Control of flowering time and growth Cessation in Arabidopsis and *Populus* trees

Henrik Böhlenius

Faculty of Forest Sciences
Department of Forest Genetics and Plant Physiology
Umeå

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Abstract

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Transitions from vegetative to reproductive states are among the most critical and highly regulated changes plants undergo during their life cycles. Trees are amongst the latest flowering plants, and can stay in a juvenile state for many years before initiating flowering. Intense efforts have been made, over many years, to shorten the juvenile phase of trees, and to elucidate the mechanisms that regulate their floral transitions. In the studies underlying this thesis, *Populus* homologs (*PtFT1, PtCO2 PtGI*) of genes involved in the photoperiodic regulation of flowering time in Arabidopsis were identified using a comparative genomics approach. Analysis of transgenic trees over- or under-expressing these genes showed that these genes are responsible for controlling both flowering induction and the timing of short-day induced growth cessation in *Populus*. A model based on these phenotypes and gene expression analysis was proposed, explaining how *Populus* trees measure daylength and regulate the critical daylength, thus accounting for the variation in this parameter in trees originating from different latitudes.

In addition, GA_4 was identified as the most important endogenous gibberellin growth hormone involved in regulating the flower meristem gene *LEAFY* and flowering initiation in the facultative long-day plant Arabidopsis (in which flowering is initiated early by long photoperiods, while under short photoperiods flowering occurs later and is dependent on gibberellin). Further data acquired showed that in short days flowering initiation was preceded by dramatic increases in gibberellin and sugar levels in the shoot apex, and that gibberellin activates *LEAFY* expression independently of protein synthesis. The results provide corroborative evidence that *LEAFY* is regulated by a labile negative regulator, which possibly represses an apex-specific activator.

Keywords: Flowering initiation, growth cessation, bud set, photoperiodic regulation, circadian clock, phase, period, rhythm, gibberellin, *LEAFY*, protein synthesis, Cycloheximide (CHX).

Author's address: Henrik Böhlenius, Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, SLU, S-90183, Umeå, Sweden

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Appendixes

The presented thesis is based on the following papers, which will be reffered to by their roman numerals

List of Papers

I. Böhlenius, H. &Nilsson, O. The flowering meristem gene LEAFY is regulated by gibberellins and a labile repressor. *Manuscript*.

II. Eriksson, S. Böhlenius, H. Moritz, T. & Nilsson O. GA_4 is the active gibberellin in the regulation of *LEAFY* transcription and Arabidopsis floral initiation. *Plant Cell* 2007 18, 2172-2181

III. Böhlenius, H. Huang, T. Campa, L. Straus, S. Broumer, A. Jansson, S. & Nilsson, O. *FT/CO* Regulatory module controls flowering and short day-induced bud cessation in *Populus* trees. *Science* 2006 312, 1040-43

IV. Böhlenius, H. Eriksson, M. & Nilsson, O. The *Populus* tree homolog of *GIGANTEA* controls short day-induced growth cessation through the expression of *CONSTANS*. *Manuscript*

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Introduction

In contrast to animals, plants can not move from their growth location. To survive the harsh growth conditions in winter, plants growing in the temperate zones of the world have evolved a mechanism whereby they can switch between active growth during summer and dormancy in winter. During an annual growth period, environmental conditions such as temperature, nutrient status and water supply can vary from day to day and year to year but changes in photoperiodic length (daylength) are predictable year after year. Plants have therefore developed a mechanism to measure these changes and coordinate developmental processes with them, thereby ensuring that developmental events occur at appropriate times during the growth season. Two processes that are under such control are the short day-induced growth cessation in *Populus* trees and the long day-induced flowering in *Arabidopsis*. Almost 90 years ago, in the 1910s, Wightman Garner and Henry Allard discovered that shortening the daylength induced early flowering in tobacco plants (Garner and Allard, 1920) They then investigated the flowering time of other species in response to changes in daylength and proposed that daylength was a major determinant of flowering initiation. They introduced the terms photoperiod (daily patterns of light and dark) and photoperiodism (the response to photoperiods) and classified plants according to their photoperiodic response into long day plants in which flowering is accelerated in long days, short-day plants in which flowering occurs in shorter days and day-neutral plants in which flowering is not regulated by photoperiod. However, in trees flowering is preceded by an extended juvenile phase in which they are unable to respond to environmental signals such as increases in daylength. Therefore, flowering in trees it is not obvious under photoperiodic control. It is generally believed that trees have to reach a certain developmental stage (age or size) in order to make the transition from juvenile to adult state. Similarly, it is considered that *Arabidopsis* plants grown under non-inductive short-day conditions have to reach a certain developmental stage before entering their reproductive phase.

From a commercial perspective, the long juvenile phase of trees is one of several features limiting efficient tree breeding programs. Therefore, improving our knowledge of the ways in which trees regulate their flowering time is of great interest, to provide opportunities to induce flowering earlier. Furthermore, in many countries fast-growing tree species are being increasingly used in plantations, and in the future genetically modified trees may be widely are grown for pulp and bioenergy production. In such scenarios it is may be equally important to prevent flowering, or to ensure that any flowers produced are sterile to prevent genetically modified pollen spreading over hundreds of kilometers to natural populations. In addition, improved knowledge of the ways in which trees measure daylength and control growth cessation in the fall would provide opportunities to adapt specific tree varieties to specific climates or climatic changes.

Background

Flower initiation in Arabidopsis

One of the most widely studied plants is *Arabidopsis thaliana* because of its small genome and short generation time. In these plants flowering is controlled by environmental signals such as light, temperature, and daylength (Bernier and Perilleux, 2005). These signals are integrated into three pathways: the long day pathway that controls flowering in response to changes in daylength (described in later sections), the autonomous pathway that controls flowering under both long and short days and the gibberellin pathway that is necessary for flowering under short days (Figure 1) (Boss and Thomas, 2002; Mouradov *et al.*, 2002; Putterill *et al.*, 2004; Simpson, 2004). The activity of the autonomous pathway genes is mediated through the floral repressor *FLOWERING LOCUS C* (*FLC*), which represses expression of the floral activators *SUPPRESSOR OF CONSTANS OVER EXPRESSION 1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*) (Michaels and Amasino, 1999; Lee et al., 2000; Michaels and Amasino, 2001; Michaels et al., 2005; Searle et al., 2006). *Arabidopsis* plants that are unable to synthesize the plant hormone gibberellin (GA), such as the *ga1-3* mutant, fail to initiate flowering under short day conditions, showing that GA plays a central role in determining the time of flower initiation in short days (Wilson *et al.*, 1992).

The floral transition involves both physiological changes and changes in gene expression in the shoot apex. The vegetative leaf-producing program is turned off, and the reproductive program, in which newly formed primordia develop into flowers, is turned on. For this transition to take place the expression of genes involved in flower formation needs to be activated. One of the genes that play a key role during flower development is the flower meristem identity gene *LEAFY* (*LFY*). Loss-of-function mutations in *LFY* lead to plants in which shoots replace most flowers (Weigel et al., 1992). *LFY* expression is first detectable in leaf primordia and reaches maximal levels in young floral meristems (Blazquez et al., 1997; Blazquez et al., 1998a).

