 ºC in darkness.

2. Co-Cultivation

A two-day culture of *Agrobacteria* containing appropriate binary vectors grown on LB agar plates with appropriate antibiotics was used to prepare 25ml LB medium with corresponding antibiotics. The 25-ml culture was shaken at 28 ºC until OD_{660} reached about 0.3. The cell suspension was centrifuged at 12,000RPM for 10 min and the supernatant was discarded. The cells were resuspended in 100ml M liquid that consists of 457mg of basal medium MS, 0.1mg of L-cysteine, 20mg of L-glutamine, 3g sucrose and appropriate antibiotics. Forty explants (stems and petioles) were dipped into 25ml of the bacteria suspension in Petri dishes and stirred slowly. After 16 hours, the explants were blotted on sterile paper towel to remove excess bacteria and cultured on solidified M1 medium supplemented with 10µlM NAA and 5µM 2iP. The explants were culture at room temperature on M1 medium for 48 hours in darkness.

3. Decontamination

The explants were washed 4 times (5min/each time) in an antibiotic solution that included sterile water, 250mg/L cefotaxime, 500mg/L carbenicillin and 25mg/L tetracycline with vigorous stirring. After the antibiotic wash, the explants were washed 4 more times (5min/each) in sterile water, also vortexed vigorously.

After washing, the explants were transferred to M2 medium consisting of 4.57g/L basal medium MS, 1mg/L L-cysteine, 200mg/L L-glutamine, 30g/L sucrose and 4g/L phytagar, supplemented with 10μM NAA, 5μM 2iP, 500mg/L carbenicillin, and 250mg/L cefotaxime. The explants were cultured on M2 medium for 10 to 15 days for stems and about 25 days for leaves in darkness at 24 ºC.

4. Regeneration

The explants were transferred from darkness to light and cultured on solidified M3 medium consisting of 4.57g/L basal MS medium, 1mg/L L-cysteine, 200mg/L L-glutamine, 30g/L sucrose, 4g/L phytagar, supplemented with 500mg/L carbenicillin, 250mg/L cefotaxime, 0.1μM thidiazuron.

5. Rooting

The shoots were transferred to M1/2 medium that contains the same ingredients as M medium but only half the concentration of the macro-nutrients and 15g/L sucrose.

J. DNase Treatment of RNA Samples Prior to RT-PCR

RNA samples were treated with DNase to remove DNA. RNA was mixed with RQ1 RNase-Free DNase buffer, RQ1 RNase-Free DNase (1u/μg RNA) and Nuclease-free water to a final volume of 10μl (Promega), incubated at 37 ºC for 30 min. After DNA digestion, the reaction was terminated by adding 1μl of Stop Solution and incubating at 65 ºC for 10 min.

K. cDNA Synthesis

First strand cDNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega) according to the manufacture's instructions. Approximately 0.4µg of total RNA (in 4.5µl or less) was mixed with 0.5µl Oligo(dT), preheated at 70ºC for 5 min and immediately chilled on ice for at least 5 min. After incubation on ice, 4µl ImProm-II™ 5XReaction Buffer, 4.8µl 25mM $MgCl_2$ (6mM final conc.), 1µl 10mM dNTP Mix (0.5mM/each final conc.), 0.5µl Recombinant RNasin® Ribonuclease Inhibitor (20 units) and ImProm-II™ Reverse Transcriptase (1µl /reaction) (Promega) were added to the cDNA and reaction volume adjusted to 20µl with nuclease-free water. The reaction was first incubated at 25 ºC for 5 min for annealing followed by 42 ºC for 1 hour for cDNA synthesis. After synthesis, the reaction was stopped by incubation at 70 ºC for 15 min. The inactivated reverse transcription reaction was used directly for PCR amplification.

L. Tissue Culture Media Preparation

Media was prepared with nano pure water, autoclaved at 120 ºC for 15 min. Vitamins, growth regulators, antibiotics and herbicides were added by filter sterilization after the media was cooled to 50 ºC. After adding filter sterilized ingredients, the media was poured to sterile Petri dishes or baby food jars and allowed to cool before use.

M. DNA and RNA Quantification

DNA and RNA were quantified by measuring absorbance at 260nm (A_{260}). The DNA or RNA samples were diluted with water and loaded in a 96-well Costar low UV plate for absorbance detection by μQuant™ Microplate Spectrophotometer (Bio-Tek Instruments, Inc) following the manufacturer's instruction. For reliable quantification, A_{260} readings should lie between 0.1 and 1.0.

Absorbance at 280nm (A_{280}) was also measured to calculate the ration of A_{260} to A_{280} (A_{260}/A_{280}) ratio, a measure of nucleic acid purity. A ratio between 1.8 and 2.0 is acceptable.

N. DNA Sequencing

All DNA sequencing was performed by the DNA Sequencing Facility operated by the Center for Biosystem Research, University of Maryland Biotechnology Institute, College Park, MD. DNA samples were prepared according to the instructions from facility and M13 Forward and Reverse primers were used for all sequencing.

O. Sequence Analysis

Sequence similarity searches were performed using BLAST in the NIH website (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990). Clustal W alignments were performed using Lasergene® (DNASTAR Inc.) or GeneDoc 2.6.02 from GeneDoc HomePage (http://www.psc.edu/biomed/genedoc/). Restriction site

analysis used NEBcutter V2.0 from the website of New England Biolabs, Inc. (http://tools.neb.com/NEBcutter2/index.php).

III. Experimentation

A. Construction of PtFD1 RNAi and Overexpression (OE) Vectors

For the functional characterization of function of *PtFD1* during poplar apical bud formation and dormancy, transgenic poplars that either overexpress PtFD1 or transgenic poplars with reduced or silence *PtFD1* using RNAi were created. DNA sequences were first cloned into the entry vector, pENTR/D-TOPO®. After cloning into the entry vector, DNA of interest was transferred to the binary T-DNA binary vectors pB7GWIWG2 (II) for RNAi and pB7WG2 for overexpression.

PtFD1 DNA used for chimeric gene construction was produced by PCR using 4w PCR2-14 PTBF1, a PCR product that contains the full length of *PtFD1* cDNA sequence. The primers are listed below:

Table 4. Primers for PCR of 4w PCR2-14 PTBF1

Direction	Primer Name	Primer Sequence
Forward	PTBF1-ATG	(CACC)[1] ATG TGG TCA TCG CCA GGA GCA
Reverse	PTBF1+TGA	TCA AAA TGG AGC TGT TGA GGT TCT ATA GAG
Reverse	PTBF1-Rev51	GCC AGA GAC ATC ACC GTT TTC TTG AG

[1] (CACC) were added to the 5' end of the primer for directional cloning of the PCR product to the pENTR-TOPO® vector.

PCR reactions were carried out using 200ng of template DNA, 1μM of each primer, 1Xreaction Buffer, 2mM $MgCl_2$, 0.2mM/each dNTPs, and 0.25 unit Takara

ExTaq DNA polymerase. PCR amplification with PTBF1-ATG and PTBF1+TGA primer set produced the coding region of *PtFD1* (+TGA) while PTBF1-ATG and PTBF1-Rev51 primer combination gave a *PtFD1* fragment that terminates prior to the bZIP basic region (Rev51). PCR products (+TGA and Rev51) were separated on a 1% agarose gel. Bands of interest were excised and purified using Concert™ Rapid Gel Extraction System kit (Gibco BRL). Purified fragments were cloned into the pENTR/D-TOPO entry vector (Invitrogen, MD). Competent *E. coli* (TOP 10) cells were transformed with the entry vector and transformed bacteria selected by their resistance to kanamycin (50µg/ml) on solidified LB agar medium. Plasmid DNA from kanamycin resistant colonies was extracted, digested with restriction enzymes (*Asc*I and *Not*I) and visualized in agarose gels. Plasmids that produced the desired restriction patterns were sequenced. After confirmation of the DNA sequence, it was transferred to the appropriate Gateway™ binary vector by *in vitro* recombination (Invitrogen) (Karimi et al., 2002).

Construction of PtFD1 RNAi Chimeric Gene

PtFD1 inserts in pENTR/D-TOPO clones, +TGA and Rev51 were transferred to the destination binary vector pB7GWIWG2 (II) using the Gateway™ LR Recombination Reaction (Invitrogen, MD). When transcribed, this construct will produce a double-stranded RNA (hairpin RNA) from the inserted sequence of *PTFD1*, which then triggers post-transcriptional gene silencing. Competent *E. coli* (TOP 10) cells were transferred with RNAi expression vectors and selected by resistance to spectinomycin (50µg/ml) and chloramphenicol (50µg/ml).

Construction of PtFD1 Overexpression Chimeric Gene

PtFD1 DNA sequences in pENTR/D-TOPO clones, +TGA, were transferred to the destination binary vector pB7WG2 using Gateway™ LR Recombination Reaction (Invitrogen, MD). This results in a chimeric gene where the full length *PtFD1* cDNA was inserted downstream of the CaMV 35S promoter. The vector pB7WG2::+TGA was transformed into competent *E. coli* (TOP 10) cells and grown on LB agar plates containing spectinomycin (50µg/ml).

Spectinomycin-resistant colonies were selected and cultured in 5ml LB broth supplemented with appropriate antibiotics overnight at 37 °C. Plasmids DNA were purified and digested with restriction enzymes (RNAi/+TGA and RNAi/Rev51 vectors: *Eco*RI; Overexpression/+TGA: *Spe*I ans *Xba*I) and visualized in agarose gel to confirm the recombination.

B. Generation of *A. tumefaciens* with T-DNAs

After the PtFD1 RNAi [pB7GWIWG2 (II)::+TGA, pB7GWIWG2 (II)::Rev51] and overexpression [pB7WG2::+TGA] vectors were verified, bacteria stocks were made by mixing 850µl of the cell suspension and 150µl sterile glycerol and directly frozen in liquid nitrogen. The bacteria stocks were stored in -80 °C.

To transfer the binary T-DNA plasmids to *Agrobacterium*, TOP10 cells transformed with the binary T-DNA plasmids were grown on LB agar plates supplemented with the antibiotics including spectinomycin (50µg/ml), chloramphenicol (50µg/ml) for RNAi or spectinomycin (50µg/ml) for overexpression. Single colonies were selected and cultured in 5ml LB broth with

the same antibiotics overnight at 37 °C. After overnight incubation, plasmids DNAs were extracted and quantified. The *Agrobacterium* strain used in this experiment is C58/pMP90. Approximately 1µg of plasmid DNAs were transformed to C58/pMP90 competent cells via the freeze-thaw method. C58/pMP90 cells transformed with the PtFD1 RNAi vectors [pB7GWIWG2 (II)::+TGA, pB7GWIWG2 (II)::Rev51] were selected on LB agar plates supplemented with gentamicin (20µg/ml), spectinomycin (50µg/ml) and chloramphenicol (50µg/ml) while cells transformed with PtFD1 overexpression vectors [pB7WG2::+TGA] were selected using gentamicin (20µg/ml) and spectinomycin (50µg/ml). The plates were incubated at room temperature in darkness.

Single colonies were selected from the plates and cultured overnight at 28°C in 5ml LB broth supplemented with antibiotics including gentamicin (20µg/ml), spectinomycin (50µg/ml), chloramphenicol (50µg/ml) for RNAi and gentamicin (20µg/ml), spectinomycin (50µg/ml) for overexpression. Purified plasmids DNA were digested with restriction enzymes (RNAi/+TGA and RNAi/Rev51 vectors: *Eco*RI; Overexpression/+TGA: *Spe*I ans *Xba*I) and analyzed by agarose gel electrophoresis to confirm the presence and organization of the vectors.

C. Propagation of Transgenic Poplars

The protocols used to generate transgenic poplars were adopted from Leple et al. 1992. *Agrobacterium* mediated transformation of poplar (*Populus alba* × *Populus tremula*) clone 717-1B4 was performed by co-cultivating sterile explants with *A. tumefaciens* containing the RNAi or overexpression binary vectors.

Explants used for transformation consisted of stems and petiole sections approximately 8mm in length with the stems split longitudinally. The explants were first preconditioned on M1 medium for 48 hours before co-cultivation. After co-cultivation, the explants were de-contaminated and cultured on M2 medium with carbenicillin (500mg/L) and cefotaxime (250mg/L). The explants were transferred to M3 medium for regeneration after 2 weeks of culture on M2. Regenerated shoots were excised from calli when they were approximately 1cm in length and transferred to M1/2 medium for rooting (Leple et al., 1992). The regenerated shoots were sub-cultured on M1/2 medium.

