Plant Mol Biol (2010) 73:143–156
DOI 10.1007/s11103-010-9619-2

Alteration of *PHYA* expression change circadian rhythms and timing of bud set in *Populus*

Iwanka Kozarewa · Cristian Ibáñez · Mikael Johansson · Erling Ögren · David Mozley · Eva Nylander · Makiko Chono · Thomas Moritz · Maria E. Eriksson

Received: 31 August 2009 / Accepted: 20 February 2010 / Published online: 13 March 2010

Abstract In many temperate woody species, dormancy is induced by short photoperiods. Earlier studies have shown that the photoreceptor phytochrome A (phyA) promotes growth. Specifically, *Populus* plants that over-express the oat *PHYA* gene (*oatPHYAox*) show daylength-independent growth and do not become dormant. However, we show that *oatPHYAox* plants could be induced to set bud and become cold hardy by exposure to a shorter, non-24 h diurnal cycle that significantly alters the relative position between endogenous rhythms and perceived light/dark cycles. Furthermore, we describe studies in which the expression of endogenous *Populus tremula* × *P. tremuloides PHYTOCHROME A* (*PttPHYA*) was reduced in *Populus* trees by antisense inhibition. The antisense plants showed altered photoperiodic requirements, resulting in earlier growth cessation and bud formation in response to daylength shortening, an effect that was explained by an altered innate period that leads to phase changes of clock-associated genes such as *PttCO2*. Moreover, gene expression studies following far-red light pulses show a phyA-mediated repression of *PttLHY1* and an induction of *PttFKF1* and *PttFT*. We conclude that the level of *PttPHYA* expression strongly influences seasonally regulated growth in *Populus* and is central to co-ordination between internal clock-regulated rhythms and external light/dark cycles through its dual effect on the pace of clock rhythms and in light signaling.

Keywords Growth cessation · Dormancy · Circadian clock · Photoperiodism · Phytochrome · Populus

Abbreviations

CaMV	Cauliflower mosaic virus
CDL	Critical daylength
LL	Constant light
DD	Constant dark
FR	Far-red
LD	Long day

Iwanka Kozarewa and Cristian Ibáñez authors contributed equally to this manuscript.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-010-9619-2) contains supplementary material, which is available to authorized users.

I. Kozarewa · C. Ibáñez · M. Johansson · E. Nylander · M. E. Eriksson (✉)
Department of Plant Physiology, Umeå Plant Science Centre, Umeå University, 901 87 Umeå, Sweden
e-mail: maria.eriksson@plantphys.umu.se

E. Ögren · D. Mozley · M. Chono · T. Moritz
Department of Forest Genetics and Plant Physiology, Umeå Plant Science Centre, Swedish University of Agricultural Sciences, 901 83 Umeå, Sweden

Present Address:
I. Kozarewa
Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire CB10 1SA, UK

Present Address:
C. Ibáñez
Facultad de Ciencias, Departamento de Biología, Universidad de La Serena, Casilla 599, Benavente 980, La Serena, Chile

Present Address:
M. Chono
Research Team for Wheat and Barley Biotechnology, National Institute of Crop Science, National Agriculture and Food Research Organization, 2-1-18 Kannondai, Tsukuba 305-8518, Japan

SD Short day
ZT Zeitgeber time

Introduction

Circadian rhythms persist in constant conditions with a period close to 24 h (Dunlap 1999). To track the time of the year accurately, plants measure daylength. According to the external coincidence model proposed by Bünning (1936), these measurements require an endogenous circadian oscillator that is reset daily to local light conditions (entrained), and decides a light-sensitive phase of the day (reviewed in Thomas and Vince-Prue 1997). In plants that are induced to flower under long days (LDs), a long photoperiod will illuminate this critical phase and result in flowering, while under short days (SDs) flowering is inhibited when darkness is perceived at this point.

The timing of the peak expression (phase) of *CONSTANS* (*CO*) in relation to light follows the external coincidence model and determines the flowering response in *Arabidopsis*: if expressed in light it triggers the expression of *FLOWERING LOCUS T* (*FT*), which induces flowering (Putterill et al. 1995; Samach et al. 2000; Suárez-López et al. 2001; Roden et al. 2002; Yanovsky and Kay 2002; Valverde et al. 2004; Corbesier et al. 2007). In hybrid aspen (*Populus tremula* × *P. tremuloides, Ptt*), LDs sustain shoot elongation and trees stop growth and set bud only when daylength falls below the critical daylength (CDL). In our study, CDL is the photoperiod that marks the transition between active growth to cessation of growth and bud set. Growth cessation refers to the stopping of internode elongation before bud set; in deciduous trees, growth cessation is induced by shortening daylength and down-regulation of active gibberellins (Olsen et al. 1995, 1997; Eriksson and Moritz 2002). Growth cessation and the formation of terminal buds is a prerequisite for cold-acclimation and freezing tolerance (Olsen et al. 1997). *PttCO* and *PttFT* have recently been shown to promote growth in aspen (Böhlenius et al. 2006), when their expression coincide with light, providing a molecular frame work for further understanding daylength control of growth.

Rhythmic outputs from the endogenous oscillator(s), such as gene expression or leaf movement under constant conditions, are used to monitor the function of the internal clock (Millar et al. 1995; Swarup et al. 1999). In *Arabidopsis*, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1,* Wang and Tobin 1998) and *LATE ELONGATED HYPOCOTYL* (*LHY*, Schaffer et al. 1998) encode single-Myb domain transcription factors that oscillate in a circadian fashion, with peaks of mRNA and protein abundance in the early morning (Salome and McClung 2005). They form part of a negative-feedback loop with *TIMING OF CAB EXPRESSION 1 (TOC1)* (Alabadí et al. 2001; Perales and Más 2007). *TOC1* encodes a nuclear protein containing a receiver domain similar to those found in plant response regulators (Strayer et al. 2000). The peaks in the abundance of both mRNA and protein expressed from *TOC1* occur at dusk, approximately 180° out of phase with *CCA1* and *LHY* (Salome and McClung 2005).

Mutations in genes that are constituents of the circadian clock often result in changes in flowering time in *Arabidopsis*. Such effects have been attributed to changes in the timing of *CO* expression (Roden et al. 2002; Yanovsky and Kay 2002). In studies on the *toc1-1* mutant, the circadian period was shortened to 21 h, resulting in *CO* expression occurring during light periods under both LD and SD, and consequently, photoperiod-independent flowering (Yanovsky and Kay 2002). However, when grown under light with dark cycles matching the endogenous cycle of 21 h, timing of flowering in *toc1-1* did not differ from wild type (WT) plants (Yanovsky and Kay 2002). Roden et al. (2002) showed that growing *Arabidopsis* WT plants with external cycles shorter or longer than 24 h resulted in later and earlier phasing of *CO* expression, with flowering accelerated only when *CO* expression coincided with the light period. Together these data illustrate the importance of the circadian system in synchronization of seasonal events.

In *Arabidopsis*, the light dependent *FLAVIN-BINDING KELCH DOMAIN F BOX PROTEIN 1* (*FKF1*) was found to control flowering time by regulation of *CO* expression through degradation of a CO repressor (CDF1; Imaizumi et al. 2005). In addition, FKF1 and GIGANTEA (GI) interact in response to blue light and to regulate the timing of daytime *CO* expression (Sawa et al. 2007). However, recent studies (Salazar et al. 2009) suggest a *CO transcription independent* role of FKF1 in regulation of the level of expressed *FT*. Both *FKF1* and *GI* expression is under circadian clock regulation, possibly by a binding motif called an evening element (EE, AAAATATCT) in their promoters (Harmer et al. 2000; Imaizumi et al. 2003). The EE is sufficient for *CCA1/LHY* binding (Harmer et al. 2000; Perales and Más 2007).