It has been shown that one reason why the *ga1-3* mutant fails to initiate flowering under short-day conditions is its inability to upregulate *LFY* expression (Blazquez et al., 1998b). The GA signal was shown to be integrated through a promoter element with similarities to a GA-myb binding site. When this site is mutated in a *LFY* construct with a minimal promoter, its transcription is not upregulated in short days, but it still responds to long-day signals (Blazquez and Weigel, 2000). Furthermore, *LFY* expression from a constitutive promoter restores the late flowering of a *ga1-3* mutant in short days, confirming that *LFY* acts downstream of GA (Blazquez et al., 1998b). Like *LFY*, *SOC1* is downregulated in *ga1-3* mutants, and can restore short-day flowering in a *ga1-3* background when constitutively expressed (Moon et al., 2003).

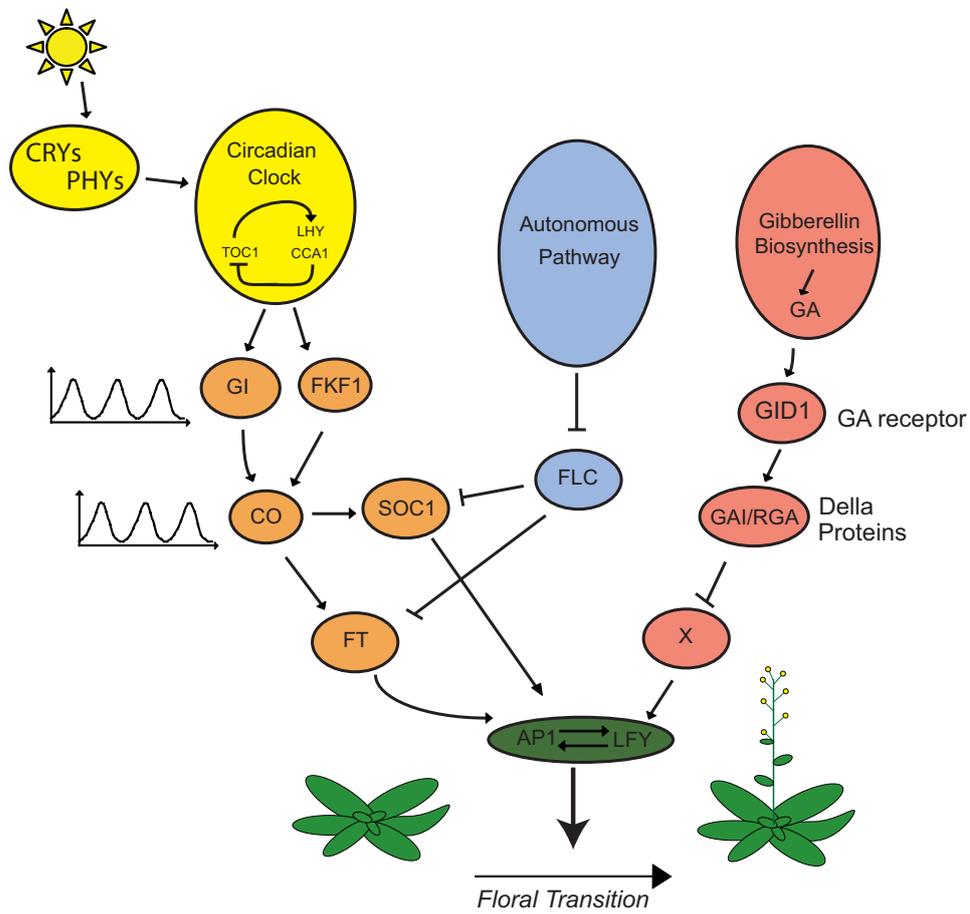**Figure 1. Simplified model of flowering pathways in *Arabidopsis***
Flowering initiation in ***Arabidopsis*** is controlled by three different pathways, the
Long day pathway, the Autonomous pathway and the Gibberellin pathway.

Synthesis of Bioactive GAs and GA signalling

The last steps in the synthesis of bioactive gibberellins are catalyzed in parallel pathways by a set of 2-oxoglutarate-dependent dioxygenases. The precursors of bioactive gibberellins GA_{53} and GA_{12} are converted in two parallel pathways to GA_9 and GA_{20} by three consecutive oxidations at C-20 catalyzed by GA20-oxidase (*GA20OX*). GA_9 and GA_{20} are further oxidized at C-3 by GA3-oxidase to form the bioactive GA_1 and GA_4 (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000; Olszewski et al., 2002). The biologically active gibberellins GA_3, GA_5 and GA_6 can be further synthesized from GA_{20}. The bioactive gibberellins GA_1 and GA_4 can be inactivated by oxidation to GA_{53} and GA_8, respectively, by GA2-oxidases (Hedden and Phillips, 2000). The ways in which plants perceive GA and the GA signal is transduced to induce a hormone response were recently discovered. GA signaling has been proposed to be repressed by the action of DELLA proteins, and in response to GA, DELLA proteins are targeted for degradation by the 26S proteasome (Peng et al., 1997; Dill and Sun, 2001; King et al., 2001a). A recent study in rice (*Oryza sativa*) identified the GIBBERELLIN DWARF1 (GID1) protein as a soluble receptor of GA that interacts with the DELLA protein SLENDER RICE1 (SLN1) upon binding GA (Ueguchi-Tanaka et al., 2005). GID1 showed high binding activity to bioactive gibberellins, whereas its binding activities for biologically inactive gibberellins were low (Ueguchi-Tanaka et al., 2005). In the *Arabidopsis* genome there are three orthologs of GID1, all of which display higher binding activity to GA_4 than other bioactive gibberellins (Nakajima et al., 2006). This finding corresponds well with observations that GA_4 is the active gibberellin in cell elongation and shoot growth in *Arabidopsis* (Talon et al., 1990; Xu et al., 1997; Cowling et al., 1998). However, in the monocot *Lolium temulentum*, GA_5 and GA_6 have been found to be the bioactive gibberellins in flowering, although their activity in stem elongation was low (King et al., 2001b; King et al., 2003). It could therefore be speculated that gibberellins other than GA_4 may be important in the regulation of flowering initiation.