BASTA was added to the medium M3 and M1/2 at 5mg/L to select transformed cells. Both the pB7GWIWG2 (II) T-DNA and the pB7WG2 T-DNA contain a *Bar* gene that confers resistance to glufosinate ammonium (Karimi et al., 2002). Therefore, cells that were not transformed with the *Bar* gene were killed by the herbicide and failed to grow.

D. RT-PCR of *PtFD1*

To determine the expression of PtFD1 in shoot tips or apical buds at different development stages, buds or shoot tips were collected from both the transgenic and control plants that were grown in either LD or after 3, 6, 8, 12 weeks of SD treatment for RT-PCR. *PtFD1* specific primers (ATG and Rev51) were used to detect PtFD1 mRNA. Total RNA was extracted, precipitated and quantified. The RNAs were first treated with DNase to remove the DNAs that might occur in the samples. First strand cDNAs were synthesized from 0.4µg of total RNA using the

ImProm-II™ Reverse Transcription System kit (Promega). PCR amplification was performed using Takara Extaq polymerase (Takara Biomedicals, Japan). PCR products were separated and visualized by agarose gel electrophoresis. The primers used for PCR amplification are listed below:

Table 5. *PtFD1* specific primers for PCR amplification

Direction	Primer Name	Primer Sequence
Forward	ATG	ATG TGG TCA TCG CCA GGA GCA
Reverse	Rev51	GCC AGA GAC ATC ACC CTT TTC TTG AG

Total RNA from leaves of the 717-1B4 and two overexpression transgenic lines (OE2-1; OE2-3) treated with LD or after 8 weeks of SD treatment were also analyzed by RT-PCR. In addition, flowers produced in two of the overexpression lines were also collected and used for RT-PCR experiments with *PtFD1* specific primers to detect PtFD1 expression.

E. Histological Analysis of Apical Buds

For PtFD1 RNAi plants, shoot tips were collected every five days after the photoperiod was changed to SD up to 25 days. For PtFD1 overexpression plants, shoot tips or apical buds were collected after 3, 6 or 8 weeks of SD treatment. Corresponding shoot tips were also collected from control 717-1B4 poplars for comparison.

The collected tissues were fixed immediately in fresh FAA [50% EtOH, 5% glacial acetic acid, 10% formaldehyde, 35% water (v/v)]. Vacuum infiltrated for 2 hours. Fixed tissues were dehydrated and infiltrated according to Table 6.

Table 6. Paraffin/TBA method (Ruzin, 1999)

Step	95% EtOH	100% EtOH	TBA	Mineral Oil	Duration
1	50		50		1 day
2		25	75		1 day
3		25	75		1 day
4			100		1 day
5			100		1 day
6			100		1 day
7			67	33	1 day

After TBA infiltration, about 1/3 volume of the mixture was poured off and replaced with an equal volume of melted paraplast. The vials were placed in an oven (58 °C) without caps. At 12-hour intervals, 1/2 volume was removed and replaced with an equal volume of the melted paraffin. This process was repeated 2-3 times. As a final step the entire mixture was poured off and replaced with melted paraplast. This step was repeated 4-5 times over a 12-hour interval and left overnight after the last change of paraplast. When no residual TBA can be detected, the tissues were embedded.

Embedding was performed using the LEICA EG 1160 Paraffin Embedding Center. Selected tissues were placed at the center of a mold and melted paraplast was added to the mold until it reached the top edge of the plastic ring. The melted paraffin in the mold was then solidified on a cooling plate with -5 °C. The paraffin block was released from the mold when the paraffin was completely hardened.

15µm sections were prepared using disposable microtome knife and mounted onto microscope slides with Sass's adhesive. Sections were first deparaffinized in

xylene followed by hydration in a graded EtOH series and water (Ruzin, 1999). The sections were stained in Safranin O (1% w/v in water) for 1 min, destained with water and then dehydrated in a graded EtOH series to 95% EtOH, followed with staining in Fast Green FCF (0.1% w/v in 95% EtOH) for 2 min and destained in 100% EtOH, 2 times at 2 minutes each time (Table 7).

Sections were cleared with 1:1 xylene and methyl salicylate for 5 sec, then dipped 2 times in 100% xylene. Coverslides were mounted with Permount®.

Table 7. Staining processes for the slides

Step	Name	Procedure
1	Deparaffinization	10min in 100% Xylene (2 times)
		15min in acetone
2	Hydration	2min in 100% EtOH (3 times)
		1min in 95% EtOH
		1min in 85% EtOH
		1min in 70% EtOH
		1min in 50% EtOH
		1min in 30% EtOH
		2min in H_2O
3	Staining	1min in Safranin O (1% w/v in H_2O)
4	Destaining	3-4 times in H_2O till the water is clear
5	Dehydration	2min in 30% EtOH
		2min in 50% EtOH
		2min in 70% EtOH
		2min in 85% EtOH
		2min in 95% EtOH
6	Staining	2min in Fast Green FCF (0.1% w/v in 95% EtOH)
7	Destaining	2min in 100% EtOH (2 times)

Results

I. Construction of Transgenic Vectors

A. PCR Amplification of *PtFD1* Fragments

Two sets of primers (Figure 1) were used to amplify *PtFD1* fragments by using a full length *PtFD1* cDNA (4w PCR2-14 *PTBF1*) as the PCR template. PCR amplification with PTBF1-ATG and PTBF1+TGA primer set produced an 820bp DNA fragment consisting of the coding region of *PtFD1* while PTBF1-ATG and PTBF1-Rev51 primer combination results in a 600bp *PtFD1* fragment consisting of the 5' region of the cDNA that terminates prior to the bZIP basic region. The PCR products were analyzed on a 1% agarose gel. Figure 2 shows the results of the PCR amplification and two bands approximately 820bp and 600bp are detected. After gel electrophoresis, the 820bp and 600bp bands were cut from the gel and purified. To further verify that the correct fragments were amplified, the purified fragments were digested with endonuclease restriction enzymes. For the 820bp product, *Eco*RI digestion was expected to produce 2 bands approximately 400bp while *Stu*I gives 2 bands of 580bp and 240bp. For the 600bp product, *Eco*RI digestion was expected to produce 2 bands of 400bp and 200bp while *Nsi*I gives 2 bands of 470bp and 130bp. The PCR products were digested with these enzymes and separated by agarose gel electrophoresis. The gel image showed bands of these predicted size

(data not shown), confirming that the PCR products were consistent with the *PtFD1* cDNA sequence.

Figure 1. *PtFD1* cDNA sequence. The locations of forward and reverse primers are indicated by arrows.

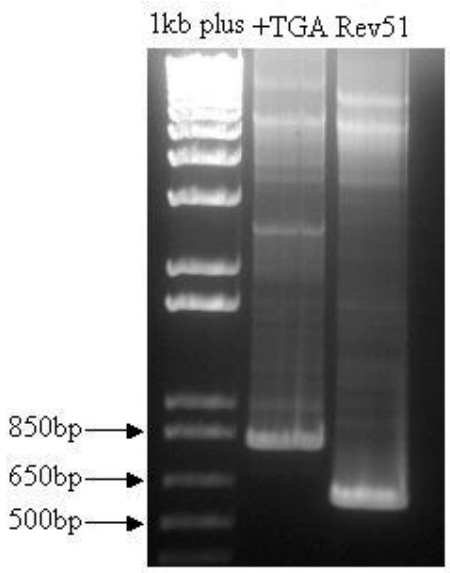

Figure 2. PCR of 4wk PCR 2-14 PTBF1. Primer combinations are as following: lane +TGA, PTBF1-ATG and PTBF1+TGA; lane Rev51, PTBF1-ATG and PTBF1-Rev51. PCR products were resolved through a 1% agarose gel containing ethidium bromide..

B. Cloning of PCR Products into pENTR/D-TOPO Vector

The purified PCR products (+TGA and Rev51) were cloned into pENTR/D-TOPO vector, transformed into competent *E. coli* (TOP 10) cells and grown overnight on LB plates containing 50µg/ml kanamycin. Individual colonies from the plates were picked and grown in LB broth containing kanamycin overnight at 37°C. Plasmid DNA was extracted from bacteria culture, digested with *Asc*I and *Not*I and separated through a 1% agarose gel. For the +TGA clone, digestion with *Asc*I and *Not*I should produce two bands of approximately 2580bp and 840bp. Two of the seven colonies tested showed the predicted bands. For the Rev51 clone, digestion with *Asc*I and *Not*I should result in two bands of 2580bp and 620bp. Of all the 16 colonies tested, 3 of them showed the predicted bands (data not shown). Plasmids containing the predicted bands were sequenced to verify the sequences of the cloned PCR products.

The sequences of the PCR clones were aligned to the *PtFD1* cDNA sequence and proved to be identical. Two clones (+TGA1 and Rev51-2) were selected for use in the LR recombination reaction for producing the binary T-DNA vectors.

C. Transfer of the *PtFD1* Fragments to the Binary Vectors

The *PtFD1* fragments (+TGA1 and Rev51-2) cloned into the pENTR/D-TOPO vector were transferred to the RNAi and Overexpression binary vectors to generate 3 chimeric genes. These include two RNAi clones (pB7GWIWG2(II)::+TGA) and (pB7GWIWG2(II)::Rev51) and one

overexpression clone (pB7WG2::+TGA). Transfer to the pB7GWIWG2(II) and pB7WG2 binary vectors was accomplished using the GATEWAY™ LR recombination reaction. The entry vector pENTR/D-TOPO contains *att*L sites (*att*L1 and *att*L2) that will recombine with the *att*R sites (*att*R1 and *att*R2) in the destination binary vector resulting in the transfer of the cloned *PtFD1* fragments in the entry vector to the destination vector. The recombination reaction used the LR Clonase™ enzyme mix. Topoisomerase I was added to relax the DNA of the destination vector and increase the efficiency of the LR reaction (GATEWAY™ Technology Instruction Manual, Invitrogen). The binary vectors were transferred to *E. coli* competent cells (TOP10) and grown overnight on LB agar plates supplemented with spectinomycin and chloramphenicol for RNAi and only spectinomycin for overexpression. After overnight culture at 37°C, colonies were picked from the plates and cultured overnight with shaking in 5ml of LB broth containing the appropriate antibiotics. Plasmid DNA was extracted from the cultured bacteria cells, digested with restriction enzymes and separated through 1% agarose gel electrophesis containing ethidium bromide. Table 8 listed the restriction enzymes used to analyze the purified plasmids.

Table 8. Restriction enzymes and predicted digestion production of *PtFD1* binary plasmids

	pB7GWIWG2 (II)::Rev51 RNAi	pB7GWIWG2 (II)::+TGA RNAi	pB7WG2::+TGA overexpression
*Eco*RI	880bp, 1.2kb, 10.2kb	10.8kb, 1.4kb, 1.2kb	
*Spe*I + *Xba*I			1.3kb, 8.9kb

As shown in Figures 3 and 4, clones digested with the respective restriction enzymes produced predicted bands, confirming that the *PtFD1* fragments had been transferred to the T-DNA binary vector.

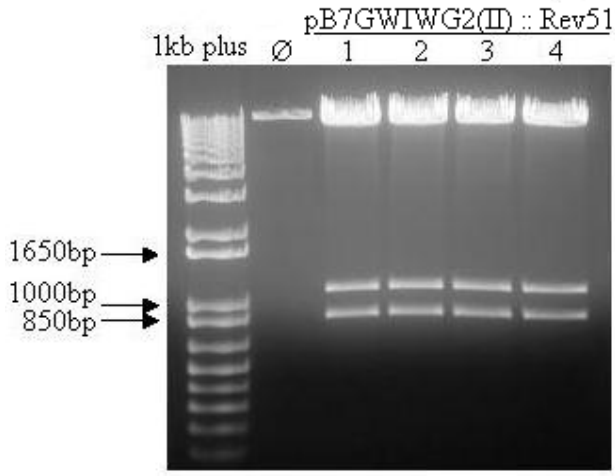

Figure 3. Digestion of the RNAi vector pB7GWIWG2 (II)::Rev51 with *Eco*RI. Lanes 1 to 4 contains pB7GWIWG2 (II)::Rev51 plasmid DNA extracted from 4 different colonies. Lane Ø is a control pB7GWIWG2 (II) vector without an insert. The digestion reaction was carried out at 37 °C for 1 hour and resolved through a 1% agarose gel contains ethidium bromide. 1kb DNA plus ladder was used as a size marker.