Photoreceptors are essential for the plant's detection of its light environment. There are several families of photoreceptors in *Arabidopsis*, including the red (R)/far-red (FR) monitoring phytochromes (phy) A through E (Clack et al. 1994) and the blue light absorbing cryptochromes (cry) 1 and 2 (Lin et al. 1996; Lin et al. 1998). Light received by crys and phys synchronizes the circadian clock to that of the solar day (Devlin 2002; Somers et al. 1998).

Decreasing levels of individual receptors will result in less light being received by the circadian oscillator and hence a lengthening in period. *Arabidopsis* plants deficient in phyA have longer periods of *CHLOROPHYLL A/B*

BINDING PROTEIN (*CAB2*) expression than WT in low red and blue light (Somers et al. 1998). *phyA* mutants also flower later than WT plants when SDs of fluorescent light are extended with several hours of light rich in FR light, which promotes flowering (Johnson et al. 1994; Yanovsky and Kay 2002). Thus, a reduction in phyA level results in changes in period length of daily clock-controlled rhythms, as well as seasonally regulated traits such as flowering. Phytochrome A also regulates the binding of the CCA1 protein to the *CAB2* promoter in order for it to robustly cycle in a circadian manner (Wang and Tobin 1998; Tepperman et al. 2001), providing a direct molecular link between the light reception and light modification of output clock rhythms.

Populus has three phys: phyA, phyB1, and phyB2 (Howe et al. 1998). Over-expression studies of oat phyA have implicated phyA in growth cessation and bud set (Olsen et al. 1997). Growth in hybrid aspen is controlled in a manner reminiscent of flowering in *Arabidopsis*, which is supported by the loss of short day induction of bud set in oat phyA over-expressers, night break experiments (Olsen et al. 1997), and the function of *CO/FT* (Böhlenius et al. 2006). These findings, together with the fact that many genes involved in seasonal regulation of flowering and bud set are regulated in a circadian fashion, led us to investigate circadian clock function and seasonal regulation of growth in hybrid aspen with reduced expression of *PttPHYA*. Such plants showed lengthening of circadian clock-controlled gene expression and leaf movements rhythms under constant conditions. We also found that a FR pulse induced a significantly higher *PttLHY1* expression in antisense *PttPHYA* lines compared to WT, suggesting that period lengthening is due to modulated *PttLHY* expression. The increase in the length of the circadian period changed the coincidence of light with the *PttCO2* expression and consequently led to an altered *PttCO2* expression in response to SD as well as a reduced induction of *PttFT* upon FR treatment. Thus, a reduction in phyA signaling fail to support growth under SD and FR supplemented light and is sufficient to explain the earlier growth cessation and bud set detected in the antisense lines.

Results

Identification of antisense lines with reduced expression of *PttPHYA*

PttPHYA was introduced into hybrid aspen in reversed orientation (antisense) behind the 35S cauliflower mosaic virus (CaMV) using *Agrobacterium*-mediated transformation; seven independent lines were obtained. The critical day length (CDL) for growth cessation and bud formation for hybrid aspen clone T89 is 15.5 h (Olsen et al. 1997). Strong lines featured compact growth and apical bud formation under tissue culture conditions (16 h light/8 h dark; Supplemental Fig. 1a). To accurately assess the level of expressed *PttPHYA* in the obtained putatively down-regulated lines, denoted *aPttPHY*A, the expression of remaining sense *PttPHYA* was quantified in leaves harvested under long day conditions (LD, 18 h light/6 h dark) by anti-sense RNA ribo probes using Northern blot. The transcript levels were reduced in all lines and ranging from just 30–80% of WT levels in *aPttPHYA-7* and *-5* (Supplemental Fig. 1b). The expression of endogenous *PttPHYA* was low, but readily detectable throughout the plant in all tissues and photoperiods assayed (Supplemental Figure 2a, b). For initial growth experiments, all lines were used. For additional analyses, we selected lines based upon their *PttPHYA* expression and growth performance, and later focused on *aPttPHYA-1* and *-5*, which represented strong and moderate growth phenotypes.

Strong down-regulation of *PttPHYA* causes earlier timing of growth cessation and bud set under CDL

The effects of photoperiod on height increment under growth-permissive (LD) conditions were first tested using all seven lines (Supplemental Table 1). In general, *aPttPHYA* lines did not differ from WT under LD with respect to elongation.

In response to a decreasing photoperiod, two growth stages were recorded: cessation of internode elongation and then bud set (Figure 1). The time to bud set at CDL conditions (15 h light/9 h dark) was compared between the selected *aPttPHYA* lines *-1*, *-2*, *-9*, and WT. All three transgenic lines formed buds significantly earlier than WT (Supplemental Table 2). The timing of bud set in this experiment and the expression level of *PttPHYA* in these lines had a correlation coefficient (R^2) of 0.985. In addition, an experiment with *aPttPHYA-1* and *-5* and WT confirmed that *aPttPHYA-1* ceased growth and formed buds earlier than WT and *aPttPHYA-5* at CDL (Supplemental Table 2).

Under SD even moderate down-regulation of *PttPHYA* leads to an earlier growth cessation

The timing of growth cessation/bud set, as well as height increment and increase in leaf number, was examined in *aPttPHYA-1*, *aPttPHYA-5*, and WT after a shift from near CDL (16 h light/8 h dark) to SD conditions (12 h light/12 h dark). Under these conditions, both lines show growth cessation and bud set significantly earlier than WT (Table 1). Height increment and leaf number were slightly but significantly reduced in *aPttPHYA-5* compared to WT, whereas there were no statistical difference between the two transgenic lines.

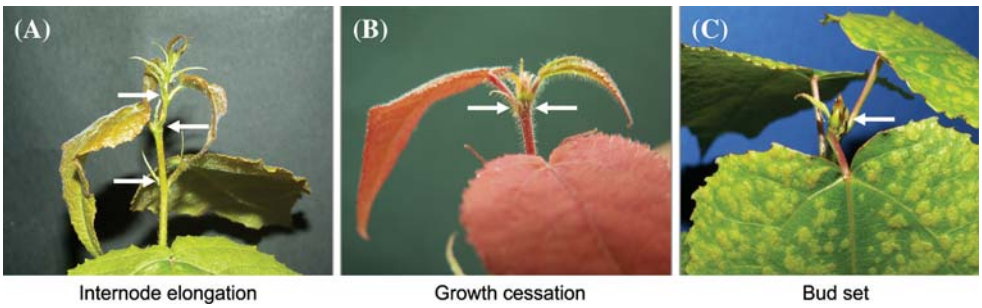

Fig. 1 Stages of growth in response toward SDs. An actively growing plant showing internode elongation (*arrows* **a**), growth cessation where internode elongation has stopped (*arrows* **b**), and bud set (bud indicated by *arrow* **c**)

Table 1 Growth response of *PttPHYA*-antisense and wild type plants under SD (12 h light/12 h dark) or SD followed by 3 h enriched in FR (SD + FR)

Line	Days to stop		Days to bud set		Height increment		Leaf number	
	SD	SD + FR	SD	SD + FR	SD	SD + FR	SD	SD + FR
WT	20.0	26.3	29.2	31.2	10.2	23.2	9.2	6.7
a-1	14.0***	16.4*	20.0***	20.0*	8.4[§]	22.5	8.0[§]	6.0
a-5	14.0***	16.4*	20.0***	22.6[§]	7.8*	27.6	6.8*	6.0

Height increment (cm), increase in leaf number, time to growth cessation (time to stop), and visible bud formation (time to bud set) were recorded. Before the shift, plants were grown at 16 h light/8 h dark (both treatments), and initially 4 h and 30 min of FR enriched light (only SD + FR treatment)