Flowering in *Populus* trees

Flowering in woody plants, such as *Populus* trees, differs from flowering in *Arabidopsis* in several respects. Firstly, flowering occurs after a long juvenile phase in trees that can last for decades, during which time they are unable to produce flowers and fruits, while the juvenile phase in *Arabidopsis* is much shorter (weeks). Secondly, when *Arabidopsis* flowers the shoot apical meristem is transformed into an inflorescence meristem that forms flowers on its flanks, and is not normally capable of reverting to a vegetative meristem, so once *Arabidopsis* plants have started to flower, they are committed to reproductive development
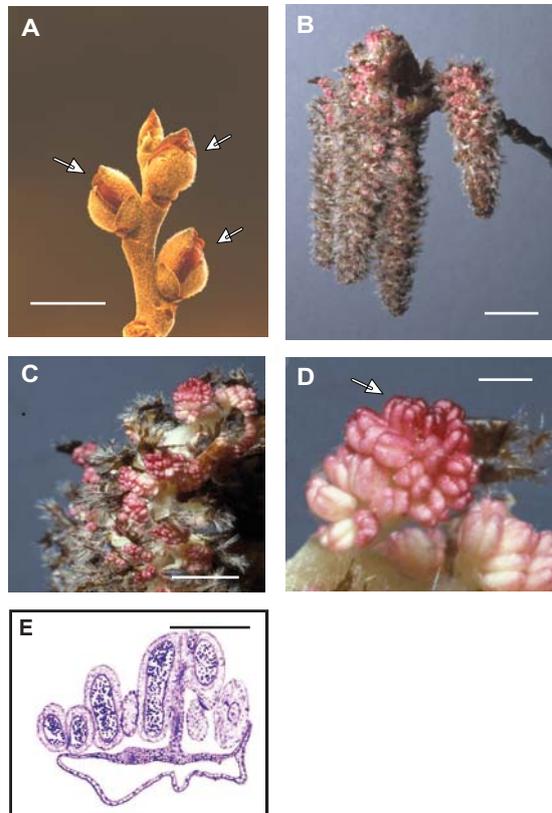
Figure 2. *Populus* flower phenotypes
In *Populus* individual flowers are attached to inflorescences (catkins). A, dormant flower buds in winter indicated by arrows. B, elongated male catkin in spring. C, close-up of male catkin. D, single male flower, the arrow indicates pollen sac. E, section of male flower. Scale bars: A and B, 1 cm; C and E, 1mm.

until they senesce. In contrast, in trees such as *Populus* all apical mersitems and many lateral meristems are maintained in a vegetative state once flowering has been induced, allowing them to continue vegetative growth over many years after flowering. Floral initiation in *Populus* occurs in late spring or early summer with the formation of flower buds in the axes of the current year leaves and the stem. During summer, the flower buds develop further before entering dormancy in the fall (Figure 2). In early spring the flower buds start to elongate and individual flowers form on axillary inflorescences (called catkins) before vegetative bud

burst (Figure 2). The genus *Populus* is dioecious, i.e. male and female flowers are found on separate trees. After the pollen or seeds are released, the catkins are shed.

In *Arabidopsis*, genetic and molecular approaches have identified several key regulatory genes that play important roles in flower initiation and development. Two of these genes, *LFY* and *APETALA1* (*AP1*), are essential for correct flower initiation and flower development (Schultz and Haughn, 1991; Mandel et al., 1992; Weigel et al., 1992). When *LFY* was expressed from a constitutive promoter in male hybrid aspen (*Populus tremula x P. tremuloides*), in a study by (Weigel and Nilsson, 1995), early flowering was induced, but unfortunately these transgenic hybrid aspens produced abnormal terminal flowers as well as single flowers that did not shed any pollen. Furthermore, (Rottmann et al., 2000) found that early flowering could not be induced by *LFY* overexpression in some *Populus* genotypes. However, in *Citrus* early flowering and normal fruit development was observed when *AP1* and *LFY* were overexpressed, whereas in hybrid aspen *AP1* had no stimulatory effect on flowering (Nilsson and Weigel, 1997; Pena et al., 2001).

In annual plants GA often stimulates flowering, but GA inhibits flowering in a number of hardwood tree species (Metzger, 1995; Meilan, 1997), including fruit trees such as cherry, peach, apricot, almond and lemon, in which GA inhibitors have been used to induce early flowering (Zeevaart, 1983). In contrast to these trees GA is frequently used in conifers to promote flowering for breeding purposes (Meilan, 1997). However, while the GA inhibitor paclobutrazol induces flowering in *Citrus* it appears to be ineffective in inducing flowering of juvenile *Eucalyptus nitens* and *Populus deltoides* (Williams *et al.*, 1999; Yuceer *et al.*, 2003). Therefore it has been hypothesized, based on the results of GA or GA inhibitor treatments (with no genetic evidence), that GA inhibits flowering in trees. However, a recent report has provided genetic evidence that GAs are involved in regulating flowering in grapevines, since an early flowering dwarfed mutant has been found to be deficient in the grapevine homolog of the Arabidopsis gene *GAI*, involved in the GA signaling pathway (Boss and Thomas, 2002).

Short Day-Induced Growth Cessation in *Populus*

In temperate regions of the world, trees cycle between active growth during the summer and dormancy in the winter. This involves short day-induced growth cessation and bud set in the fall, followed by a dormant state characterized by enhanced tolerance to cold. During the growth period the daylength gradually increases in the spring and early summer, then declines in the late summer and fall. When the length of the day drops below a certain threshold level (the critical daylength), plants respond by initiating growth cessation, marked by a reduction in internode elongation followed by the formation of terminal buds (autumn buds). During the first weeks of short days, all primordia developed before the onset of short days will develop into leaves, and the last primordia formed before the onset of short days is the last leaf to mature, often not to full size (Rohde et al., 2002).

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This means that the developmental program will only change to the production of bud scales and embryonic leaves in primordia initiated after the transition to short days (Rohde et al., 2002).

Typically, trees originating from more northern latitudes stop growing at longer critical daylengths, and hence set buds earlier in the fall, than southern ecotypes (Pauley and Perry, 1954). This is a highly adaptive trait, under strong genetic control, since it ensures that growth cessation and bud set are induced before the winter, that is maintained when trees are transferred between latitudes (Howe et al., 1996b; Frewen, 2000).

In several woody species the need for a long day signal to maintain active growth can be replaced by addition of gibberellins, and there is further evidence that GA biosynthesis is reduced after short-day treatment (Olsen et al., 1995b; Olsen et al., 1995a; Olsen et al., 1997a). In addition, overexpression of the oat phytocrome A (*OPHYA*) in hybrid aspen resulted in trees that were insensitive to changes in daylength, and GA levels remained constant in *35S:OPHYA* plants after short-day treatment, which could be one reason for the inability of short-day conditions to induce growth cessation (Olsen et al., 1997a). However, the genes involved in the molecular mechanisms that measure daylength and control growth cessation and bud set in trees are largely unknown.

Daylength measurement

Several models have been suggested to explain the mechanism whereby information on day and night length is integrated into the regulation of plant development. At present, the coincidence model is the most consistent with available genetic evidence. This model was originally proposed by Erwin Bűnning in 1936, based on studies of circadian and photoperiodic responses in soybean. According to this model light has two important roles: one in resetting the circadian clock, which is important for generating the daily oscillation of a key regulatory component with an expression peak in the late afternoon, and the other in regulating the activity of this component. A photoperiodic response is only triggered when the expression of the regulatory component reaches a threshold level during daylight (Figure 3). In the long-day plant *Arabidopsis*, the main function of these regulators is to promote flowering.