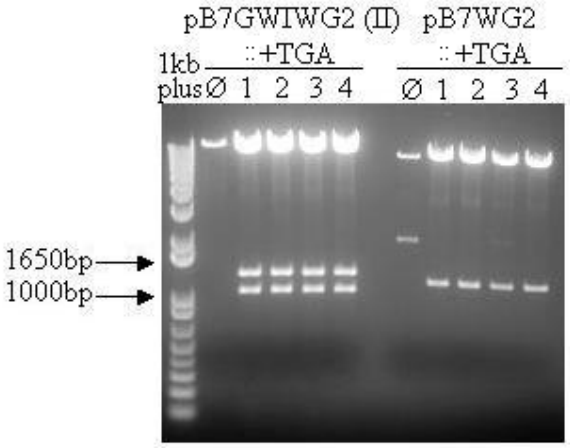

Figure 4. Digestion of RNAi vector pB7GWIWG2 (II)::+TGA and overexpression vector pB7WG2::+TGA. Lanes 1 to 4 (left side) are four independent colonies containing pB7WIWG2(II)::+TGA digested with *Eco*RI. Lane Ø is the pB7WIWG2(II) vector without an insert. Lanes 1 to 4 (right side) are four independent colonies containing pB7WG2::+TGA digested with *Spe*I and *Xba*I.

D. Transfer of the Binary Vectors to *A. tumefaciens*

Approximately 1μg of the binary RNAi and overexpression plasmids were individually transferred to *A. tumefaciens* competent cells (C58/pMP90) using the freeze-thaw method. Transformed *A. tumefaciens* RNAi [pB7GWIWG (II)::+TGA, pB7GWIWG (II)::Rev51] cells were selected on LB plates supplemented with gentamicin, spectinomycin, chloramphenicol and LB plates with gentamicin and spectinomycin for overexpression [pB7WG2::+TGA]. The plates were incubated at room temperature in darkness. After 3-4 days transformed colonies were visible. After 4 days of growth, colonies transformed with pB7GWIWG (II)::+TGA, pB7GWIWG (II)::Rev51 and pB7WG2::+TGA were picked and cultured overnight in 5ml LB broth supplemented with corresponding antibiotics. Plasmids was extracted, digested by restriction enzymes digestion as previously described and separated through a 1% agarose gel containing ethidium bromide. Figure 5 shows that the predicted bands (Table 8) for all of the plasmids were detected. Agrobacteria with RNAi and overexpression binary T-DNA were then used for poplar transformation.

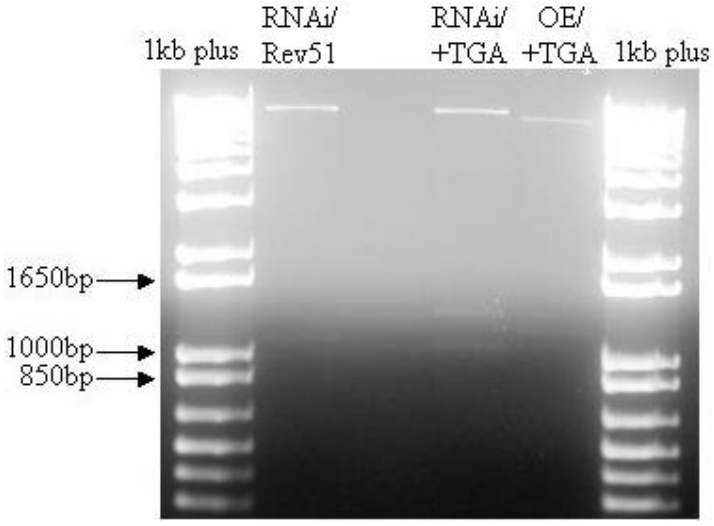

Figure 5. Digestion of T-DNA binary plasmids extracted from *A. tumefaciens*. RNAi/Rev51 and RNAi/+TGA were digested with EcoRI. OE/+TGA was digested with *Spe*I and *Xba*I. 1kb plus DNA ladder was used as the marker.

II. Propagation of Transgenic Poplars

The hybrid poplar clone 717-1B4 (*Populus tremula* X *P. alba*) was used for the transgenic studies because of its efficiency in adventitious shoot regeneration. Shoot cultures of the clone were cultured *in vitro* on M1/2 MS medium. Sterile explants (stems and petioles) were dissected from the 717-1B4 shoot cultures. Regenerated transgenic shoots were cultured in M1/2 MS medium supplemented with 5mg/L BASTA while non-transgenic 717-1B4 seedlings were grown in M1/2 MS medium without BASTA.

Of the 160 explants transformed with pB7WG::+TGA (OE/+TGA) chimeric gene, 12 explants regenerated shoots. Among 200 explants transformed with pB7WIWG2 (II)::+TGA (RNAi/+TGA), 6 explants regenerated shoots. After repeated selection on BASTA, 4 individual lines of each of the transgenic types (OE/+TGA and RNAi/+TGA) were obtained. No RNAi/Rev51 plants were obtained from the 200 explants transformed with pB7WIWG2(II)::Rev51 (RNAi/Rev51). Plantlets from individual transgenic lines were transferred to fresh M1/2 medium with BASTA in a certain time interval, usually 1-2 months. Cuttings were made to propagate more plantlets. It can be noticed that the plantlets of OE/+TGA had a thicker stem, a smaller leaf and not as green as RNAi/+TGA and the control.

III. Morphological Characteristics of the Transgenic Poplars

When enough plants were generated by tissue culture, seedlings with 5-6cm stems, 4-6 leaves and complete roots from several transgenic lines and the control were transferred to soil pots and grown in plant growth chambers (30 plantlets /each line).

After 2-3 weeks of growth in LDs, morphological differences between PtFD1 overexpressing and control plants were observed. PtFD1 overexpression plants failed to grow with an upright habit and instead the stems grow with a prostrate habit (Figure 6). This growth habit was observed in all of the PtFD1 overexpressing lines. In addition to the change in growth habit, the leaves of PtFD1 overexpressing plants were smaller and coiled upward compared to non-transgenic control plants. There were no significant morphological differences between the RNAi/+TGA and the control plants.

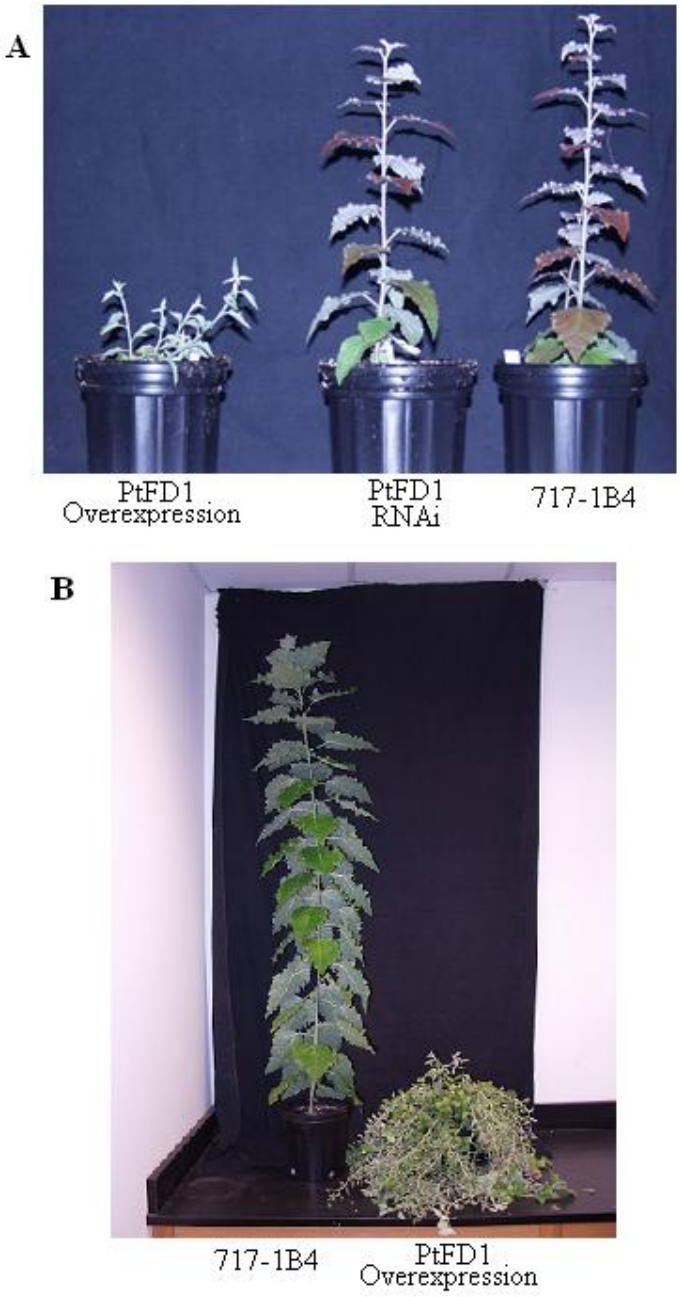

Figure 6. PtFD1 transgenic plants (Overexpression and RNAi) and control (717-1B4) plants in growth chamber (A) and greenhouse (B).

After 5-6 weeks of growth in LD, plants were transferred to SD. For control 717-1B4 poplars, apical bud morphogenesis began after 3 weeks of SD treatment. Compared to control plants, apical bud development appeared to be accelerated for the PtFD1 RNAi expressing plants but the difference was not significant. Although both control and PtFD1 RNAi plants developed apical buds at similar rate after 56 days of SD, the apical buds of the PtFD1 RNAi expressing poplar were visibly smaller in size. In contrast to PtFD1 RNAi expressing plant and control plants, poplars overexpressing PtFD1 failed to form apical buds when treated with SD even after 8 weeks. Because the shoot apices failed to develop apical buds, shoot growth continued in SD (Figure 7). It was also noticeable that the shoot elongation of PtFD1 overexpression plants was slower both in tissue culture and in growth chambers. After 8 weeks of SD treatment, the plants were transferred to SD plus LT. Leaf abscission occurred for both control and PtFD1 RNAi expressing plants while leaf abscission for PtFD1 overexpressing plants failed to occur.

In addition, flowers were induced in both tissue cultured plantlets and greenhouse grown plants of all PtFD1 overexpressing lines (Figure 8). Wild type poplars usually do not form flower buds during the first several years of their life cycle (Hsu et al., 2006).

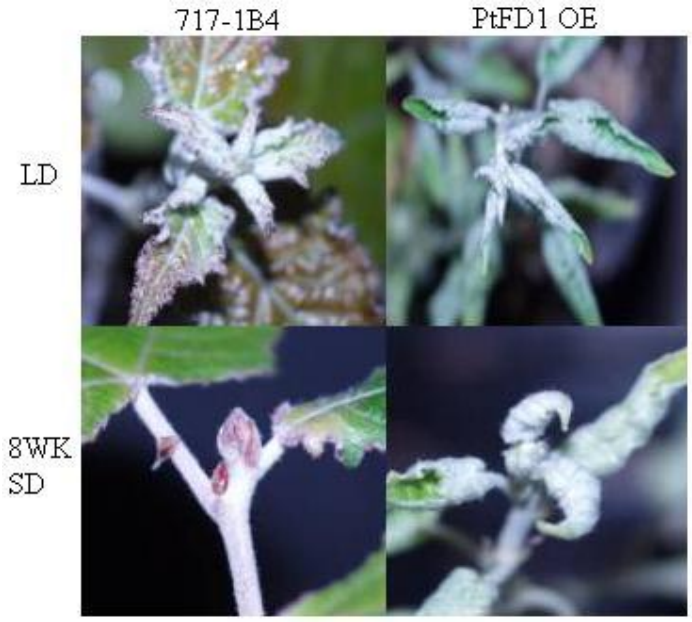

Figure 7. Shoot apices of 717-1B4 and PtFD1 overexpression plants in LD or after 8 weeks of SD treatment.