WT wild type

aPttPHYA lines *a-1*, *a-5*

Data were analyzed using ANOVA. Means were significantly different from WT according to Fisher's protected LSD tests at confidence level 0.001 (***), 0.01 (**), and 0.05 (*) as noted. [§] Indicates lack of difference between the two *aPttPHYA*, $n = 5$–6

When SDs were extended with 4.5 h of FR-enriched light (SD + FR), all genotypes responded to this end-of-day (EOD) FR treatment with fast elongation and reduced leaf number. However, as no genotype showed signs of stopping growth, the extension was shortened to 3 h. There were no differences between genotypes in height increment under either extension (Table 1). However, the 3 h extension led to a significantly earlier growth cessation in both the strong *aPttPHYA-1* and the more moderately down-regulated *aPttPHYA-5* line, with a significantly early bud set in *aPttPHYA-1*. In addition to the differential growth cessation response between genotypes, all plants independent of their growth cessation and bud set phenotype showed an increased height and lower number of leaves under SD + FR, suggesting a still functional shade avoidance response in these plants, which is mediated by stable phys (Table 1). A similar EOD FR response was found previously to be functionally independent of the insensitivity towards photoperiod in *oatPHYAox* plants (Olsen et al. 1997; Olsen and Junttila 2002).

This experiment demonstrated that under SD conditions or SD with EOD FR, there were significant difference between *aPttPHYA* lines and WT in the timing of growth cessation, and the response of strong *aPttPHYA-1* line and the moderately down-regulated *aPttPHYA*-5 were comparable under both conditions. However, when SD is combined with an EOD FR extension, growth of WT plants is better sustained, as shown by the increase in days required to stop growth or set buds when receiving EOD FR. In contrast, the *aPttPHYA* require about the same number of days under both treatments. Hence, there is dependence on *PttPHYA* expression and putatively phyA activity in maintaining growth, which varies with light period and quality as shown by the response of plants with down-regulated *PttPHYA* expression towards SD and SD with EOD FR.

A 6 h light/6 h dark cycle induces growth cessation and installs winter hardiness in *oatPHYA* over-expressing line

To explore the dependence on the coincidence between endogenous rhythms and external light conditions for growth, we tested if short light/dark cycles would induce growth cessation in aspen trees. We first entrained WT, *aPttPHYA-1*, *aPttPHYA-5*, and *oatPHYA* over-expressing line 22 (*oatPHYAox*; Olsen et al. 1997) to a 12 h light/12 h dark (24 h cycle), followed by a 6 h light/6 h dark (12 h

cycle) regime. We hypothesized that the phasing of endogenous rhythms and the external light/dark will be drastically changed under these conditions and may lead to growth cessation and bud set even in the *oatPHYAox* line, which were shown not to terminate growth in a 24 h cycle of even 6 h light/18 h dark (Olsen et al. 1997). In particular, any phase between a clock regulated endogenous factor in the *oatPHYAox* and the external 24 h light/dark cycles that may be required for growth could be significantly altered (disrupted) under the shorter 12 h cycle and perhaps permit growth cessation and bud set to occur. Similar schemes have been successful in modifying flowering response in *Arabidopsis* (Roden et al. 2002; Yanovsky and Kay 2002).

Initially, all plants were growing, while already after 63 days WT and *asPHYA-1* were showing clear signs of growth cessation and bud formation, in contrast to *oatPHYAox* plants (Fig. 2; Supplemental Fig. 3). The speed of growth cessation and bud set response depends on the daylength conditions and under SD (12 h light/12 h dark) WT took 29 days in our conditions (Table 1) to reach complete bud set. When grown under near CDL conditions all WT plants had fully formed and hardened buds after 40 days (Supplemental Table 2). We found that this process took about 80 days under 6 h light/6 h dark for WT plants.

Within 112 days under these conditions, all plants, including *oatPHYAox*, were able to stop growth and 80% of *oatPHYAox* to set an apical bud (Fig. 2). Overexpressers were significantly shorter and produced less leaves than the other genotypes. Overall, genotype significantly effected height and leaf number (ANOVA height; genotype: $F = 37{,}1$, 2 *df*, $P = 0$; ANOVA leaf number; genotype: $F = 168{,}1$, 2 *df*, $P = 0$). There were no significant differences between WT and *aPttPHYA-1*; however, *oatPHYAox* were significantly different from both ($P = 0$). A significantly reduced stature of *oatPHYAox* was also reported under external cycles of 24 h (Olsen et al. 1997); therefore, this phenotype was apparently not much affected by the change in external light/dark cycle.

Since proper growth cessation and bud set is required to acquire freezing resistance, we tested if *oatPHYAox* that had stopped growing were also capable of cold hardening. Indeed, under the 6 h light/6 h dark regime, these plants developed marginally inferior levels of cold hardiness compared to WT and *aPttPHYA-1* lines (Fig. 3). In contrast, under the 12 h light/12 h dark regime, *oatPHYAox* did not seem to undergo any cold hardening at all, whereas WT and anti-sense lines were equally competent (cf. Fig. 3a, b). Thus, the growth cessation/bud set and hardiness properties depend on accurate timing between an endogenous rhythm and external light/dark conditions, results that are clearly evident in the way *oatPHYAox* plants responded.

The expression of genes involved in daylength detection is altered in *aPttPHYA* plants under SD

The circadian clock is likely to be functioning in the detection of daylength, specifically by timing the regulation of down-stream components that directly control such events. In *Populus*, there are two *LHY*- like and one *TOC1*-like genes, identified by sequence homology of *Arabidopsis* counter parts (Takata et al. 2009; Ramírez-Carvajal et al. 2008), that may be involved in the central clock mechanism. Due to the strong sequence and expression

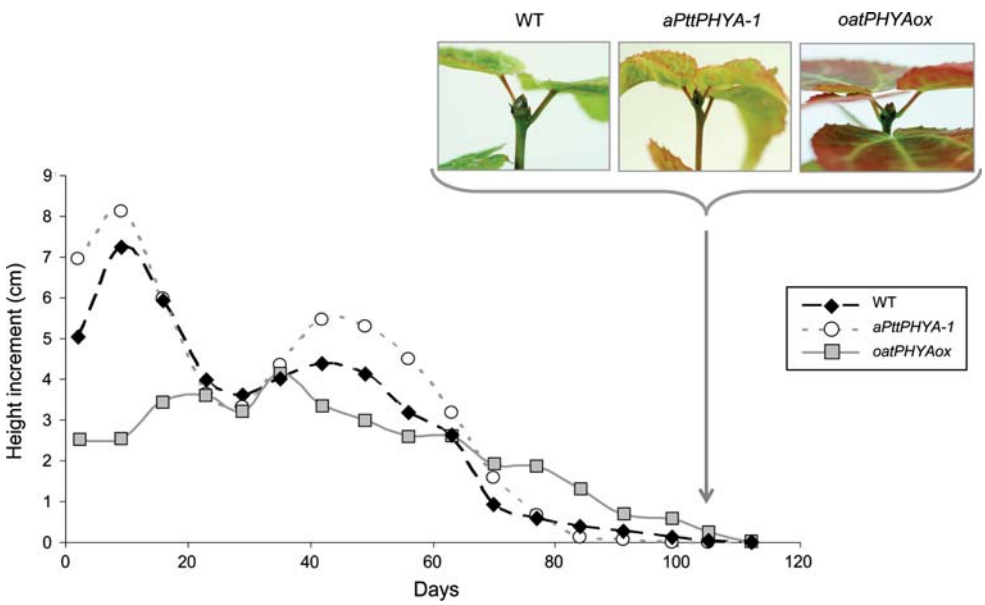

Fig. 2 Mean of weekly height increment of WT, *aPttPHYA-1*, and *oatPHYAox* lines grown under a 6 h light/6 h dark cycle. Plants were grown under 12 h light/12 h dark for 12 days, followed by 4 days in continuous light before the shift to 6 h light/6 h dark. Increments shown are mean of 7–11 plants per genotype