Since the circadian clock always sets the expression peak of the regulator in the late afternoon, a certain number of hours after dawn, it will coincidence more completely with daylight under long days and less fully (or not at all) under short days. Thus, it has a more active role in long days, resulting in the acceleration of flowering.
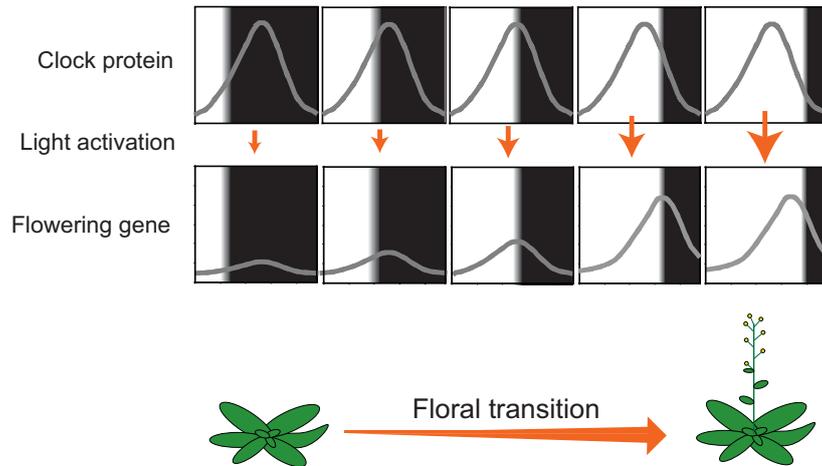
Figure 3 The external coincidence model
The function of the clock-regulated protein that activates the expression of the
flowering gene is regulated by light. The expression peak of this protein needs to
coincide with light in order to activate the expression of the flowering gene,
resulting in flowering as the daylength is increased.

The *Arabidopsis* Circadian clock

In its simplest representation, the circadian clock can be said to consist of input,
output and the core central oscillator, as reviewed by (McClung, 2001b; Más,
2005). The circadian clock is reset by light signals mediated through the
phytochrome red and far-red light receptors (PHYA to E) and the cryptochrome,
blue-light receptors (CRY1 and CRY2), reviewed by (Fankhauser and Staiger,
2002; Yanovsky and Kay, 2003).

   In Arabidopsis, the core oscillator consists of a positive/negative feedback loop
including the genes *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE
ELONGATED HYPOCOTYL* (*LHY*), *TIMING OF CAB EXPRESSION* (*TOC1*)
and   *EARLY FLOWERING 4* (*ELF4*) (Schaffer et al., 1998; Wang and Tobin,
1998a; Strayer et al., 2000; Alabadi et al., 2001; Doyle et al., 2002b). The
expression of these genes is regulated by the circadian clock, with mRNA
accumulating either in the morning (*CCA1* and *LHY*) or the evening (*TOC1* and
ELF4) (Schaffer et al., 1998; Wang and Tobin, 1998b; Covington et al., 2001).
The circadian clock is involved in regulating a number of processes that include
flowering, leaf movement and timing of gene expression (Barak et al., 2000;
Mcclung, 2001a). Since the circadian clock participates in daylength-measurement
mechanisms, mutations altering its rhythm often influence photoperiodic responses
such as flowering. (Suarez-Lopez et al., 2001; Blazquez et al., 2002; Yanovsky
and Kay, 2002; Mizoguchi et al., 2005).

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The daylength measurement mechanism in *Arabidopsis*

The key processes of the mechanism whereby plants measure daylength are the circadian regulation of *CONSTANS* (*CO*) expression and light regulation of the CO protein's stability (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004). *CO* is a B-box type zinc finger transcription factor that activates expression of the flower activator *FT* in a light-dependent manner (Yanovsky and Kay, 2002). Given that the circadian clock sets the *CO* expression peak in the late afternoon, CO protein production will coincide with light in long days, which stabilizes the CO protein, leading to activation of *FT* expression and acceleration of flowering (Figure 4) (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004). However, in short days the diurnal expression peak of *CO* occurs in darkness, low levels of CO protein is accumulated and *FT* expression is not activated, resulting in later flowering (Figure 4) (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004). The *CO* and *FT* genes display tissue-specific expression patterns, in which *CO* is expressed in both leaf and stem phloem while *FT* expression is restricted to leaf phloem (Takada and Goto, 2003; An et al., 2004). It was recently shown that the FT protein moves from the leaf to the shoot apex, where it interacts with FD and activates the expression of the flower meristem identity gene *AP1* (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007; Jaeger KE, 2007; Mathieu J, 2007; Tamaki et al., 2007). This indicates that the FT protein could be the signalling molecule referred to as "florigen" in the literature.

Regulation of *CONSTANS* by the circadian clock

In recent years an increasing number of factors affecting *CO* expression have been identified. These include *FLAVIN-BINDING KELCH REPEAT, AND F-BOX 1* (*FKF1*), *GIGANTEA* (*GI*), *EARLY FLOWERING 3* (*ELF3*), *CYCLING DOF FACTOR1* (*CDF 1*), *RED AND FAR-RED INSENSITIVE 2* (*RFI2*), and can be divided into activators (*GI* and *FKF1*) and repressors (*ELF3*, *CDF1* and *RFI2*) of *CO* expression (Fowler et al., 1999; Suarez-Lopez et al., 2001; Imaizumi et al., 2003b; Imaizumi et al., 2005; Chen and Ni, 2006b). FKF1 is an F-box protein that regulates *CO* expression in a light-dependent manner. In an *fkf1* mutant the daytime expression of *CO* is absent, explaining the late flowering phenotype (Imaizumi et al., 2003a). One of the molecular mechanisms whereby FKF1 regulates *CO* expression is by interacting with CDF1 and targeting it for degradation (Imaizumi et al., 2005). CDF1 is a repressor of *CO*, and the only protein identified to date that binds to the *CO* promoter (Imaizumi et al., 2005). *GI* shows a similar diurnal expression pattern to *FKF1,* although in a *gi* mutant *CO* expression is repressed during the entire day under both long and short day conditions, showing that GI is necessary for *CO* expression, independently of the photoperiod (Suarez-Lopez et al., 2001). Moreover, in the *elf-3* mutant, the expression of both *GI* and *FKF1* is affected, thereby increasing *CO* and *FT* expression levels, which results in earlier flowering and suggests that *ELF3* acts upstream of *GI* and *FKF1* in the regulation of flowering (Woe-Yeon *et al.*, 2005).