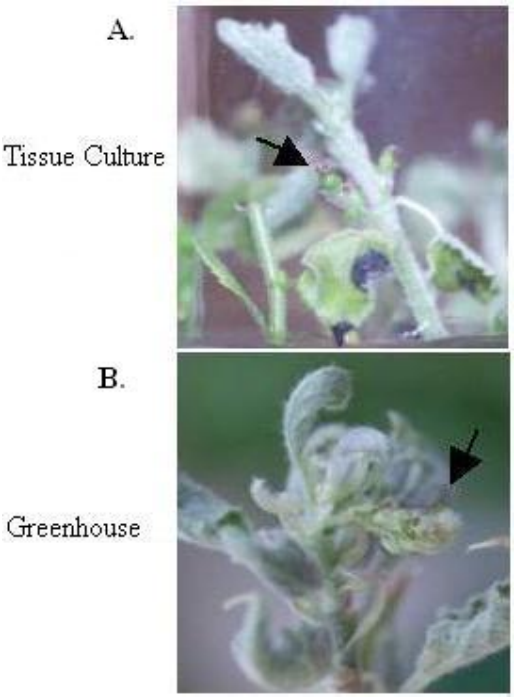

Figure 8. Flower buds on PtFD1 overexpression plantlets. The plantlets are from (A) tissue culture in continuous light and (B) a one month old green house grown plant in LD treatment.

IV. RT-PCR of PtFD1 in Transgenic Poplars

Two RNAi lines (RNAi 1-2, RNAi 1-6), two overexpression lines (OE 2-1, OE 2-3) and control 717-1B4 were transferred from tissue culture to growth chambers and treated with SD after LD. Plants were treated for 8weeks in SD at 18 ºC and an additional 4weeks in SD with 10 ºC in the day and 4 ºC at night.

A. PtFD1 Expression in Shoot Tips and Apical Buds

Shoot tips or apical buds were collected from LD and after 3, 6, 12 weeks of SD treatment. RNA was extracted and used for RT-PCR with *PtFD1* gene specific primers. As shown in Figure 9, PtFD1 mRNA was not detected in control plants in LD and after 3 weeks of SD treatment, but was detected after 6 weeks of SD treatment and with continued SD (8 weeks of SD followed by 4 weeks of SD+LT) treatment, PtFD1 mRNA abundance decrease to undetectable levels. This expression is consistent with that previously observed (Gnewikow, 2001). For the two RNAi lines, PtFD1 mRNA levels were similar to that observed in control plants. PtFD1 mRNA was detected in all treatments for the PtFD1 overexpression lines.

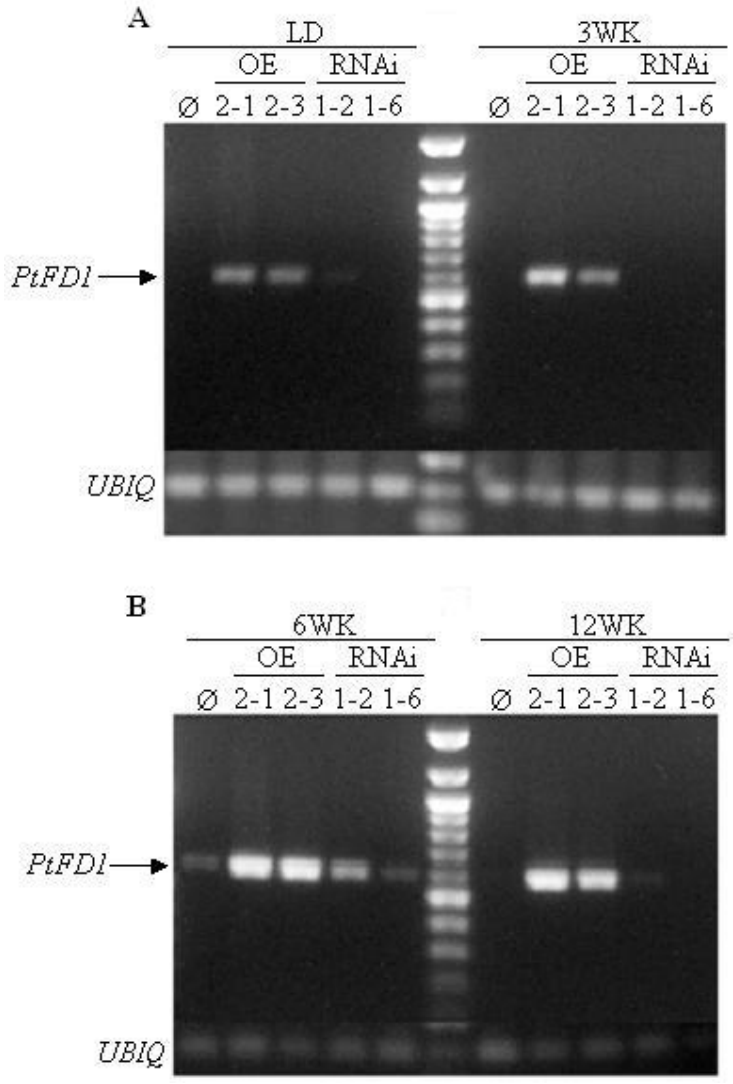

Figure 9. RT-PCR of *PtFD1*. RNA was extracted from shoot tips or apical buds of control 717-1B4 (Ø), overexpression (OE) and RNAi plants under LD or after 3 weeks (A), 6 and 12 weeks (B) of SD treatment. Primers PTBF1-ATG and PTBF1-Rev51 were used for PCR amplification. RT-PCR of UBIQ is shown as an equal loading control.

A second experiment was performed using three RNAi lines (RNAi 1-2, RNAi 1-3, RNAi 1-4), two overexpression lines (OE 2-2, OE T3) and control 717-1B4. Shoot tips and apical buds were collected after 3, 6 and 8 weeks of SD treatment for the 3 RNAi lines and control plants. For both control and the 2 overexpression lines, shoot tips were collected after 3, 6, 8 and 12 weeks of SD treatment. RNA samples from the previous experiment were combined with this experiment and RT-PCR using *PtFD1* primers was performed (Figure 10).

In the control 717-1B4 plants, PtFD1 was not expressed in LD. The expression of PtFD1 peaks after 8 weeks of SD treatment. After 12 weeks of SD treatment, the expression of PtFD1 diminishes.

In the overexpression plants, PtFD1 was expressed in a fairly high level ever since in LD. The abundance of PtFD1 mRNA after 12 weeks of SD treatment was not reduced compared to the PtFD1 mRNA level after 6 weeks of SD treatment.

In the RNAi plants, the expression of PtFD1 was higher in two of the lines, RNAi 1-2 and RNAi 1-6 compared to the control 717-1B4 plants. Reduced PtFD1 expression was found in two other lines, RNAi 1-3 and RNAi 1-4 after 8 weeks SD treatment.

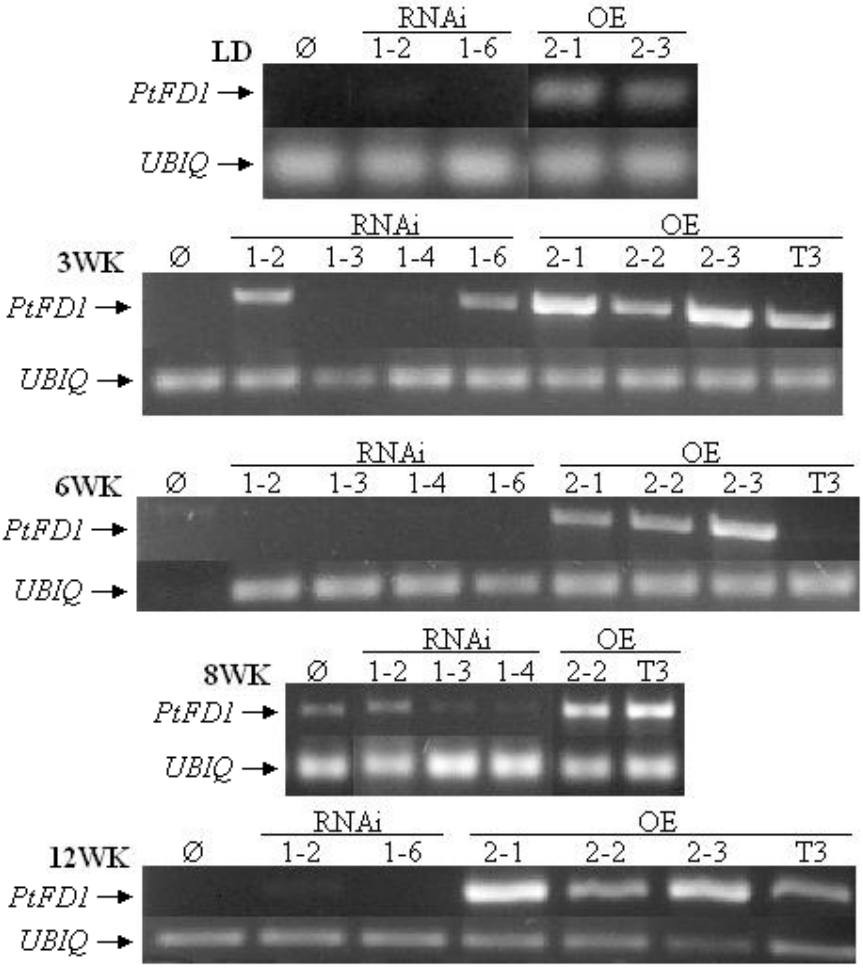

Figure 10. RT-PCR of *PtFD1*. RNA was extracted from shoot tips or apical buds of control 717-1B4 (Ø), overexpression (OE) and RNAi plants under LD or after 3, 6, 8 and 12 weeks of SD treatment. Primers PTBF1-ATG and PTBF1-Rev51 were used for PCR amplification. RT-PCR of UBIQ is shown as an equal loading control.

B. PtFD1 Expression in other Tissues

RT-PCR was also performed on RNA extracted from the leaves of control and 2 overexpression lines (OE 2-1, OE2-3) growing in LD or after 8 weeks of SD treatment. In addition, RNA from flower that developed on the overexpression lines was also analyzed. Primers ptbf1+ATG and ptbf1-Rev51 were used for PT-PCR of the RNA samples. Figure 11 shows that PtFD1 mRNA was not detected in the leaves of control plants in LD or after 8 weeks of SD treatment. This is consistent with earlier reports of PtFD1 mRNA expression (Gnewikow, 2001). PtFD1 mRNA was detected in the leaves of both overexpression lines in LD and after 8 weeks of SD treatment. Besides, PtFD1 mRNA was also detected in the flower buds of overexpression plants.

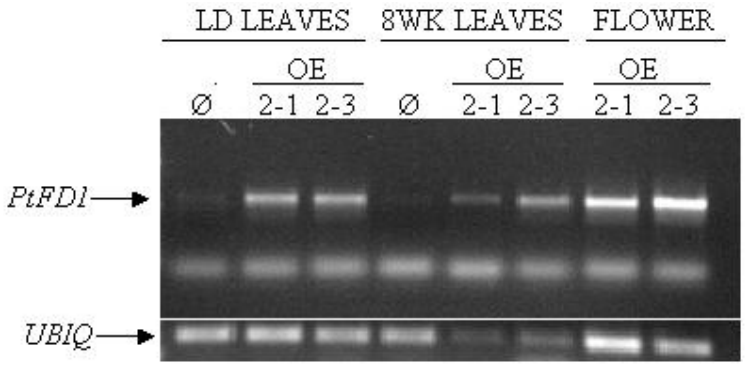

Figure 11. RT-PCR of *PtFD1*. RNA was either extracted from leaves of control 717-1B4 (Ø), overexpression (OE) and RNAi plants under LD or after 8 weeks of SD treatment or from flower buds of overexpression seedlings in tissue culture. Primers PTBF1-ATG and PTBF1-Rev51 were used for PCR amplification. RT-PCR of UBIQ is shown as an equal loading control.

V. Histological Analysis of Transgenic Shoot Tips or Apical Buds

For RNAi plants, shoot tips and apical buds were collected at 5-day intervals after being transferred to SD for 25 days. For overexpression plants, shoot tips were collected after 3, 6, 8 and 12 weeks of SD treatment. The tissues were fixed, dehydrated, infiltrated, embedded and sectioned. Tissue sections were stained with Safranin O and Fast Green FCF. Safranin O stains lignin, cutin, suberin, chitin, chromosomes and nucleoli while Fast Green FCF was used as a counterstain to reveal tissues that were not labeled by Safranin O (Ruzin, 1999).