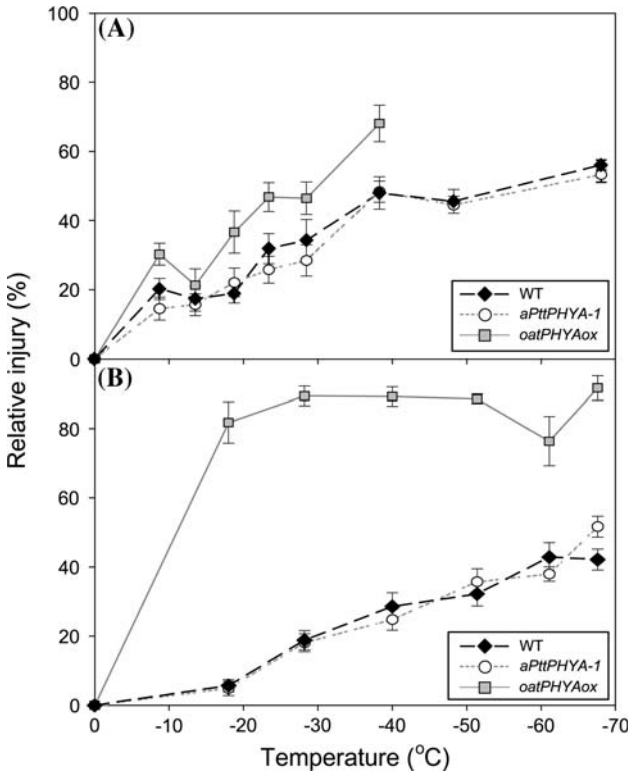

Fig. 3 Results of freeze tests using the electrolyte leakage method to assess injury of stems in WT (*closed diamond*), *aPttPHYA-1* (*open circle*), and *oatPHYAox* (*open square*) plants following cold hardening at **a** 6 h day/6 h night and **b** 12 h day/12 h night. Mean ± SE values are shown for seven replicate plants

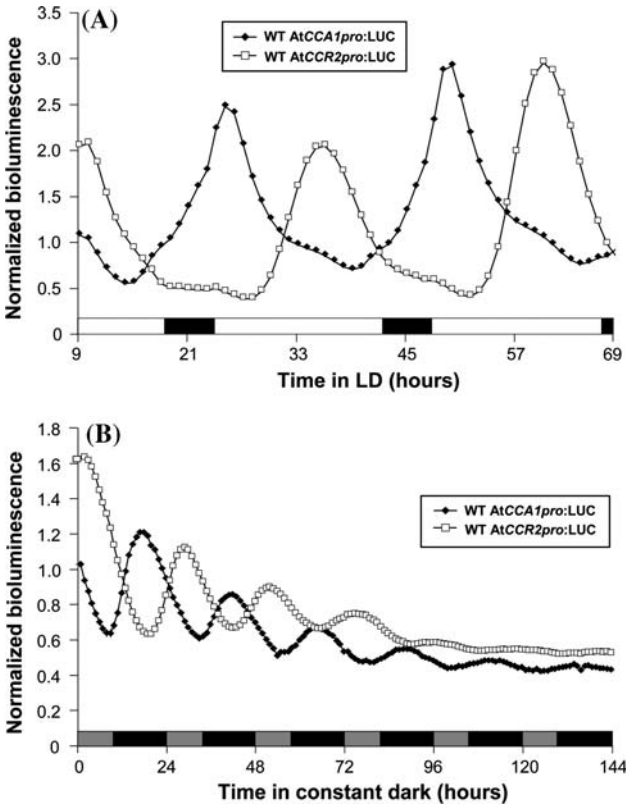

Fig. 4 Expression of the *AtCCA1pro:LUC* and *AtCCR2pro:LUC* transgene in WT. Luminescence from WT plants carrying the transgenes was detected under LD (18 h light/6 h dark, *red* and *blue* LEDs 20 μmol m^{-2} s^{-1}) (**a**) and following a shift to constant dark after entrainment under SD (8 h light/16 h dark, *red* and *blue* LEDs 20 μmol m^{-2} s^{-1}) (**b**). Normalized average luminescence from at least two independently transformed lines and cuttings ($n = 2$–4 replicates per line) is shown for each genotype. *Grey bars* indicate subjected light period and *black bars* indicate subjective dark period

profile similarity between *PttLHY1* and *PttLHY2* and their combined expression, *PttLHY* was examined with primers binding to both genes.

Clock-controlled gene expression provides an out-put that can be used to follow the innate period of rhythms in plants. Two robust promoter:reporter constructs that have previously been used in *Arabidopsis* for this purpose are the *Arabidopsis thaliana CIRCADIAN CLOCK ASSOCIATED 1* (*AtCCA1*) and *COLD CIRCADIAN RHYTHM RNA BINDING 2* (*AtCCR2*) promoter fused to firefly *LUCIFERASE (LUC)* (Wang and Tobin 1998; Strayer et al. 2000). We introduced both *AtCCA1pro:LUC* and *AtCCR2pro:LUC* transgenes into WT *Populus* plants by *Agrobacterium* mediated transformation. To investigate if they function in *Populus*, the resulting luminescence was followed under LD and under constant dark following SD entrainment (Fig. 4a, b). We found that under LD the expression of *AtCCA1pro:LUC* and *AtCCR2pro:LUC* expression, as determined by BRASS on the second peak under LD, was found at 2 h after dawn (Zeitgeber time; ZT2) and ZT13 similar to previous studies in *Arabidopsis* (McWatters et al. 2000; Kreps and Simon 1997). Moreover, under constant darkness (DD) *AtCCA1pro:LUC* and *AtCCR2pro:LUC* reported an internal mean period from 3 and 2 independently transformed lines ($n = 2$–4 per line) of 23.7 h ± 0.1 (SEM) and 23.5 h ± 0.2 as expected for a circadian (~24 h) rhythm.

In response to two short days (SD; 12 h light/12 h dark), the expression of *PttLHY* was highest immediately after lights-on and lowest at ~12 h at ZT12 for all genotypes (Fig. 5a). The lowest point of expression was in the *aPttPHYA* lines adjusted to last longer (increase later), suggesting a slight delay of *PttLHY* expression in these lines.

Two peaks of expression of *PttTOC1* were found at ZT4 and ZT12 in WT and only at ZT8 for *aPttPHYA-5* with a low and arrhythmic expression in the stronger anti-sense line *aPttPHYA-1* (Fig. 5b).

Daily peaks of *PttLHY* and *PttTOC1* expression under SD were out of phase with each other, results that were similar to what was reported for chestnut (Ramos et al. 2005). However, the double peak of *PttTOC1* expression in WT suggests that those plants may be still entraining and were not yet fixed to a single phase (Fig. 5b).