The *rif1-2* mutant has been identified as a red- and far red-light insensitive mutant that flowers early, especially under long-day conditions. It has also been shown that the early flowering phenotype of *rif1-2* mutants correlates with alterations in their *CO* and *FT* expression patterns (Chen and Ni, 2006b). Although the *rif1-2* mutation enhances *CO* expression, it does not alter *GI* expression, either in long or short days, suggesting that *RIF2* regulates *CO* expression through a mechanism that does not involve *GI* (Chen and Ni, 2006a).
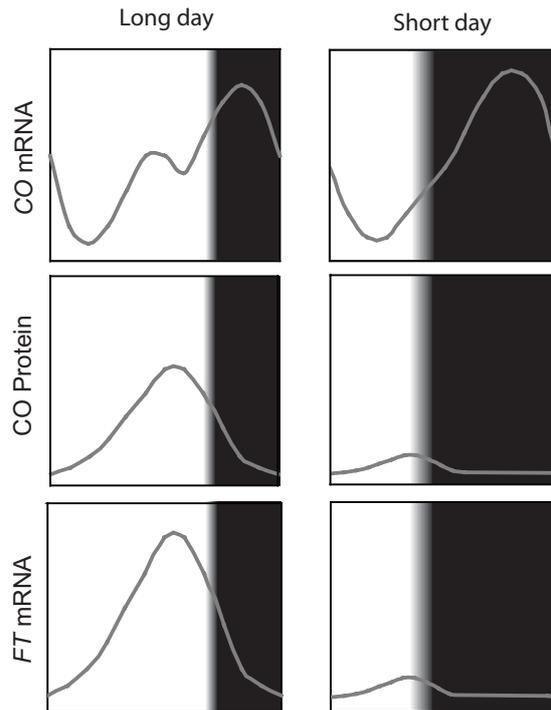

Figure 4 Regulation of *FT* expression by the CO protein
CO has a diurnal expression pattern with the highest transcript levels accumulating in the afternoon. In long days the light stable CO protein activates *FT* expression, but in short days, when no CO protein is present, *FT* displays a very low diurnal expression.

Post-transcriptional regulation of CO

The signals from light and the circadian clock are integrated through the regulation of *CO*. The CO protein is most stable in the late afternoon in long days, but in short days it is unstable throughout the entire day (Valverde et al., 2004). In the regulation of CO stability CRYs and PHYA act in an antagonistic manner to PHYB, where PHYA and CRYs is protecting the CO protein from degradation while PHYB promotes its degradation (Valverde et al., 2004). Furthermore, there is recent evidence indicating that a negative regulator of PHYA signaling, *SUPPRESSOR OF PHYA-105 (SPA1)* and its homologs *SPA2* and *SPA4*, are involved in the regulation of CO stability. The *spa1* mutant is early flowering under short days but not under long days (Ishikawa, 2006; Laubinger et al., 2006). In short-day conditions, the *spa1* mutation causes increases in *FT* transcript levels without shifting the diurnal expression peak of *CO* mRNA into light (Ishikawa, 2006; Laubinger *et al.*, 2006). Moreover, the SPA1 protein physically interacts with the CO protein, and CO protein levels are increased in a *spa1 spa2 spa4* triple mutant, indicating that SPA1 is involved in the light regulation of CO stability (Laubinger et al., 2006).

Regulation of *FT* expression

Besides the light-dependent regulation of *FT* by CO, chromatin remodeling appears to affect the regulation of *FT* expression. Several mutants have been identified that appears to be involved in chromatin structure that affects *FT* expression. The *early bolting in short day* (*ebs*) mutation accelerates flowering, especially under short day conditions, and *terminal flower 2* (*tfl2*) mutants are early flowering in both long and short days (Gomez-Mena et al., 2001; Kotake et al., 2003). It has been revealed that the early flowering phenotype in both *ebs* and *tfl2* mutants is connected to increased expression of *FT* (Gomez-Mena et a*l.*, 2001; Kotake et al., 2003; Takada and Goto, 2003). In addition to light-dependent *CO* activation, *FT* was found to be repressed in plants with high FLOWERING LOCUS C (FLC) expression (Michaels et al., 2005). FLC is a MADS box transcription factor, whose expression is strongly suppressed by vernalization and shows a specific expression pattern in the shoot apex and vascular tissues (Michaels and Amasino, 1999; Sheldon et al., 1999). This type of flowering regulation ensures that flowering takes place under favorable conditions in spring.

Objectives

The aims of the studies underlying this thesis were to improve and deepen our knowledge of the daylength measurement mechanism and regulation of flowering time in plants, especially in *Populus* trees (Papers III and IV). In addition, we obtained further information on the role of gibberellins in the regulation of *Arabidopsis* flowering initiation under short-day conditions (Papers I and II). The main questions addressed were:

Which endogenous gibberellin is most important for flowering initiation in *Arabidopsis* plants grown under short-day conditions and do metabolic changes occur in the shoot apex before flower initiation?

How is *LEAFY* transcription regulated by gibberellin, and are other, unknown factors involved in the regulation of its expression?

How is flowering time controlled in *Populus* trees and what gene(s) play important roles in regulating their transition from juvenile to adult phase?

What is the mechanism underlying daylength measurements, and how is it connected to the regulation of short day-induced growth cessation?

Methodological overview

In this section I reflect on the most important methods and plant materials used to obtain the results presented in this thesis.

Plant material

Trees originating from different latitudes display different critical daylengths for growth cessation. We therefore collected *Populus tremula* trees from various latitudes in northern Europe, including: Umeå, Sweden (latitude 63ºN); Brunnsberg, Sweden (latitude 59ºN); Ronneby, Sweden (latitude 56ºN) and Dresden, Germany (latitude 51ºN) (Papers III and IV). Not all *Populus* species are easily transformable, but the male hybrid aspen *P. tremula x tremuloides* (T89) can be transformed with high efficiency using *Agrobacterium* (Nilsson O et al., 1992). T89 was used to generate transgenic plants in which the expression of genes of interest was reduced by RNAi or increased by overexpression from a constitutively active promoter (*35S*). *Populus* trees are dioecious, therefore a female *P. tremula* clone (Brauna) was used for studies of female flower initiation and development.

Plant transformation

Various methods can be used for transferring genes into a plant's genome. One of the most commonly used is *Agrobacterium*-mediated DNA transfer. *Populus* transformation is performed by soaking stem segments in an *Agrobacterium* solution, allowing the cut ends of the stem segments to be infected. The stem segments are then placed on media containing growth hormones to stimulate shoot formation and antibiotics to select transformed shoots (Nilsson et al., 1992). This is a time-consuming process requiring 6-8 months from transformation to the production of transgenic plants ready for transfer to the greenhouse. In contrast, *Arabidopsis* can be readily transformed by dipping flowers in an *Agrobacterium* suspension (Clough and Bent, 1998). However, not all seeds are transformed in this process, so antibiotics are then usually used to select for transgenic plants.

Methods for analyzing gene expression.

Reverse transcriptase PCR (RT-PCR) is a routinely used method for analyzing gene expression since it allows the expression of genes that are very weakly expressed to be analyzed, even in small tissue samples. It involves isolating total RNA or mRNA then subjecting it to a reverse transcriptase reaction to generate cDNA, which is used as a template in PCR amplification. In the studies reported in

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Papers I and II, semi-quantitative RT-PCR was performed to detect gene expression, and 18S rRNA was amplified as an internal control in the same reaction tube as the gene product under investigation. Semi-quantitative RT-PCR means that the reaction is terminated when the PCR amplification of the gene product investigated is within the linear range. The amplified PCR products are visualized following separation on an agarose gel, or further processed for detection. This is done to allow the ratio between the intensities of the investigated and control gene products to be calculated and, thus obtain relative expression values. However, in order to compare relative expression values between samples, the expression level of the gene investigated has to be within the same linear amplification range.