SD induced apical bud formation was shown to be accelerated in PtFD1 RNAi plants (Figure 12A). After 15 days of SD treatment, obvious bud scale formation was observed in PtFD1 RNAi plants compared to control plants. Buds collected from PtFD1 RNAi plants after 56 days of SD treatment were more compact and smaller compared to the control plants.

Noteworthy differences occur between the PtFD1 overexpression and control plants. Bud scales fail to develop after 8 weeks of SD treatment in PtFD1 overexpression plants (Figure 12B).

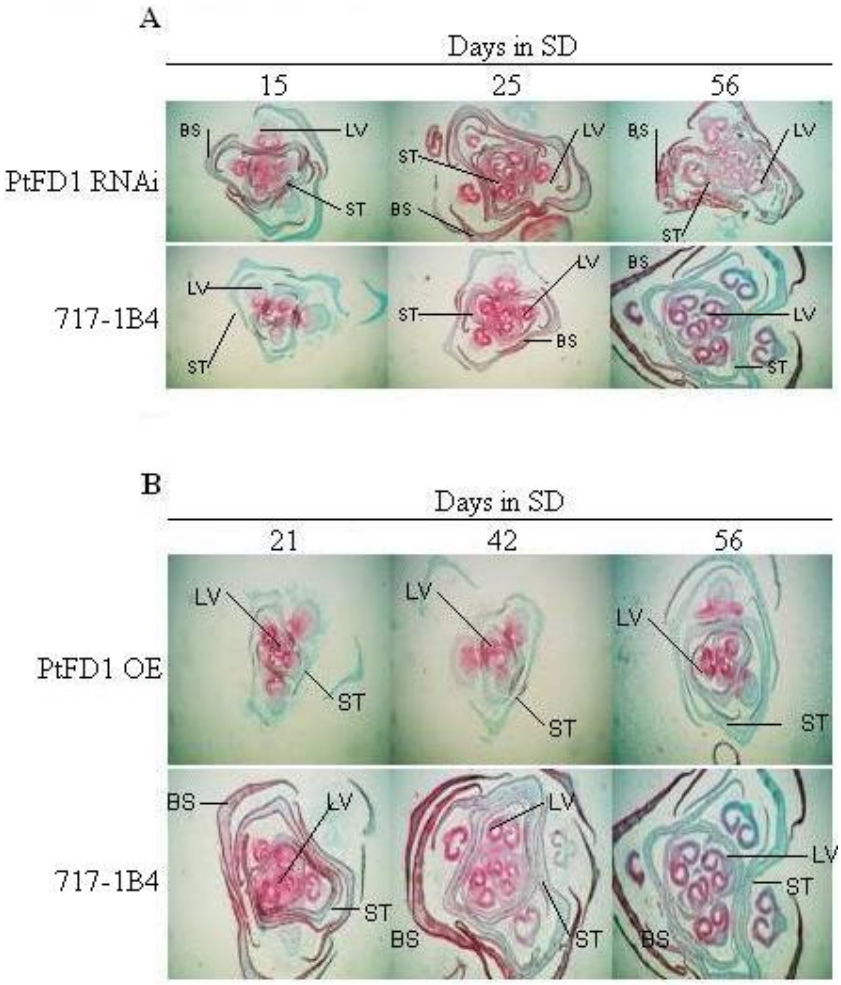

Figure 12.Anatomy of control and transgenic shoot tips or apical buds. (LV=leaves, ST=stipules, BS=bud scales)

Discussion

I. Origin of the Study of *PtFD1*

PtFD1 was first isolated in our lab from the hybrid poplar clone 545-4183 (*Populus deltoids X Populus trichocarpa*) during attempts to isolate *ABI3* from apical buds (Gnewikow, 2001). Instead of amplifying *ABI3* cDNA, a putative bZIP transcription factor was obtained. It was shown to be related to plant bZIP proteins and was named PTBF1 (Poplar Terminal Bud Factor-1) (Gnewikow, 2001). Because of the potential role of signaling and gene activation, PTBF1 was selected for further study (Schwechheimer et al., 1998).

Sequence analysis shows that this bZIP protein is related closely to two members of the group A *Arabidopsis* bZIP transcription factors, AtbZIP14 and AtbZIP27 (Figure 13). Further alignments revealed PTBF1 a homologue of AtbZIP14 (Figure 14), which was recently identified as FD in *Arabidopsis* (Abe et al., 2005). So PTBF1 was re-named as PtFD1 for consistency.

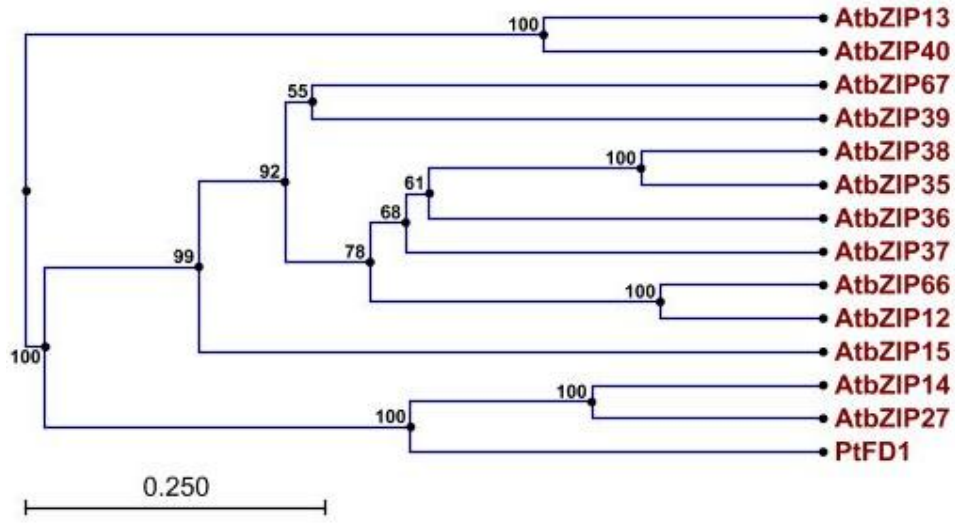

Figure 13. Sequence analysis of PtFD1 and the Group A *Arabidopsis* bZIP transcription factors. The analysis was accomplished by using GeneDoc 2.6.02 from GeneDoc HomePage (http://www.psc.edu/biomed/genedoc/)

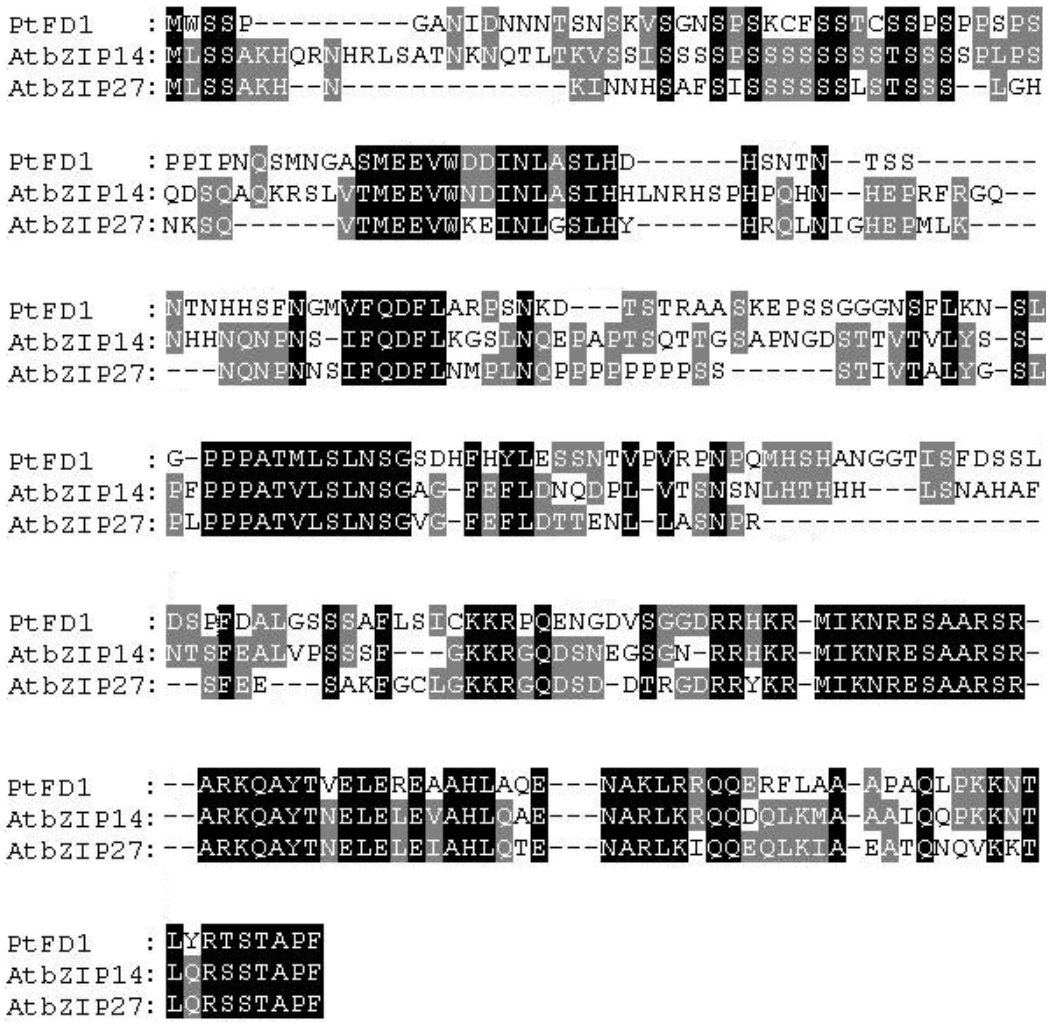

Figure 14. ClustalW alignment of PtFD1, AtbZIP14 and AtbZIP27 sequences. The alignment was accomplished by using GeneDoc 2.6.02 from GeneDoc HomePage (http://www.psc.edu/biomed/genedoc/). Residues on black, dark gray, and light gray backgrounds indicate 100%, 80%, and 60% amino acid similarity, respectively.

II. Sequence Analysis of PtFD1

PtFD1 is composed of 272 amino acids with a predicted molecular weight of 29.5 kDa (Figure 15). It contains a basic domain common to bZIP transcription factors. Located in the N-terminus of PtFD1 is a proline rich region from amino acids 35-45, which may function as a transactivation domain.

Among bZIP proteins, the most conserved sequence is the DNA-binding basic region. It contains several residues that almost locate at the same relative positions among different bZIP proteins. The conserved residues form a consensus sequence of (-18) N XXX A A X X(C/S) R (-10) in which the negative number is labeled according to the +1 leucine (Hurst, 1996). Two amino acids are conserved in most bZIP proteins, the asparagine (N) at position 211 (of the PtFD1) and the arginine (R) at position 219. Two other amino acids, serine (S) at position 214 (of the PtFD1) and alanine (A) at position 215 are also found in most most of the known bZIP proteins.

The leucine zipper domain of PtFD1 contains three heptad leucine repeats with the leucines locate at position 229, 236 and 243. Three heptad is thought to be the minimum number of heptad leucine repeats that can form a zipper although the heptad repeats can be as many as seven (Landschultz et al., 1988).

bZIP proteins can form both homodimers and heterodimers. Forming dimers with different bZIP proteins may change protein conformation and DNA binding affinity, which would allow a combinatorial level of signaling (Alberts et al., 1994).