There are two putative homologues of *PttFKF1* in *P. trichocarpa* and only one was detected under our

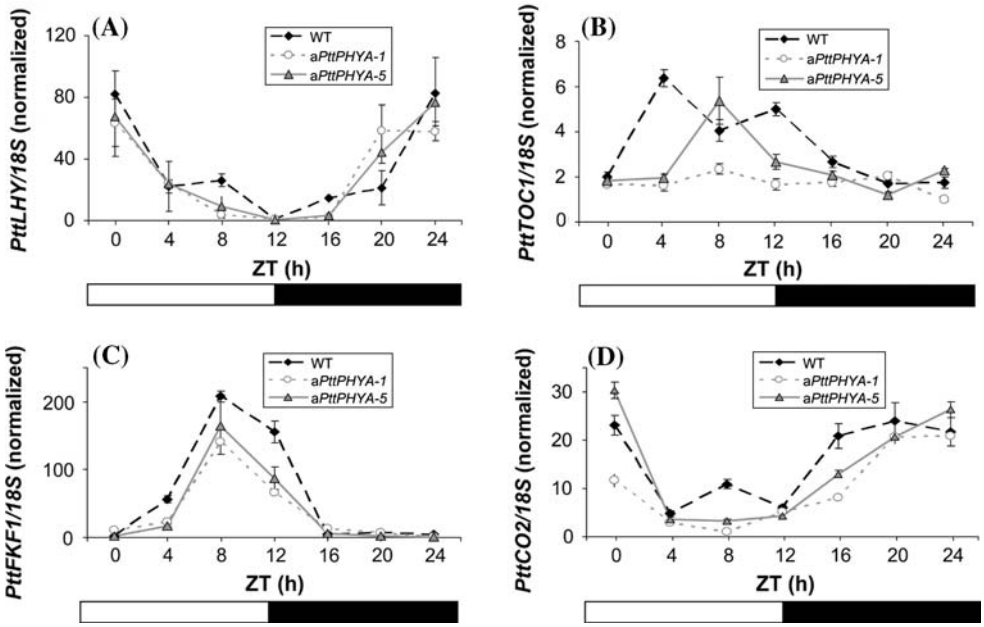

Fig. 5 Expression profiles of putative circadian clock and clock-controlled genes in leaves from plants under SD conditions (12 h light/12 h dark). WT and *aPttPHYA* plants were shifted to SD 2 days before sampling. Expression of *PttLHY* (**a**), *PttTOC1* (**b**), *PttFKF1* (**c**), and *PttCO2* (**d**) is presented as a proportion of the lowest value after standardization with respect to *18S rRNA* (*18S*). Mean ± SE of three biological replicates are shown

conditions. Analysis of its promoter revealed two evening elements, prompting us to investigate its expression using quantitative real-time PCR (qRT-PCR). *PttFKF1* expression peaked at ZT8 in all genotypes, but its level of expression was reduced in the *aPttPHYA* lines (Fig. 5c).

To investigate if the pattern of Ptt*CO2* could explain the altered photoperiodic response of the *aPttPHYA* plants (Table 1), we investigated the expression of *PttCO2* in WT and *aPttPHYA* plants under SD conditions (Fig. 5d). Our results showed that while WT *PttCO2* expression peaked at ZT0/24 and ZT8, *aPttPHYA* lines only showed expression at ZT0/24, suggesting that the *aPttPHYA* plants had down-regulated *PttCO2* only after two SDs. The peak at ZT8 is reminiscent of the FKF1 dependent peak that is detected in *Arabidopsis* following SD shifts (Salazar et al. 2009). The ZT8 peak is completely lacking and the phase of *PttCO2* expression shifted later in the *aPttPHYA* lines, results that suggest *PttPHYA* expression is needed to support its expression. These results demonstrate that the altered photoperiodic timing of growth cessation and bud set in the antisense lines is accompanied by a reduced *PttTOC1*, *PttCO2*, and *PttFKF1* expression, most notably following two SDs.

The circadian period varies with expressed *PHYA* levels

Since the circadian clock is likely to control the phase of expression of the photoperiodic genes *PttCO2* and *PttFKF1*, we probed the circadian clock function. Circadian period can be estimated by measurements of clock-controlled rhythms in the movements of *Arabidopsis* leaves (Swarup et al. 1999). We measured such movements from *Populus* leaves under continuous light (LL) using WT, *oatPHYAox* line 22, and *aPttPHYA-1*. Using one way ANOVA, these studies revealed that the mean period of leaf movements was significantly shorter in *oatPHYAox* ($F = 5.2$, 1 *df*, $P < 0.05$) and longer in *aPttPHYA-1* ($F = 9.2$, 1 *df*, $P < 0.01$) relative WT plants (Fig. 6a). The robustness of rhythmic leaf movements is shown (Relative Amplitude Errors, RAE > 0.6; Fig. 6b).

To confirm the lengthening of circadian periodicity found in *aPttPHYA-1* plants, the *PttLHY* expression was assayed under LL. We found there were delayed peaks in the expression of *PttLHY* in both *aPttPHYA-1* and *aPttPHYA-5*, compared with WT (Fig. 7). When the expression of *PttLHY1* and *PttLHY2* was assessed independently, similar profiles and delays were detected for both genes (data not shown). The phase changes in *PttLHY* expression found under these conditions suggest a lengthening of the internal period in lines with reduced levels of *PttPHYA* under light and supports the long period of leaf movements. We also found *PttFKF1* to be altered under these conditions, supporting a link between *PttLHY* and *PttFKF1* (Supplemental Fig. 4).

To follow a circadian out-put in constant darkness, the *AtCCR2pro:LUC* transgene was also introduced to the transgenic *aPttPHY-1* and *aPttPHY-5* lines. Following entrainment of LD, the expression of *AtCCR2*, reported as bioluminescence, was followed in plants in constant dark (DD). The period length was found to increase in *aPttPHYA-1* and *aPttPHYA-5* in DD, compared to WT (Table 2). The detected lengthening of the internal period in *aPttPHYA* lines can explain the late phase of *PttCO2* found under SD.

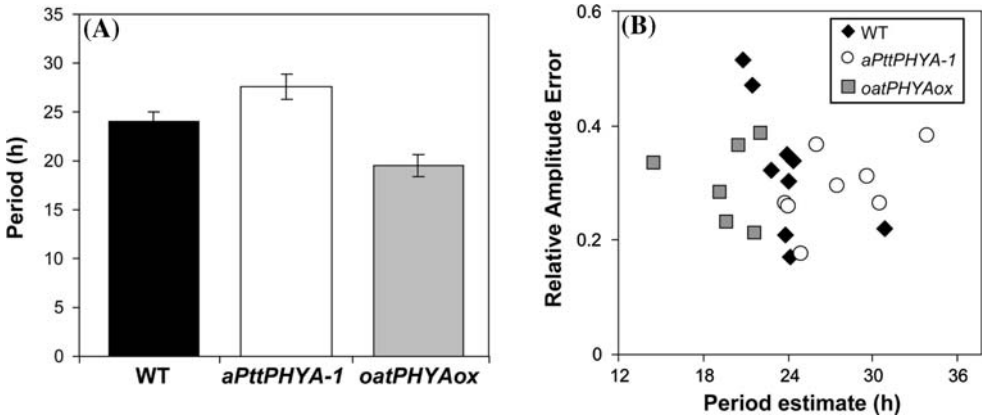

Fig. 6 Period estimates of leaf movement rhythms of WT and *aPttPHYA-1*. Plant cuttings were free-run in continuous light after entrainment to seven short days (12 h light/12 h night). For each genotype $n = 7$. **a** Mean period and SEM for each genotype. **b** Period estimates for individual leaves plotted against their relative amplitude errors (*RAE*). *Closed diamonds* WT, *open circles* *aPttPHYA-1*, *open squares* *oatPHYAox*

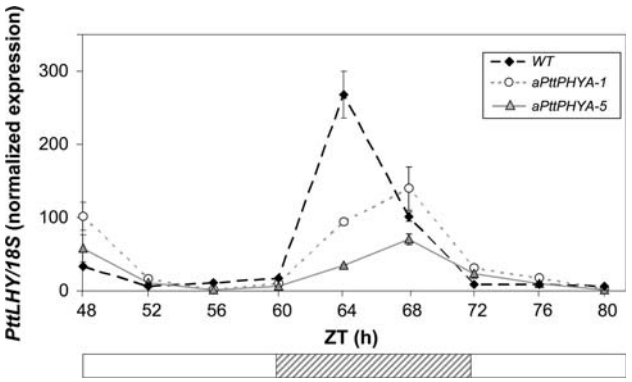

Fig. 7 Expression of *PttLHY* in WT, and *aPttPHYA*-lines under continuous light. The subjective night length used for entrainment is shown in *grey*. The results are presented relative to the lowest value after standardization with respect to *18S* rRNA. Mean ± SE denotes technical replication of pooled leaf material from three plants