A more sensitive approach for detecting gene expression is Real-time PCR (Papers I-IV). Here the amplification of the target gene product is monitored cycle-by-cycle (in real time) and the increase in the PCR product is visualized in terms of increased incorporation of a fluorescent dye into the PCR product (SYBRgreen). In this scenario it is necessary to include quality control measures for the PCR reaction, since all PCR products (both specific and unspecific) will affect the signal intensity. This is usually done by melting the PCR product while measuring the fluorescence, which decreases as the double-stranded PCR products become single-stranded. This should result in a single peak of decreased fluorescent signal if one PCR product is amplified and several peaks if there are multiple PCR products. The fluorescence values used for calculations are called Ct (cycle thresholds) and correspond to the amount of starting transcript. The Ct values for the genes analyzed have to be normalized to the expression of a reference gene reflecting the amount of cDNA in the sample. In all cases here, the reference gene used was the 18S rRNA gene, which is stably expressed in the tissues and conditions we analyzed. The expression of the genes of interest are normalized to the expression of 18S (Ct gene-Ct18S=dCt), using the formula, relative expression = 2^{-dCt}.

Studies of gene function

A gene's function can be investigated using several different methods. Random mutagenesis of seeds followed by a mutant screen of phenotypes is a commonly used method in *Arabidopsis*. However, in *Populus* species this is not possible due to their long generation times and the fact that the trees are dioecious. Therefore transgenic technologies have been developed that reduce the expression of specific genes (RNA interference) or increase their expression by the use of a constitutive promoter (overexpression).

Gene expression is reduced by introducing a single-stranded *RNAi* molecule driven by a constitutive 35S promoter into the plant's genome. This molecule has homologous sequences that will fold and create a double-stranded DNA molecule that is recognized by the plant's defense system as pathogenic RNA and degrade it to 20-25 basepair long fragments. The plant recognizes these specific sequences and targets identical endogenous sequences for degradation, thereby reducing the expression of the targeted gene. Gene expression driven by a constitutive promoter

is an efficient and well-established method for probing the function of a gene. However, transgenic plants may show artificial phenotypes due to ectopic expression, and changes in expression levels of "important" genes can be lethal, so only plants with wild-type phenotypes can be generated. In such cases an inducible system may be of interest. The advantage of inducible systems is that the expression of the genes can be induced at a specific developmental stage or in a specific tissue.

Results and discussion

Regulation of the flower meristem gene LEAFY (Paper I and II)

In *Arabidopsis* plants grown under short-day conditions the plant hormone gibberellin (GA) is required for flowering initiation (Wilson et al., 1992). One connection between gibberellins and flower initiation has been shown to be mediated through the expression of the flower meristem identity gene *LEAFY* (*LFY*), because in a GA-deficient *ga1-3* mutant carrying a *LFY::GUS* construct grown in short days, the gradual upregulation of *LFY::GUS* is repressed, but application of GA_3 increases its expression (Blazquez et al., 1998a).

There are many different gibberellins in plants, but most of them are either precursors or deactivation products and only a few have been shown to be bioactive. It has been shown in previous studies that GA_4 is the bioactive gibberellin in cell elongation and shoot growth in *Arabidopsis*. In the grass *Lolium*, it is suggested that GA_5 and GA_6 were the bioactive gibberellins in flower initiation, although their activity is lower in regulating stem elongation (Talon et al., 1990; Xu et al., 1997; Cowling et al., 1998; King et al., 2001b; King et al., 2003).

In the study reported in Paper II we investigated which endogenous gibberellin is bioactive in regulating flowering and *LFY* expression in *Arabidopsis*. The results of a dose-response experiment showed that GA_4 was 10 times more effective than GA_3 in activating *LFY::GUS* expression. When the GA levels were analyzed in the shoot apex at the time of floral initiation, GA_4 was found to be the most abundant gibberellin. Moreover, GA_4 and GA_3 were the most potent gibberellins for reducing flowering time in wild type and *ga1-3* mutant plants grown in short days. These results indicate that GA_4 is the endogenous bioactive gibberellinin regulating flower initiation and *LFY* expression. This hypothesis is further supported by a recent study, in which GA_4 reportedly showed the highest binding affinity to the gibberellin receptor GID1 (Nakajima et al., 2006).

The gibberellin signaling pathway is reported to be integrated through an 8-basepair *cis* element in the *LFY* promoter (Blazquez and Weigel, 2000). In the study described in Paper I the connection between GA and *LFY* regulation was carefully analyzed. In seedlings treated with GA_4, expression of *LFY* mRNA reached maximal levels after 1 hour. This early induction of gene expression by gibberellin is similar to the induction of other genes that are considered to be early

targets in hormone signaling (Abel et al., 1995; Gubler et al., 1995; Koshiba et al., 1995). The rapid induction of *LFY* by GA therefore indicates that *LFY* is an early target in GA signaling. In accordance with this hypothesis, *LFY* expression was induced at higher levels in seedlings treated with both the translational inhibitor cycloheximide (CHX) and GA_4 compared to CHX alone, again indicating that *LFY* is an early target in GA signaling.

Most interestingly, in seedlings treated with only CHX, *LFY* expression was induced to higher levels than in untreated seedlings, suggesting that *LFY* expression is under the control of a labile negative regulator. Further experiments with promoters carrying deletions revealed that this negative regulator represses *LFY* expression via interaction with an element in the first 246 base pairs and that the 26S proteasome is involved in the degradation of the labile repressor. These experiments were performed on whole seedlings, and a possible function of the repressor could be to restrict *LFY* expression to the shoot apex. However, when shoot apices and leaves were treated separately with CHX, *LFY* expression was only induced in the shoot apex, suggesting that the function of the repressor could rather be to repress the activity of an apex-specific activator. These results further suggest that the activator is present in the plant at the time of translation inhibition, since no new proteins could be synthesized. In an attempt to identify the flowering pathway in which the negative regulator or the activator is active, we analyzed *LFY* expression in response to CHX treatment in various mutant backgrounds with lesions in each of the three major flower-inductive pathways. In all tested mutants *LFY* expression was induced by CHX treatment, showing in conjunction with the results of the *LFY* promoter deletion experiments, that the negative regulator and GA signaling act through different pathways in regulating the expression of *LFY*.

Gibberellin and flower initiation in short days (Paper II)

Flowering in trees has similarities to the late flowering phenotype of Arabidopsis grown under short day conditions in the way that the plants have to reach a certain size or age before flowering is initiated. In order to investigate if metabolic changes in Arabidopsis grown in short days could be correlated to the time of flowering initiation micro-dissected shoot apices were sampled at various time points, then the levels of GA and sugars in them were analyzed.

In the short-day conditions applied, flower initiation occurred between days 42 and 49 after germination, based on the expression of *AP1* and *AP3*. Analysis of GA and sucrose levels in the shoot apex showed that GA_4 levels increased 30-fold between days 35 and 42, and sucrose levels 10-fold between days 35 and 42. In contrast to sucrose, glucose and fructose levels remained unchanged during the floral transition period. Thus, flower initiation in these *Arabidopsis* plants was preceded by dramatic increases in both GA_4 and sucrose levels in the shoot apex, which is interesting since they were grown in constant short-day conditions with no change in photoperiod, implying that *Arabidopsis* plants have to reach a certain size or age before gibberellin and sucrose levels start to increase in the shoot apex. It should be noted that there was no clear correlation between the increase in GA content and *LFY* expression. In young plants (14-21 days) GA levels were high

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while *LFY* expression remained at low levels, and at the time when GA levels increased there was almost no change in *LFY* expression. These findings could be explained by changes in *LFY* expression in the shoot apex being masked by *LFY* expression from the base of young leaves.