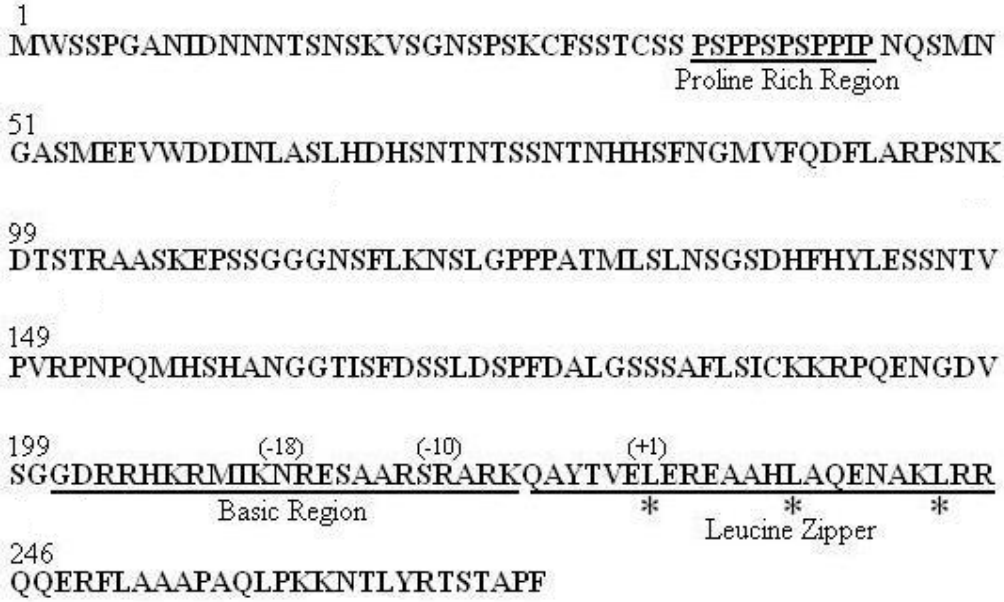

Figure 15. Amino acid sequence of PtFD1 bZIP protein. Regions showing significant homology to conserved motifs are underlined and labeled. The asterisks below the leucine residues indicate the presence of leucine repeats every 7 residues.

III. Characterization of PtFD1

A. Involvement of *PtFD1* in Bud Formation and Development

The expression of PtFD1 is induced by SD photoperiod and is coincident with apical bud formation. Prior research showed that PtFD1 mRNA levels are at their highest in apical buds when plants were exposed to SD and at their lowest when plants were exposed to SD-NB (Gnewikow, 2001). This expression pattern is similar in both apical and auxillary buds. Similar results of photoperiod regulation were observed for poplar genotypes of both 717-1B4 and 545-4183. PtFD1 expression coincides with bud formation and maturation, suggesting an involvement of PtFD1 in these processes. To test the role of *PtFD1* in bud development, PtFD1 expression was altered in transgenic poplars and any alterations in bud formation and maturation were observed.

Bud formation is characterized by the presence of bud scales. In poplar, bud scales develop from leaf-subtending stipules that enlarge to enclose the leaf primordia (Goffinet and Larson, 1981 & 1982). Bud formation usually occurs after about 6 weeks of SD treatment, which is also when PtFD1 expression is at its highest level, after which PtFD1 mRNA levels decline. It has been proposed that *PtFD1* may somehow influence bud scale growth (Gnewikow, 2001). In transgenic poplars that overexpress PtFD1, apical bud development was inhibited even after a considerable length of SD treatment. Anatomic studies showed overexpression of PtFD1 impinged on the formation of bud scales (Figure 12). Besides, poplar

homologue of *ABI3*, *PtABI3* also impinges on apical bud formation. Overexpession of *PtABI3* promoted the growth and differentiation of embryonic leaves while suppressing the development of bud scales (Rohde et al., 2002). This is similar to the effect of PtFD1 overexpression. This raises the possibility that *PtABI3* and *PtFD1* may interact in this process.

For the RNAi transgenic plants, bud formation and development was similar to that of the control plants. According to the RT-PCR results, the PtFD1 levels in the RNAi lines (RNAi1-2 and RNAi 1-6) were not significantly reduced compared to the controls. Among available RNAi transgenic lines, RNAi 1-3 and RNAi 1-4 showed reduced PtFD1 expression than RNAi 1-2 and RNAi 1-6. Regenerating plants with RNAi chimeric genes was not efficient compared to PtFD1 overexpressing construct, which could indicate the *PtFD1* is required for shoot regeneration. Obtaining strong PtFD1 RNAi lines will probably require an inducible promoter. This may be why the RNAi transgenic lines in this experiment did not show significant differences to the wild type plants in bud formation.

B. Involvement of *PtFD1* in Flowering

In the annual plant *Arabidopsis*, flowering is regulated through four major genetic pathways that mediate responses to either environmental or endogenous signals (reviewed by Parcy, 2005). These genetic pathways converge on the activation of a set of floral pathway integrators including *LEAFY* (*LFY*), *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF CO OVEREXPRESSION* (*SOC1*). These integrators convert multiple input signals to regulate floral meristem

identity (FMI) genes, which in turn initiate the transition from vegetative to reproductive development at the shoot apical meristem (Simpson and Dean, 2002). In the photoperiodic pathway, a circadian-clock mediator, *CONSTANS* (*CO*) plays a key role. *CO* encodes a transcriptional regulator that promotes flowering in LD through direct upregulation of *FT*, a conserved promoter of flowering (Kardailsky et al., 1999; Kobayashi et al., 1999; Onouchi et al., 2000; Samach et al., 2000). FD, a bZIP protein that is expressed in the shoot apex is required for FT activity. FT mRNA moves from leaf phloem to the shoot apical meristem (SAM) where it forms a transcriptional complex with FD to activate FMI genes such as *APETALA1* (*AP1*) (Huang et al., 2005; Abe et al., 2005; Wigge et al., 2005).

In contrast to the short life cycles of annual plants, woody plants such as poplars have life spans of hundreds of years and a long juvenile phase of about 7 to 10 years (Braatne et al., 1996). During the juvenile phase, the plants lack reproductive capacity and must reach maturity to be able to form flower buds (Kozlowski and Pallardy, 1997; reviewed by Poethig, 1990). Flower buds were observed in all PtFD1 overexpression transgenic lines indicating that *PtFD1* has a role in flowering and potentially the transition from juvenility to maturity (Figure 8.). Recently, the role of two poplar FT family members, PtFT1 and PtFT2 in poplar flowering was reported (Böhlenius et al., 2006; Hsu et al., 2006).

FT2 transcripts were rare in juvenile trees while levels were abundant during reproductive growth in mature trees in long days. Overexpression of FT2 was found to induce flowering in juvenile poplars within one year (Hsu et al., 2006). Poplars

transformed with *35S::PtFT1* were found to generate flower-like structures directly from the *Agrobacterium*-infected explants. Furthermore, trees overexpressing PtFT1 did not cease growth when transferred to SD treatment (Böhlenius et al., 2006). These phenotypes are very similar to what observed for the PtFD1 overexpression poplars. Unlike the PtFD1 RNAi plants, PtFT1 RNAi plants were reported to be much more sensitive to SD treatment (Böhlenius et al., 2006). Thus it appears that the CO/FT regulon controls both the flower timing and seasonal growth cessation and bud set by regulating photoperiod output signal (Böhlenius et al., 2006). Since similar phenotypes between PtFD1 and PtFT1 plants were observed, it seems that both flowering and bud development share similar regulatory features.

It is likely that flowering in poplar was regulated through similar pathways as those in *Arabidopsis*. In *Arabidopsis*, FD mRNA appears to be transported from leaves to shoot apex where its regulatory role occurs. In poplars, PtFD1 is also expressed in the shoot apex. High levels of FD1 transcripts occur in both leaves and shoot apices of PtFD1 overexpression plants. Since flowering occurs in these plants suggests that FD alone can induce flowering.

C. *PtFD1* and ABA Pathways

Arabidopsis Group-A bZIP proteins are thought to play an important role in ABA signal transduction in both seeds and vegetative tissues (Jakoby et al., 2002). In poplar, exogenous ABA caused a small increase in PtFD1 expression in shoot tips (Gnewikow, 2001).

The leaves of PtFD1 overexpression lines were smaller than the control plants and the margins of the leaves showed a curling shape, which usually occur when plants were in low humidity. This phenotype may represent an altered response to ABA.

The similar phenotype was observed in *aba1* mutants in *Arabidopsis*. The ABA deficient mutants showed a semi-dwarf phenotype, leaves of reduced size and curly leaf margins, which were thought to be related to impaired stomatal closure (Barrero et al., 2005). This similarity suggests that *PtFD1* may be involved in ABA biosynthesis or act as a component of the ABA signal transduction pathway. The phenotypes observed in PtFD1 overexpression transgenic plants may be caused by defects in stomata function. The malfunctions lead to the failure of stomata closure so that water loss through transpiration is greatly increased. The plants then develop smaller leaves.

D. Possible Roles of *PtFD1*

From the morphological and anatomic results, it is clear that *PtFD1* plays a role in suppression of the formation of apical buds. This role appears to be related to bud scale formation. *PtFD1* acts as a negative inhibitor in the processes of bud formation and maturation. Excessive amount of PtFD1 impairs the plant's responses to SD treatment including growth cessation and bud formation. The signal transduction pathway of growth cessation and bud formation remains unknown. Poplars overexpressing PtFD1, PtFT1, PtABI3 or PHYA all displayed defects in growth cessation and bud formation indicating a role for these genes in

this pathway. All these factors may be involved in bud formation through one or multiple pathways.

PtFD1 is also a positive regulator of flowering and overexpression of PtFD1 causes early flowering in juvenile plants. The mechanism is likely to be similar with that of *Arabidopsis*. Growth cessation, bud set and flowering may share some common regulators, including the circadian-clock mediator (*CO*), bZIP transcription factor (*FD*), "florigen" (*FT*), phytochromes (*PHYA*).

In *Arabidopsis*, *FT* act as an activator of flowering while its homologue *TERMINAL FLOWER1* (*TFL1*) represses flowering (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Kardailsky et al., 1999; Searle and Coupland, 2004; Wigge et al., 2005). The antagonistic functions can be converteds by swapping a single amino acid (Hanzawa et al., 2005). The bZIP transcription factor FD can interact with both FT and TFL1 (Abe et al., 2005; Wigge et al., 2005). Accumulation of CO in LD induces the transcription of FT, which converts FD into a strong activator by forming a complex with FD and bind to the promoter of the floral identity gene *AP1* (Valverde et al., 2004; Wigge et al., 2005; Ahn et al., 2006). TFL1 competes with FT to react with FD, converts FD into a strong repressor and delays the transition from the vegetative growth to flowering (Hanzawa et al., 2005; Ahn et al., 2006). Thus by interacting with different components, FD can change between activator and repressor. In poplar, it is very likely that the balance between *FT* and *TFL1* regulates the responses to floral inductive signals (Kardailsky et al., 1999). During the long juvenile period, a chromatin structure-based repression of

PtFT1 prevent the plants from entering reproductive phase too early (Böhlenius et al., 2006).

IV. Suggestions for Future Study

*PtFD*1 has been proven to play roles in bud formation, bud maturation, vegetative growth and flowering. It may also take part in ABA and signal transduction pathway. How it functions in all of the events remains elusive.

Since *PtFD1* is thought to be involved in several genetic regulatory pathways, its direct targets are very likely to be components of the transduction pathways. With the available transgenic lines, suppression subtractive hybridization (SSH) can be used to isolate differentially expressed transcripts. DNA microarray can be used for gene expression profiling. Isolated genes are candidate components in the regulation of the physiological events.

Evidence shows that *PtFD1* functions by interacting with other factors of the regulatory pathways, forming either a modulating complex or a dimer. Identifying these components is another important work that needs to be done. Yeast two hybrid system can be applied to identify factors that interact with PtFD1, such as PtFT. This may help to elucidate the networks of the pathways.