Down-regulation of *PttPHYA* induces *PttLHY1* expression and reduces *PttFKF1* and *PttFT* expression in response to a FR light pulse

To investigate the effect of FR light pulses on selected clock and photoperiodic genes' expression, *aPttPHYA-1* and WT plants were adapted to constant dark for 4 days before 1 h of FR treatment. The activation of expression in *aPttPHYA-1* and WT in leaf tissues were monitored by qRT-PCR and primers detecting *PttLHY1*, *PttLHY2*, *PttTOC1*, *PttFKF1*, *PttCO2*, and *PttFT* relative control primers *18 s RNA*. We found that the only genes showing significant difference in response to a FR pulse were *PttLHY1*, *PttFKF1*, and *PttFT* (Fig. 8). The expression of *PttLHY1* increased in *aPttPHYA-1* relative WT, suggesting that phyA mediated FR light acts to repress the expression of this gene.

In contrast, *PttFKF1* and *PttFT* expression was reduced in *aPttPHYA-1*, indicating a promoting effect of phyA mediated FR signaling on these genes' expression. These changes are in line with the notion that de-repression of *PttLHY1* expression in response to reduced phyA signaling may lead to a lengthening of period of the circadian clock regulated gene expression and physiological out-puts such as leaf movements (Fig. 6; Table 2). The lower induction of *PttFKF1* and *PttFT* expression in *aPttPHYA-1* upon FR treatment suggests a regulatory link between *PttPHYA* expression, and these genes' expression in the seasonal regulation of growth cessation. It is possible that the effect on *PttFKF1* could be a consequence of the repression of PttLHY1 or a direct effect given the 1-h time frame. In particular, this regulation is relevant for understanding the phyA mediated regulation of growth cessation and bud set following EOD FR treatment (Table 1), where growth terminates faster both in strong and moderately down-regulated lines. Together, these data suggest molecular links between FR light signaling, expressed *PttPHYA* levels, and downstream targets of regulation of the circadian clock and growth.

Discussion

The internal circadian clock keeps the organism in tune with the daily changes in light/dark and the seasonal cycles of the year. This is important for synchronized flowering and timely cessation of growth and bud set of perennials at high latitudes (Eriksson and Millar 2003; Rohde and Bhalerao 2007). Changes in light perception affect the accuracy of this daylength discrimination and may lead to alterations in the clock function, which alters photoperiodic responses. The present investigation found that plants with reduced levels of the endogenous *PttPHYA* responded faster to daylength shortening than WT plants did, with earlier growth cessation and bud set. The severity of the phenotype correlated with the level of *PttPHYA* expressed and was more apparent in response to SD and SD with EOD FR extensions of the photoperiod, which acts through phyA since EOD sustain growth for longer in WT plants (Table 1). In addition, plants with moderate down-regulation were significantly earlier to

Table 2 Period estimates for rhythmic *PttPHYA*-antisense and wild type plants carrying the *AtCCR2pro:LUCIFERASE (LUC)* transgene under constant darkness

Genotype	*AtCCR2pro:LUC* line	Period (h)	SEM	Number of cuttings (rhythmic/total)
WT	Line 5	22.9	±1.2	8/8
	Line 7	22.5	±1.0	8/9
	Line 8	23.3	±1.2	9/9
a-1	Line 1	26.0	±0.7	9/9
	Line 2	27.0	±0.4	7/9
	Line 3	25.7	±0.3	8/9
a-5	Line 1	26.0	±0.2	9/9
	Line 3	26.3	±0.2	9/9

WT wild type

aPttPHYA lines *a-1*, *a-5*

Aspen cuttings were grown under 18 h white light/6 h dark cycles under about 50 µmol m^{-2} s^{-1} and then moved to 15 µmol m^{-2} s^{-1} white LEDs for 3 days before receiving constant darkness (DD) at dusk. Rhythm analysis on DD traces was performed by BRASS

stop growth under these conditions as compared to the SD treatment, showing a strong sensitivity for *PttPHYA* levels in order to sustain growth under EOD FR. Thus, these results led to the conclusion that earlier timing of growth cessation and bud set is due to reduced levels of *PttPHYA*.

Genes required for dormancy induction and maintenance were induced by SDs alone (Ruttink et al. 2007), and phyA has previously been implicated in the SD induction of growth cessation and cold acclimation by studies of *oatPHYA* over-expressing plants (Olsen et al. 1997; Welling et al. 2002). Leaves are the main receptors of daylength information, while the apex is the organ that responds with growth cessation and bud formation. Ruonala et al. (2008) showed that *CENL1* expression in the apex of *oatPHYAox* plants continues to increase during SD exposure and may be important for the sustained growth in these *PHYA* over expressers.

We studied the timing of putative clock components (*PttLHY*, *PttTOC1*) and likely clock regulated genes, which may be important for photoperiodic regulation (*PttFKF1*, *PttCO2)*. Following two SDs, *PttLHY* showed a slight delay in its lowest level of expression, while *PttTOC1*, *PttFKF1*, and *PttCO2* showed a reduced expression in the *aPttPHYA* lines (Fig. 5). The expression pattern of *PttCO2* was most changed, and in *aPttPHYA* plants showed no overlap in its expression with the light period at ZT8, suggesting that an earlier disruption of the coincidence between light and *PttCO2* can be responsible for the higher sensitivity to SD detected in plants with reduced *PttPHYA* levels (Table 1; Fig. 5d). The regulation of *PttCO2* probably depends on circadian clock function in these trees, so we determined whether the clock regulated control of leaf movements and gene expression was altered in *aPttPHYA* lines under constant light and dark. In constant light, a lengthening in the internal period of leaf movements was detected (Fig. 6), and we could confirm that the expression of *PttLHY* and *PttFKF1* was affected in a comparable manner (Fig. 7; Supplemental Fig. 4). In constant dark, the effect was similar with a