The dramatic increase in GA_4 levels observed in the shoot apex raises the question whether GA_4 is produced locally or imported from other tissues. Transcription of the genes controlling GA metabolism is regulated by bioactive gibberellins and can therefore give information regarding where GAs are produced. While several of the *GA20oxidases* and *GA3-oxidases* are subject to negative feedback regulation by GAs, several of the *GA2oxidases* are positively regulated. GA20oxidase and 3β-hydroxylase catalyze the last steps in the synthesis of bioactive gibberellins, while GA2oxidases inactivate bioactive gibberellins. We therefore analyzed the transcript levels of these genes in shoot apices. The transcription levels of *GA20oxidase* were unchanged from day 35 to 42, when the highest increase in GA levels were detected, and the same pattern was observed for the GA3-oxidases. The expression levels of *GA2oxidases* increased gradually from day 35 to 56, with no reduction in expression at day 42. Taken together, these results suggest that the local increase in GA_4 levels in the shoot apex cannot be explained by either increased expression of *GA20oxidases* or decreased expression levels of *GA2oxidases*, suggesting that the increase in GA_4 levels originates from sources outside the apex.

Previous analyses of events in the grass *Lolium temulentum* have shown that GA_5 and GA_6 can be transported from the leaves to shoot apices (King et al., 2001b). In order to test whether this is also the case for GA_4, deuterium-labeled GA_4 was applied to single leaves and shoot apices were analyzed for the presence of transported, labeled GA_4. Like GA_5 and GA_6, GA_4 was detected in the apex, showing that GA_4 moves from the leaf to the shoot apex. The import of gibberellin and sucrose to the shoot apex at the time of flower initiation suggests that plasmodesmata connecting the phloem to the shoot apex open before flowering is initiated, similar to events observed in long-day-induced flowering (Ormenese et al., 2000). Interestingly, sucrose in combination with GA_3 has a synergistic effect on the activation of *LFY* expression (Blazquez and Weigel, 2000). It has also been shown that application of sucrose to the shoot apex can complement the late flowering phenotype of *co*, *gi*, *fca*, *fpa*, and *fve* mutants, but the flowering phenotype of *ft* mutants could not be rescued in this way (Roldan *et al.*, 1999). One possible role for sucrose could be to act together with the FT protein to stimulate flowering.

Regulation of flowering time in *Populus* trees (Paper III).

In trees, flowering is preceded by a long juvenile phase that can last for decades, during which they are unable to respond to environmental stimuli such as increases in daylength. In an attempt to reduce the flowering time in *Populus* we identified and cloned the *Populus FT* homolog (*PtFT1*). Transgenic trees overproducing *PtFT1* and *FT* produced normal male and female catkins with normal flower development after two months in the greenhouse, showing that *PtFT1* is a powerful inducer of flowering. This finding suggests that the level of

PtFT1 is important for flower initiation. We therefore analyzed the level of *PtFT1* in *Populus* trees of different ages. The expression level of *PtFT1* gradually increased with age, reaching a threshold level at which flower initiation was induced. In *Arabidopsis*, the gene *EARLY BOLTING IN SHORT DAY*S (*EBS*) acts as a repressor of *FT*, probably by modulating chromatin structure (Pineiro et al., 2003). One possible mechanism responsible for the gradual increase of *PtFT1* expression is that the repression of flowering activators such as *PtFT1* could be gradually released during cycles of active growth and dormancy, thereby eventually activating flower initiation.

   In contrast to *PtFT1* transgenic trees, *Populus* trees transformed with the *Arabidopsis* flower meristem identity gene *LEAFY* (*LFY*) produced single flowers instead of catkins, reflecting the function of *LFY* as a flower meristem identity gene and *PtFT* as a flowering time gene (Weigel and Nilsson, 1995). Unfortunately, all attempts to perform a cross with *PtFT1* transgenic trees have been unsuccessful to date. It is still too early to draw definitive conclusions regarding the reasons for this failure, since positive controls also failed during these pollination experiments, however, the fertility of *35S::PtFT1* plants could be reduced because, in contrast to wild type plants, when flowering occurs early in *PtFT1* plants they are actively growing, and producing leaves and shoots at the same time as the flowers mature, which might compromise the flowers' maturation.

The FT/CO regulatory module (Paper III)

In aspen trees, short-day induced growth cessation is the only known daylength-regulated process, and it is induced at the end of summer when the daylength falls below a critical threshold (the critical daylength). To survive in the range of climatic conditions across northern Europe, aspen trees have evolved local adaptations. Typically, the growth of trees originating from northern latitudes terminates early in fall, while the days are still quite long, and growth cessation is induced later in the fall in trees from southern latitudes, when the days are considerably shorter.

   In the study described in Paper III we investigated differences in critical daylength for growth cessation and bud set in European aspen (*Populus tremula*) originating from different latitudes (provenances) and the regulatory mechanism controlling the variations. The critical daylength varied from 21 hours for the provenance from northern Sweden to 15 hours for the German provenance. This difference has been shown to be under strong genetic control and is maintained when trees are transferred between latitudes (Howe et al., 1996a; Frewen et al., 2000). Based on our results we proposed a model for the mechanism whereby the variation in critical daylength is controlled, in which the *PtFT/PtCO* regulon plays a central role. According to this model the Arabidopsis *CO* homolog in *Populus trichcarpa*, *PtCO2*, displays a diurnal expression pattern, expression levels peaking at the end of the day. *PtCO2* analysis showed that expression of the gene starts to increase six hours earlier in the southernmost provenance than in the northern provenance. This means that when the provenances are grown under

conditions within a certain range of daylengths, *PtCO2* expression in the northern provenance will peak in the dark, *PtFT1* expression will not be induced and growth cessation will be induced, but in the southernmost provenance the *PtCO2* expression peak will coincide with the light period, *PtFT1* diurnal expression will be activated and growth cessation will be repressed. The importance of *PtCO2* and *PtFT1* transcriptional levels for the timing of growth cessation was further corroborated by the findings that transgenic plants with reduced levels of *PtCO2* and *PtFT1* were hypersensitive to changes in daylength, and overexpression of *PtFT1* resulted in plants being insensitive to changes in daylength.

   The function of the *CO/FT* regulon in *Populus* appears to be similar to its function in *Arabidopsis*, in which the peak in *CO* expression needs to coincide with light in order to activate *FT* transcription (Yanovsky and Kay, 2002; Searle and Coupland, 2004). These results suggest that the photoperiodic pathway may have two functions in *Populus*: initiating flower development in spring as the days get longer, and controlling growth cessation in fall as the days get shorter. In *Populus*, overexpression of oat PHYA (*OPHYA*) resulted in plants insensitive to changes in daylength (Olsen et al., 1997b). Our results suggest that this effect is at least partly mediated through an inability to downregulate *PtFT1* expression (Paper III). This could be a result of a shift in the phase of *PtCO* expression or, more likely, an alteration of CO protein stability and/or activity.