Appendices

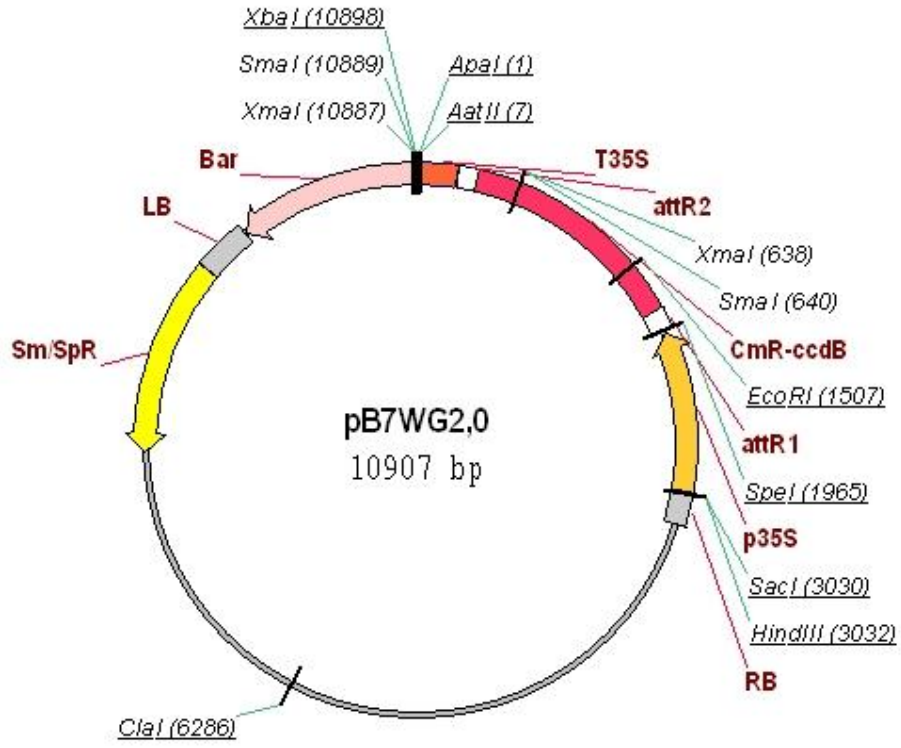

Figure A. 1. The map of over-expression T-DNA binary vector, pB7WG2. (Karimi, M., Inze, D., Depicker, A., Gateway vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci. 2002 May;7(5): 193-195.)
Source of the map:
http://www.psb.ugent.be/gateway/index.php?NAME=pB7WG2&_app=vector&_act=construct_show&

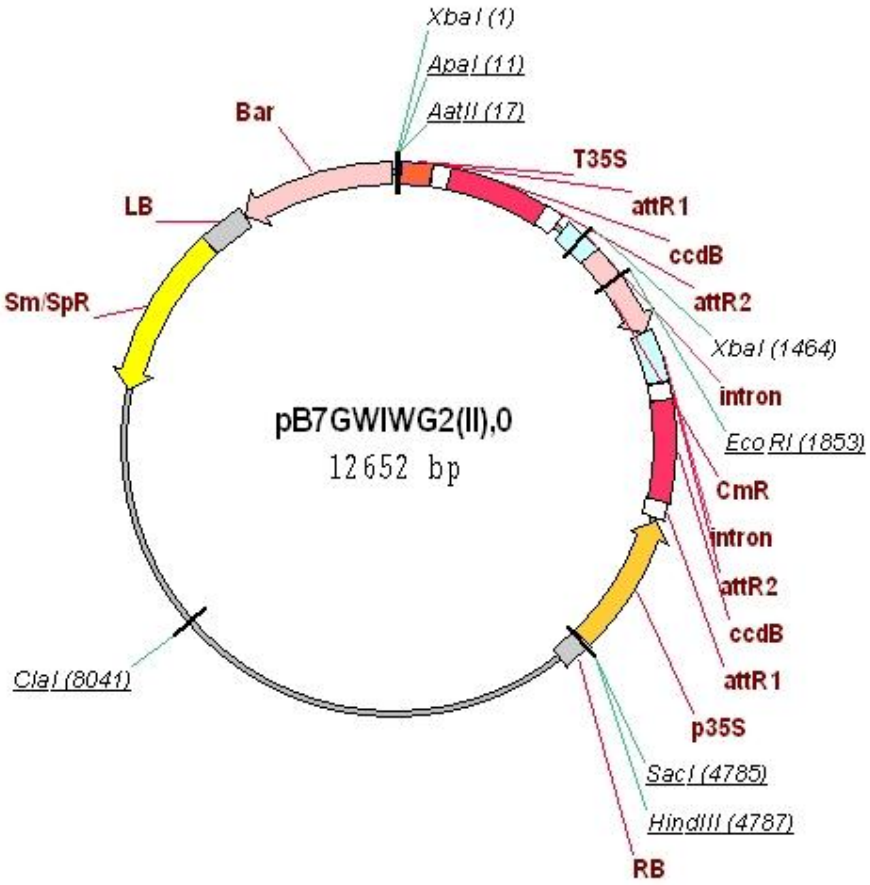

Figure A. 2. The map of RNAi T-DNA binary vector, pB7GWIWG2(II). (Karimi, M., Inze, D., Depicker, A., Gateway vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci. 2002 May;7(5): 193-195.)

Source of the map:

http://www.psb.ugent.be/gateway/index.php?NAME=pB7GWIWG2(II)&_app=vector&_act=construct_show&

References

Ahn, J. H., Miller, D., Winter, V. J., Banfield, M. J., Lee, J. H., Yoo, S. Y., Henz, S. R., Brady, R. L. and Weigel, D. 2006. A divergent external loop antagonistic activity on floral regulators FT and TFL1. *EMBO J.* 25:605–614.

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

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. 1994. Molecular Biology of the Cell. 3rd Ed. Garland: New York, NY.

Alvarez, J., Guli, C. L., Yu, X-H. and Smyth, D. R. 1992. *Terminal flower*: a gene affecting inflorescence development in *Arabidopsis thaliana*. Plant J. 2: 103-116.

Amling, H. J. and Amling, K. A. 1980. Onset, intensity and dissipation of rest in several pecan cultivars. J. Amer. Soc. Hort. Sci. 105: 536-540.

Barney, C. W. 1951. Effects of soil temperature and light intensity on root growth of loblolly pine seedlings. Plant Physiol. 26: 146-163.

Barrero, J. M., Piqueras, P., González-Guzmán, M., Serrano, R., Rodríguez, P. L., Ponce, M. R. and Micol, J. L. 2005. A mutational analysis of the ABA1 gene of *Arabidopsis thaliana* highlights the involvement of ABA in vegetative development. J. Exp. Botany 56(418):2071-2083

Bensmihen, S., Rippa, S., Lambert, G., Jublot, D., Pautot, V., Granier, F., Giraudat, J. and Parcy, F. 2002. The homologous ABI5 and EEL transcription factors function antagonistically to fine-tune gene expression during late embryogenesis. Plant Cell. 14(6): 1391-1403.

Bewley, J. D. 1997. Seed germination and dormancy. Plant Cell 9: 1055-1066.

Böhlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A. M., Jansson, S., Strauss, S. H. and Nilsson, O. 2006. *CO/FT* regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science*. 312: 1040-1043.

Bonicel, A., Haddad, G. and Gagnaire, J. 1987. Seasonal variation of starch and major soluble sugars in the different organs of young poplars. Plant Physiol. Biochem. 25: 451-459.

Braatne, J. H., Rood, S. B. and Heilman, P. E. 1996. Life history, ecology, and conservation of riparian cottonwoods in North America. In Biology of *Populus*, R.F. Stettler, H.D. Bradshaw, Jr., P.E. Heilman, and T.M. Hincley, eds (Ottawa, Canada: NRC Research Press), pp. 57-85.

Brocard, I. M., Lynch, T. J. and Finkelstein, R. R. 2002. Regulation and Role of the *Arabidopsis* Abscisic Acid-Insensitive 5 Gene in Abscisic Acid, Sugar, and Stress Response. Plant Physiol. 129(4): 1533-1543.

Carles, C., Bies-Etheve, N., Aspart, L., Leon-Kloosterziel, K. M., Koornneef, M., Echeverria, M. and Delseny, M. 2002. Regulation of *Arabidopsis thaliana* Em genes: role of ABI5. Plant J. 30(3): 373-383.

Critchfield, W. B. 1960. Leaf dimorphism in *Populus trichocarpa*. Am. J. Bot. 47. 699-711.

Coleman, G. D., Chen, T. H. H. and Fuchigami, L. H. 1992. Complementary DNA cloning of poplar bark storage protein and control of its expression by photoperiod. Plant Physiol. 98: 687-693.

Coleman, G. D. and Chen, T. H. H. 1996. Photoperiod-Associated Gene Expression during Dormancy in Woody Perennials. Plant Dormancy. G. Lang (Ed.) 301-307.

Dennis, F. G. J. 1996. A physiological comparison of seed and bud dormancy. *In* Lang G.A. ed. Plant Dormancy: Physiology, Biochemistry and Molecular Biology. Ed 1. CAB International. London. pp 47-56.

Dickmann, D. I., and Keathley, D. E. 1996. Linking physiology, molecular genetics, and the *Populus* ideotype.In Biology of *Populus* and its implications for management and conservation. Edited by R. F. Stettler, H. D.Bradshaw Jr., P. E. Heilman and T. M. Hinkley. NRC Research Press, National Research Council of Canada,Ottawa, ON. pp. 491-514.

Downs, R. J. and Borthwick, H. A. 1956. Effects of photoperiod on growth of trees. Bot. Gaz. 117: 310-326.

El-Antably, H. M. M., Wareing, P. F. and Hillman, J. 1967. Some physiological responses to D, L abscisin (dormin). Planta. 73: 74-90.

Faust, M., Liu, D., Line, M. J. and Stutte, G. W. 1995. Conversion of bound to free water in endodormant buds of apple is an incremental process. Acta. Hort. 395: 113-118.

Faust, M., Erez, A., Rowland, L. J., Wang, S. Y. and Norman, H.A. 1997. Bud dormancy in perennial fruit trees: physiological basis for dormancy induction, maintainance, and release. HortScience. 32 (4): 623-629.

Finkelstein, R. R. 1994. Mutations at two new *Arabidopsis* ABA response loci are similar to the abi3 mutations. The Plant Journal 5: 765-771.

Finkelstein, R. R. and Lynch, T. J. 2000. The *Arabidopsis* abscisic acid response gene ABI5 encodes a basic leucine zipper transcriptional factor. Plant Cell 12: 599-609.

Finkelstein, R. R., Gampala, S. S. L. and Rock, C. D. 2002. Abscisic acid signaling in seeds and seedlings. Plant Cell. Supplement: S15-S45.

Foster, R., Izawa, T. and Chua, N. H. 1994. Plant bZIP proteins gather at ACGT elements. FASEB. 8: 192-200.

Giraudat, J., Hauge, B., Valon, C., Smalle, J., Parcy, F., and Goodman, H. 1992. Isolation of the *Arabidopsis ABI3* gene by positional cloning. Plant Cell. 4: 1251-1261.

Gnewikow, H. L. 2001. Isolation and characterization of a basic leucine zipper from poplar that is involved in terminal bud formation. Master Thesis.

Goffinet, M. C. and Larson, P. R. 1981. Structural changes in *Populus deltoides* terminal buds and in the vascular transition zone of the stems during dormancy induction. Am. J. Bot. 68: 118–129.

Goffinet, M. C. and Larson, P. R. 1982. Lamina abortion in terminal bud-scale leaves of *Populus deltoides* during dormancy induction. Bot. Gaz. 143: 331–340.

Hansen, E., Olsen, J. E. and Junttila, O. 1999. Gibberellins and subapical cell divisions in relation to bud set and bud break in *Salix pentandra*. J Plant Growth Regul. 18: 167-170.

Hanzawa, Y., Money, T. and Bradley, D. 2005. A single amino acid converts a repressor to an activator of flowering. Proc. Natl. Acad. Sci. U. S. A. 102: 7748-7753.

Heide, O. M. 1974. Growth and dormancy in Norway spruce ecotypes (*Picea abies*). I. Interaction of photoperiod and temperature. Physiol. Plant. 30: 1-12.

Heide, O. M. and Prestrud, A. K. 2005. Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. Tree Physiol. 25(1): 109-114.

Howe, G., Gardner, G., Hackett, W. and Furnier, G. 1996. Phytochrome control of short-day-induced bud set in black cottonwood. Physiol. Plant. 97: 95-103.

Hsu, C-Y., Liu, Y., Luthe, D. S. and Yuceer, C. 2006. Poplar *FT2* shortens the juvenile phase and promotes seasonal flowering. Plant Cell. Online Preview.

Huang, T., Böhlenius, H., Eriksson, S., Parcy, F. and Nilsson, O. 2005. The mRNA of the *Arabidopsis* gene *FT* moves from leaf to shoot apex and induces flowering. *Science.* 309: 1694-1696.

Hummel, R. C., Ascher, P. D. and Pellett, H. M. 1982. Inheritance of the photoperiodically induced cold acclimation response in *Cornus sericea*. Theor. Appl. Genet. 62: 385-394.