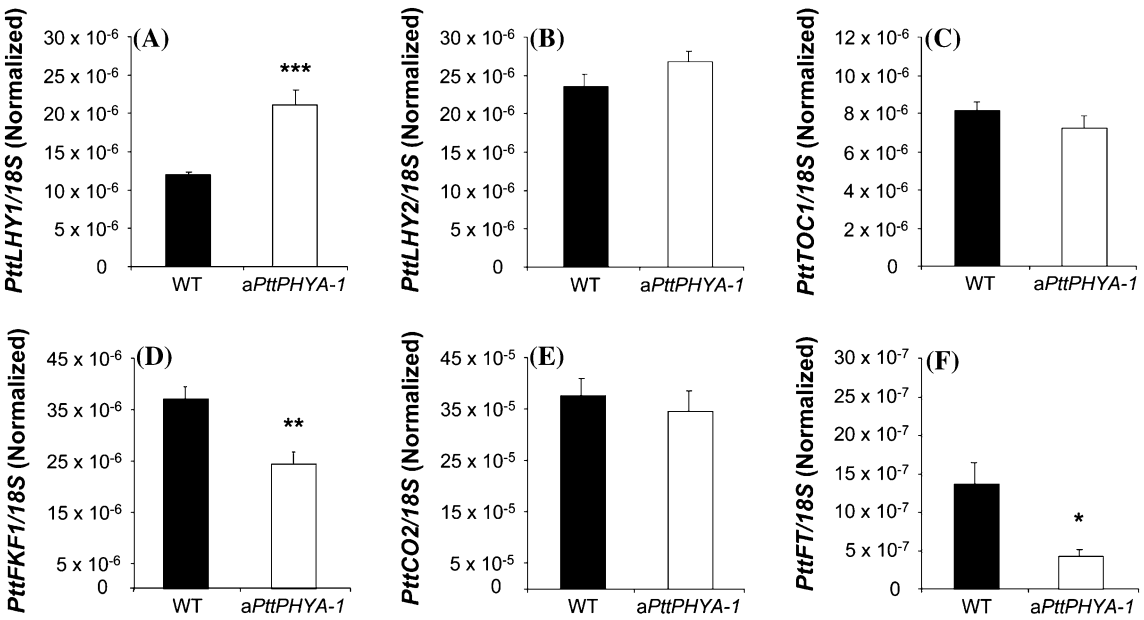

Fig. 8 FR response of gene expression in dark-adapted leaves. WT and aPttPHYA-1 plants were shifted to constant dark 4 days before a FR light pulse and sampling. Expression of *PttLHY1* (**a**), *PttLHY2* (**b**), *PttTOC1* (**c**), *PttFKF1* (**d**), *PttCO2* (**e**), and *PttFT* (**f**) is presented as standardization with respect to *18S rRNA* (*18S*). Mean ± SE of three biological replicates are shown. Data were analyzed using ANOVA. Means were significantly different with significance level 0 (***), 0.001 (**) and 0.01 (*)

slightly longer period detected for the measured heterologus *AtCCR2* promoter:*LUC* reporter (Table 2). Together these data support the notion that clock regulated periods are lengthened as levels of *PttPHYA* decrease.

It is possible that phyA Pfr levels persist in WT and act to re-set the period of the clock through *PttLHY*. This is supported by the increased *PttLHY* expression of *aPttPHYA-1* to a FR pulse and suggests that phyA acts to repress *PttLHY* expression in response to FR (Fig. 8). Therefore, phyA reduction in the antisense plants likely impaired the re-setting and resulted in a longer endogenous period. Such alterations in *PttLHY* levels will also probably affect the levels of *PttTOC1*, which were clearly reduced under SD (Fig. 5b).

Moreover, we also detected a significant dependence on phyA in response to FR of *PttFKF1* and *PttFT* (Fig. 8). Most importantly, this suggests that *PttFT* can be stimulated in a light dependent manner, but does not require increased *PttCO2* expression. This could be explained by an increased stabilization of PttCO2 protein by FR light leading to increased levels of *PttFT* or by a mechanism that acts independent of PttCO2 but requires light and PttFKF1. Both are plausible explanations since there is evidence of both in *Arabidopsis* (Salazar et al. 2009 and refs. there in).

Our studies are coherent with studies on phyA signal transduction in *Arabidopsis* hypocotyls that indicate a short signaling pathway between phyA received FR light and target genes within the circadian clock and genes important for photoperiodic measurement (Tepperman et al. 2001). Tepperman et al. found that *CO* expression was stimulated by FR in a phyA dependent manner, a finding we did not detect in our experimental set up. However, since we investigated the response of dark adapted leaves of trees that were previously actively growing under LD, while Tepperman et al. (2001) investigated hypocotyls that were not previously exposed to light cycles, it is likely that the expression pattern of specific genes are different.

Using a 6 h light/6 h dark treatment, we found that WT, *aPttPHYA-1*, and most interestingly *oatPHYAox* were able to stop growth and set bud (Fig. 2). The inability of *oatPHYAox* to stop growth and set buds under a 24 h cycle suggests that the phase of a critical and presumably circadian controlled endogenous rhythm with light (phyA) signaling is in a favorable angle with the external cycle and will support growth continuously in such plants. By introducing a light/dark cycle of 12 h, this phase is efficiently disrupted and lead to growth cessation and bud set also of *oatPHYAox* plants (Fig. 2). Moreover, we found that these plants obtained dormancy and normal freezing tolerance (Fig. 3). This result is novel in the sense that *oatPHYAox* lines were previously found to be unable to set bud and become freezing tolerant by any photoperiodic (SD) treatment alone. However, growth could be inhibited and hardiness obtained only by a combination of application of GA inhibitors together with temperature cycles (Olsen et al. 1997; Møhlmann et al. 2005).

We propose that daylength discrimination in *Populus* depends on correct function of the circadian oscillator. Its dysfunction here is manifested as lengthening of internal period resulting in earlier disruption of the coincidence of light and phase of *PttCO2* leading to altered daylength sensing and an earlier bud set (Table 1; Fig. 5d); however, dependence on light-mediated activation was shown by the 12 h cycle experiment (Fig. 2) and the FR pulse response of *asPttPHYA-1* (Fig. 8).

Hence, *Populus* will grow when the circadian clock controlled phase of *PttCO2* coincides with light and *PttFT* expression is stimulated. *PttFT* can presumably be induced both via daylength regulation of *PttCO2* or through direct FR induction perhaps through PttCO2 and PttFKF1 (Fig. 9). Most importantly, in the control of seasonal growth of this species, both modes of regulation require a functional phyA signaling.

It has been known for many years that the length of the photoperiod is an important environmental signal for various physiological processes, including the induction of dormancy in many woody species (Wareing 1956). However, our understanding of the molecular mechanisms involved in growth cessation and bud set and its induction by a CDL has been limited. In this study of hybrid aspen with low levels of *PttPHYA*, we have established a link

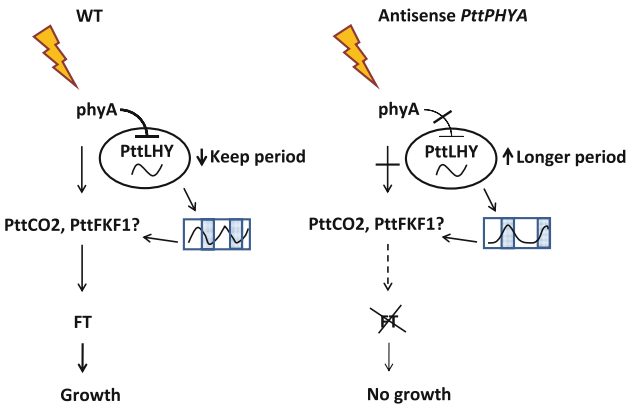

Fig. 9 A hypothetical model of phyA contribution to the regulation of growth in *Populus* under short day. WT plants with normal levels of *PttPHYA* expression repress *PttLHY* in order to re-set clock rhythms according to light/dark cycles and promote *PttFT* expression possibly via activation of PttCO2 and PttFKF1 in response to light (FR) (*left*). In antisense lines with lower levels of *PttPHYA* expression, the level of *PttLHY* is not restricted and probably leads to an increased period length and a delayed phase of clock regulated genes such as *PttCO2* so that the coincidence with light is disrupted earlier than WT in response to a perceived short day. Therefore, *PttFT* levels are not supported in response to light perceived by phyA, and low levels of *PttFT* (*dashed arrow and cross*, *right*) will hasten growth cessation. *Arrows* indicate positive effect and *bars* repression

between light perception, the circadian clock, and the photoperiodic response of growth cessation and bud set. We demonstrated the following: (1) reduction of *PttPHYA* expression levels results in earlier timing of growth cessation and bud set in hybrid aspen, (2) the effect on timing of growth cessation correlates with a lengthened internal circadian clock period in *aPttPHYA* lines, (3) FR light signaling via phyA affects *PttLHY1*, *PttFKF1*, and *PttFT* expression levels support a role in clock entrainment and growth, and (4) the integration between the endogenous timing with the environment is essential to growth since *oatPHYAox* lines with constantly activated light signaling will stop growth when external light/dark cycles are unfavorably matched with internal rhythms. Together these studies show the important role of phyA signaling in timing of seasonal regulation of growth in deciduous trees.