Regulation of *PtCO2* in *Populus* trees (Paper IV)

Knowledge about the regulation of *PtCO* is important for understanding the daylength measurement mechanism. We have identified *Populus* trees with differences in critical daylength that are controlled by variations in the *PtCO2* expression peak, but the mechanism responsible for this variation is unknown. In *Arabidopsis*, the expression of *CO* is under control of the circadian clock and alterations within the circadian clock often affect photoperiodic responses such as flowering through *CO* expression (Blazquez and Weigel, 1999; Suarez-Lopez et al., 2001; Doyle et al., 2002a; Yanovsky and Kay, 2002).

   In the study described in Paper IV we performed a detailed analysis of diurnally regulated genes in an attempt to identify genes responsible for the variations in critical daylength and the *PtCO* expression peak. Our results revealed that in northern and southern trees the circadian clock and two clock-regulated outputs that are not connected to growth cessation have similar rhythms, suggesting that the variation in the *PtCO2* expression peak is not caused by variation in the rhythms of the circadian clock.

   In *Arabidopsis* the connection between the circadian clock and the photoperiodic pathway has been suggested to be mediated through GI since a *gi* mutant is late flowering and the expression of *CO* is repressed throughout the entire day (Suarez-Lopez et al., 2001). We therefore isolated a putative *GIGANTEA* homolog in *Populus* (*PtGI*) and analyzed its expression in *P. tremula* trees originating from different latitudes. The diurnal expression pattern of *PtGI* clearly reflected the latitude of origin, since its expression started to rise earlier in the more southern provenances. Similarly, the expression of *PtCO2* displayed an

increase that was correlated with the variation in *PtGI* expression, suggesting that the timing of *PtGI* expression is important for regulation of the *PtCO2* expression peak. The role played by *PtGI* expression in the control of growth cessation and timing of *PtCO2* phasing was further investigated by examining *PtGI* RNAi plants with reduced *PtGI* expression levels. These transgenic plants initiated growth cessation and bud set in long days, in a similar fashion to *PtCO2* and *PtFT1* RNAi plants, and the expression of both *PtCO2* and *PtFT1* in the *PtGI RNAi* plants was strongly affected, with no sign of diurnal expression patterns.

To summarize, these results suggest that the variation in critical daylength displayed by *Populus tremula* trees originating from different latitudes is sequentially mediated by *PtGI*, *PtCO* and *PtFT1*, and that *PtGI* provides an important connection between the circadian clock and photoperiodic responses in *Populus* trees. Since GI is a nuclear protein with unknown function it is possible that *PtGI* plays a role in recruiting other transcription factors that are important for the regulation of *PtCO2*.

Summary and future plans

The work presented here was undertaken to deepen our understanding of the ways in which plants control flowering and their annual cycles of active growth and dormancy. In *Arabidopsis* plants grown under short days the studies show that flowering initiation is preceded by dramatic increases in GA_4 and sucrose levels in the shoot apex (Paper II). In addition, the GA measurements revealed that GA_4 was the most abundant gibberellin in the shoot apex at the time of flowering initiation and the most potent endogenous gibberellin for inducing *LEAFY* expression and flowering in short days (Papers I and II). The results indicate that GA activates *LFY* expression in the presence of a translational inhibitor, implying that all of the components required for activation are already present within the plant and that *LFY* is regulated by a labile negative regulator (Paper I). These results further suggest that the function of the negative regulator could be to repress the activity of an apex-specific activator, and that the negative regulator acts independently from the long-day pathway and the gibberellin signaling pathway. To further explore the properties of the role of GA in the regulation of flowering under short days it would be interesting to analyze the amounts of GA and sucrose in the apex during flowering initiation in some of the late flowering mutants with lesions in the autonomous pathway to discover if later upregulation of GA levels is correlated to the late flowering phenotype in these mutants. The labile negative regulator has not yet been identified, but a possible strategy to identify a "repressor mutant" would be to create a transcriptional fusion between the *LFY* promoter and luciferase, mutagenize seed from the transgenic plants by EMS treatment, and screen the resulting seedlings for individuals with high levels of luminescence.

Trees have a long juvenile phase before flowering is initiated, and the mechanism regulating this transition is unknown. In the study reported in Paper III we cloned and overexpressed the *FT* homolog (*PtFT1*) in juvenile *Populus* trees. This resulted in early flowering trees with normal flower development and transgenic trees that were insensitive to changes in daylength. Improving growth

conditions or developing alternative ways to modulate *PtFT1* expression should increase the possibility to perform successful crosses with transgenic, early flowering *PtFT1* trees.

Based on the results presented in Papers III and IV we proposed a model explaining how trees may measure daylength and control their critical daylength (and hence the variations amongst *Populus tremula* trees from different latitudes in this respect). This model involves diurnal expression of genes and light-dependent activation of gene products. In *Populus tremula* trees originating from northern and southern latitudes the circadian clock displays similar rhythmic oscillations. The circadian clock sets the diurnal expression of *PtGI*, the expression of which varies diurnally and peaks earlier in trees originating from southern latitudes than in trees from northern latitudes. *PtGI* then regulates the expression of *PtCO2*, which has a similar diurnal expression pattern to *PtGI*, with an earlier expression peak in southern trees than in northern trees. This means that when trees are grown within a certain range of daylengths, *PtCO* expression peaks in the dark in northern trees, *PtFT1* expression is not activated, and growth cessation is not repressed, while in southern trees, *PtCO* expression peak occurs in the light, *PtFT* expression is active, and growth cessation is repressed. Therefore, these results suggest that the photoperiodic pathway *PtGI-PtCO-PtFT* plays a role in the regulation of both flowering and timing of growth cessation. These results further suggest that *PtGI* provides an important connection between the circadian clock and the photoperiodic pathway in *Populus* trees.

Using trees representing the natural variation in critical daylength and growth cessation we could further investigate and explore the fine-tuning of mechanisms whereby plants measure daylength and differences in critical daylengths evolved under the selective pressure to adapt to growth at different latitudes.

One strategy to identify new components involved in the regulation of critical daylength would be to use microarrays to compare the expression of diurnally regulated genes between trees originating from different latitudes. Since *PtGI* probably plays an important role in connecting the circadian clock and photoperiodic responses, it will be of great interest to see if sequence variations in the *PtGI* promoter between different aspen tree provenances can explain some of the natural variation in the phase of *GI* expression. Further investigations of allelic distributions of genes known to be involved in the regulation of photoperiodic responses in the F1 population could possibly identify the dominant allele(s) responsible for the shifts in phase of *PtGI* and *PtCO2* expression.

Unravelling the mechanisms underlying the control of flowering time in trees should greatly accelerate current tree breeding programs, and facilitate rapid marker-assisted breeding for producing varieties with new and better wood properties. The new knowledge of the molecular mechanisms controlling the timing of growth cessation should also facilitate attempts to breed trees capable of adapting to changing climates and elite trees capable of growing at different latitudes.

Acknowledgments-Tack

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