Iwasaki, K. and Weaver, R. J. 1977. Effects of chilling, calcium cyanamide, and bud scale removal on bud break, rooting, and inhibitor content of buds of 'Zinfandel' grape *Vitus vinifera* L. J. J. Amer. Soc. Hort. Sci. 102: 584-587.

Izawa, T., Foster, R., Chua, N. H. 1993. Plant bZIP protein DNA binding specificity. J. Mol. Biol. 230(4):1131-44.

Jackson, S. and Thomas, B. 1997. Photoreceptors and signals in the photoperiodic control of development. Plant Cell Environ. 20: 790-795.

Jakoby, M., Weisshaar, B., Dröge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T. & Parcy, F. 2002. bZIP transcription factors in *Arabidopsis*. Trends Plant Sci. 7: 106-111.

Jian, L-C, Li, P. H., Sun, L-H and Chen, T. H. H. 1997. Alterations in ultrastructure and subcellular localization of Ca^{2+} in poplar apical bud cells during the induction of dormancy. J. Expt. Bot. 48: 1195-1207.

Junttila, O. 1976. Apical growth cessation and shoot tip abscission in Salix. Physiol. Plant. 38: 278-286.

Junttila, O. 1980. Effect of photoperiod and temperature on apical growth cessation in two ecotypes of Salix and Betula. Physiol Plant. 48: 347-352.

Junttila, O. 1982. The cessation of apical growth in latitudinal eco-types and ecotype crosses of *Salix pentandra* L. J. Exp. Bot. 33: 1021–1029.

Junttila, O. and Kaurin, A. 1990. Environmental control of cold acclimation in *Salix pentandra*. Scand. J. of For. Res. 5:195-204.

Junttila, O., Jensen, E. and Ernstsen, A. 1991. Effects of prohexadione (BX-112) and gibberellins on shoot growth in seedlings of *Salix pentandra*. Physiol Plant. 83: 17-21.

Junttila, O., Nilsen, J. and Igeland, B. 2003. Effect of temperature on the induction of bud dormancy in various ecotypes of *Betula pubescens* and *B. pendula*. Scand. J. For. Res. 18:208-217.

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

Karimi, M., Inzé, D. and Depicker A. 2002. GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci. 7: 193-195.

Kerstetter, R. A. and Hake, S. 1997. Shoot meristem formation in vegetative development. Plant Cell. 9: 1001-1010.

Kim, S. Y., Ma, J., Perret, P., Li, Z. and Thomas, T. L. 2002 *Arabidopsis* ABI5 subfamily members have distinct DNA-binding and transcriptional activities. Plant Physiol. 130: 688-697.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki T. 1999. A pair of related genes with antagonistic roles in mediating flowering signals. *Science*. 286: 1960-1962.

Koornneef, M., Reuling, G. and Karssen, C. M. 1984. The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Physiol. Plant. 61:377-383.

Kozlowski, T. T. and Pallardy, S. G. 1997. Physiology of Woody Plants. 2nd Ed. Academic Press, Inc., UK.

Landschulz, W. H., Johnson, P. F. and McKnight, S. L. 1988. The Leucine Zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science*. 240: 1759-1764.

Lang, G. A. 1987. Dormancy: a new universal terminology. HortScience. 22:817-820.

Lavender, D. P., Sweet, G. B., Zaerr, J. B. and Hermann, R. K. 1973. Spring shoot growth in Douglas-fir may be initiated by gibberellins exported from the roots. *Science.* 182: 838-839.

Le Bris, M., Michaux-Ferrière, N., Jacob, Y., Poupet, A., Barthe, P., Guigonis, J-M. and Le Page-Degivry, M-T. 1999. Regulation of bud dormancy by manipulation of ABA in isolated buds of *Rosa hybrida* cultured *in vitro*. Austr. J. Plant Physiol. 26: 273-281.

Leple, J. C., Brasileiro, A. C. M., Michel, M. F., Delmotte, F. and Jouanin, L. 1992. Transgenic poplars: expression of chimeric genes using four different constructs. Plant Cell Reports 11: 137-141.

Leung, J. and Giraudat, J. 1998. Abscisic acid signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 199-222.

Li, P. and Adams, W. T. 1993. Genetic control of bud phenology in pole-size trees and seedlings of coastal *Douglas-fir*. Can. J. For. Res. 23: 1043–1051.

Menkens, A. E., Schindler, U. and Cashmore, A. R. 1995. The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. Trends Biochem. Sci. 20: 506-510.

Meshi, T. and Iwabuchi, M. 1995. Plant transcription factors. Plant Cell Physiol. 36(8): 1405-1420.

Meshi, T., Moda, I., Minami, M., Okanami, M. and Iwabuchi, M. 1998. Conserved Ser residues in the basic region of the bZIP-type transcription factor HBF-1a(17): importance in DNA binding and possible targets for phosphorylation. Plant Mol. Biol. 36: 125-136.

Nambara, E., Hayama, R., Tsuchiya, Y., Nishimura, M., Kawaide, H., Kamiya, Y. and Naito, S. 2000. The role of ABI3 and FUS3 loci in *Arabidopsis thaliana* on phase transition from late embryo development to germination. Dev. Biol. 220:412–423.

Olsen, J. E., Junttila, O., Moritz, T. 1995. A localized decrease of GA1 in shoot tips of *Salix pentandra* seedlings precedes cessation of shoot elongation under short photoperiod. Physiol Plant. 95: 627-632.

Olsen, J. E., Junttila, O., Nilsen, J., Eriksson, M. E., Martinussen, I., Olsson, O., Sandberg, G. and Moritz, T. 1997. Ectopic expression of oat phytochrome A in hybrid aspen changes critical daylength for growth and prevents cold acclimatization. Plant J. 12: 1339-1350.

Olsen, J. E., Jensen, J. B., Mølmann, J., Ernstsen, A. and Junttila, O. 2004. Photoperiodic regulation of apical growth cessation in northern tree species: the role of phytochrome and gibberellin. Journal of Crop Improvement. 10: 77-112.

Onouchi, H., Igeño, M. I., Périlleux, C., Graves, K. and Coupland, G. 2000. Mutagenesis of plants overexpressing *CONSTANS* demonstrates novel interactions among *Arabidopsis* flowering-time genes. Plant Cell. 12: 885-900.

Örvar, B. L., Sangwan, V., Omann, F. and Dhindsa, R. S. 2000. Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. Plant J. 23: 785-794.

Oyama, T., Shimura, Y. and Okada, K. 1997. The *Arabidopsis* HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyls. Genes Devel. 11: 2983-2995.

Parcy, F., Valon, C., Raynal, M., Gaubier, P., Delseny, M. and Giraudat, J. 1994. Regulation of gene expression programs during *Arabidopsis* seed development : respective roles of the ABI3 locus and of endogenous abscisic acid levels. Plant Cell 6: 1567-1582.

Parcy, F. 2005. Flowering: a time for integration. Int. J. Dev. Biol. 49: 585-593.

Pathak, D. and Sigler, P. B. 1992. Updating structure-function relationships in the bZip family of transcription factors. Curr. Opin. Struct. Biol. 2: 116-123.

Perry, T. O. 1971. Dormancy of Trees in Winter. *Science*. 171: 29-36.

Poethig, R. S. 1990. Phase change and the regulation of shoot morphogenesis in plants. *Science*. 250: 923-930.

Powell, L. E. 1987. Hormonal aspects of bud and seed dormancy in temperate-zone woody plants. HortScience. Vol. 22(5): 845-850.

Rinne, P., Saarelainen, A. and Junttila, O. 1994. Growth cessation and bud dormancy in relation to ABA concentration in seedlings and coppice shoots of Betula pubescens as affected by a short photoperiod, water deficit and chilling. Physiologia Plantatrum 90: 451-458.

Rinne, P. L. H. and van der Schoot, C. 1998. Symplasmic fields in the tunica of the shoot apical meristem coordinate morphogenetic events. Development. 125: 1477–1485.

Rohde, A., Howe, G. T., Olsen, J. E., Moritz, T., Van Montagu, M., Junttila, O. and Boerjan, W. 2000. Molecular Aspects of Bud Dormancy in Trees. Molecular Biology of Woody Plants. Jain and Minocha (Eds) 1:89-134. Kluwer Academic Publishers: Netherlands.

Rohde, A., Prinsen, E., De Rycke, R., Engler, G., Van Montagu, M. and Boerjan, W. 2002. PtABI3 impinges on the growth and differentiation of embryonic leaves during bud set in poplar. Plant Cell. 14(8): 1885-1901.

Ruzin, S. E. 1999. Plant microtechnique and microscopy. Oxford University Press, Inc. New York.

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

Sauter, J. J., Wisniewski, M. and Witt, W. 1996. Interrelationships between ultrastructure, sugar levels, and frost hardiness of ray parenchyma cells during frost acclimation and deacclimation in poplar (*Populus* x *canadensis* Moench <*robusta*>) wood. J Plant Physiol. 149: 451-461.

Searle, I. and Coupland, G. 2004. Introduction of flowering by seasonal changes in photoperiod. *EMBO J*. 23: 1217-1222.

Schmidt, R. J., Burr, F. A., Aukerman, M. J. and Burr, B. 1990. Maize regulator gene opaque-2 encodes a protein with a ‘leucine zipper’ motif that binds to zein DNA. PNAS. 87: 46-50.

Shannon, S. and Meeks-Wagner, D. R. 1991. A mutation in the *Arabidopsis TFL1* gene affects inflorescence meristem development. *Plant Cell*. 3: 877-892.

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

Suttle, J. and Hultstrand, J. 1994. Role of endogenous abscisic acid in potato microtuber dormancy. Plant. Physiol. 105:891-896.

Swartz, H. J., Geyer, A. S., Powell, L. E. and Lin, S. C. 1984. The role of bud scales in the dormancy of apples. J. Amer. Soc. Hort. Sci. 109: 745-749.

Taiz, L. and Zeiger, E. 2002. Plant Physiology. 3rd ed. Sinauer Associates, Inc., Publishers. *Sunderland, Massachusetts*.

Taylor, G. 2002. *Populus*: *Arabidopsis* for forestry. Do we need a model tree? Annals of Botany 90:681-689.

Tinklin, I. G. and Schwabe, W. W. 1970. Lateral bud dormancy in blackcurrant. Ann. Bot. 84: 691-706.

Valverde, F., Mouradov, A., Soppe, M., Ravenscroft, D., Samach, A. and Coupland, G. 2004. Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science*. 303: 1003-1006.

Vegis, A. 1964. Dormancy in higher plants. An. Rev. of Plant Phys. L. Machlis (Ed.) 15: 185-224.

Villiers, T. A. 1975. Dormancy and the Survival of Plants. The Institute of Biology's Studies in Biology no. 57.

Walsch, J., Waters, C. A. and Freeling, M. 1998. The maize gene liguleless2 encodes a basic leucine zipper protein involved in the establishment of the leaf blade-sheath boundary. Genes Devel. 12: 208-218.

Walser, R. H., Walker, D. R. and Seeley, S. D. 1981. Effect of temperature, fall defoliation, and gibberellic acid on the rest period of peach leaf buds. J. Amer. Soc. Hort. Sci. 106: 91-94.

Welling, A., Moritz, T., Palva, T. and Junttila, O. 2002. Independent activation of cold acclimation by low temperature and short photoperiod in hybrid aspen. Plant Physiol. 129: 1633-1641.

Wigge, P. A., Kim, M. C., Jaeger, K. E., Busch, W., Schmid, M., Lohmann, J. U. and Weigel, D. 2005. Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science*. 30: 1056-1059.

Williams, Jr. B. J., Pellett, N. E. and Klein, R. M. 1972. Phytochrome control of growth cessation and initiation of cold acclimation in selected woody plants. Plant Physiol. 50: 262-265.

Coleman, G. D. and Chen, T. H. H. 1996. Photoperiod-associated gene expression during dormancy in woody perennials. Plant Dormancy. G. Lang (Ed.) 301-307.

Zhu, B. and Coleman, G. 2001. Phytochrome-mediated photoperiod perception, shoot growth, glutamine, Calcium, and protein phosphorylation influence the

activity of the poplar bark storage protein promoter (bspA). Plant Physiol. 126:342-351.