Materials and methods

Vector construction

The antisense *PttPHYA* vector was constructed using the *PttPHYA* full length cDNA clone (accession number AJ001318) described in Eriksson and Moritz (1997). It was cut with *Xho* I, filled in with Klenow, *Bam H*I linked, and ligated into the *Bam H*I site of pPCV702.kana (Koncz and Schell 1986). Constructs carrying the cDNA in antisense orientation were identified by enzymatic digestion and confirmed by sequencing. The resulting construct is denoted aPttPHYA and includes the full length *PttPHYA* cDNA in an inverted position driven by the CaMV 35S promoter.

Plant transformation

Hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) clone T89 was transformed with *aPttPHYA* and regenerated essentially as described (Eriksson et al. 2000). Seven independent lines were produced. Due to problems with maintenance of lines in vitro, two lines (*aPttPHYA-1* and *-5*) remained during the course of experiments.

Gene identification and qRT-PCR

Putative homologues of *Arabidopsis* circadian clock-associated genes were identified by BLAST searches against two databases (http://haddock.fysbot.umu.se and http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) followed by phylogenetic analyses (data not shown). We used two approaches: direct collection of genomic data followed by predictions using FGENESH (http://www.softberry.com/berry.phtml), and searches against databases to identify genes that had already been predicted. In all cases, the results were the same. Primers used for detection of *PttCO2* and *PttFT* were according to Böhlenius et al. (2006): *PttFKF1* forward: GCAGAAGAATCCGGCAATTAAC, reverse: GCTCCAATGCACCTGTGAC; *PttLHY1* and *2* (combined) forward: GAGAAAGAACCACGTCCTGAG, reverse: CTTCTGAGCAATTGTCATCCTG; *PttLHY1* forward: TGCAATGAATCCTTTATCAC, reverse: CTTTAACAGTGCCATTATCC; *PttLHY2* forward: AGTAATCAATCCTCTGTCACC, reverse: CTAGTGCTGTTATCCTGTTTG; *PttTOC1* forward: AAGTGCTCTTATCAAGTTACATCAGTG, reverse: TTGGTCATTGGAATGTCAACTTCAG. All annealing temperatures were at 55°C, except for *PttFT*, which annealed at 57°C.

RNA was extracted from leaf samples collected every 4 h as described in Eriksson et al. (2000), and the resulting RNA was purified by an RNeasy Plant Mini Kit (Qiagen, Hiden, Germany), including DNAse treatment as described in the manufacturer's protocol and a second DNAse treatment using a TURBO DNA-free kit (Ambion) when necessary. RNA concentration was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA); 1 μg of RNA was used as template for cDNA synthesis, using iScript™ cDNA Synthesis Kit procedures (Bio-Rad Laboratories, Inc., Hercules, CA USA).

Real-time PCR was performed with an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories) using iQ SYBR® Green Supermix (Bio-Rad Laboratories). In each reaction, 12.5 ng cDNA was used, except in *PttFT* expression analyses, where 50 ng cDNA was used.

Standards were prepared by diluting a pool of all cDNA samples to be tested. The PCR values presented here are mean of three biological repeats unless otherwise specified expressed relative to the lowest value of all samples after standardization to the 18S rRNA. The efficiency of every primer used was determined using the iCycler iQ™ Real-Time PCR Detection System Software according to this formula: Efficiency $= \{10^{(-1/\text{slope}}\} - 1$

To calculate the relative amount of transcript present in each sample, the following formula (adapted from Czechowski et al. 2004) was used: "relative transcript amount $= (1 +$ efficiency of the gene of interest$)^{-\text{threshold cycle of the gene of interest}}$ divided with $(1 +$ efficiency of the reference gene$)^{-\text{threshold cycle of the reference gene}}$"

Plant growth conditions

The photoperiod in long day (LD) conditions was 18 h light/6 h dark or where indicated 16 h light/8 h dark, under critical day length conditions (CDL) 15 h light/9 h dark, and under short days (SD) 12 h light/12 h dark. Temperature (18°C) and relative humidity (80%) were kept constant. Plants were watered daily and fertilized with a complete nutrient solution (SuperbaS, Supra Hydro AB,

Landskrona, Sweden) once a week. At the onset of SD, fertilization was discontinued.

The rooted in vitro cuttings for SD (control) and SD + FR treatments were first planted in soil and grown under 16 h light/8 h dark at 200 μmol m^{-2} s^{-1} under cool fluorescent light (Philips TLD 50W 83 OHF, Germany). After two weeks of growth, the plants were divided into separate chambers (HPS 1500.S, Heraeus Industretechnik, Germany) with white light of 100 μmol m^{-2} s^{-1} (Philips) under a 12 h light/12 h dark regime. The main photoperiod was extended to 4 h and 30 min of a mixture of R and FR light with the ratio R/FR 0.01–0.08 and 20–26 μmol m^{-2} s^{-1} total irradiation from LEDs with peak wavelength at 660 nm (R) and 730 nm (FR) (MD Electronics, UK). After a month, the R/FR extension was reduced to 3 h.

For studies of growth under 6 h light/6 h dark cycles, plants were established under LD (18 h light/6 h dark) for 7 weeks, transferred to SD (12 h light/12 h dark) for 12 days, and transferred to continuous light for 4 days before 6 h light/6 h dark.

Before the freeze tests, plants were kept under 6 h light/6 h dark for 16 weeks, or 12 h light/12 h dark for 9 weeks when transferred to cold under the respective photoperiods and dim fluorescent light (10–15 μmol m^{-2} s^{-1}, Philips TLD 36W/840, Germany) for 6 weeks, 3 weeks of 6°C followed by 1°C for 3 weeks.

Freeze test

Stem sections from seven plants per genotype were harvested at one time point. A 20–60 cm stem section beneath the shoot tip was cut into 5-cm segments. They were rinsed twice in distilled water and then placed into test tubes. The six test temperatures were run in a randomized order over a period of 10 days. The tubes were stored at 2°C with caps loosely fitted until use. The caps were then removed and small amounts of ice were added before tubes were placed in the freezing cabinet (500/80-180DU, Weiss Umwelttechnik GmbH, Germany). Samples were equilibrated for 1 h at −2°C before they were cooled at a rate of 3°C h^{-1} down to the test temperature that was kept constant for 1 h before tubes were thawed at rate of 10°C h^{-1} up to −2°C. Temperature was tracked using a thermocouple and a CR10X data logger (Campbell Scientific Inc., Logan, UT, USA). On retrieval, 2–5 slices of 5-mm lengths were cut from the midsection of each stem segment while still frozen using a sharp industrial blade. The slices were placed into 3–4 ml of 0.01 M boric acid with 0.002% Triton X-100. Samples were shaken at 200 rpm for 18 h at room temperature before the temperature-corrected conductivity was measured (cell model 6.0907.110, Metrohm, Switzerland). The measurements were repeated after samples had been exposed to 90°C for 10 min and then shaken for another 18 h. Indices of injury were calculated by linear scaling according to Flint et al. (1967).

Growth conditions and sampling for qRT-PCR assays under SD and LL and following a FR pulse

WT, *aPttPHYA-1*, and *aPttPHYA-5* lines as indicated were grown under LD at constant 18°C for 40 days as above when subjected to SD 48 h before sampling.

After seven SDs, plants were given LL and allowed to free-run for 48 h, when samples were harvested for free-running experiment. The tissues collected were leaf blades from three different plants per genotype harvested at 4-h intervals from internodes 9–11 (counting from the first internode below the first leaf at least 1 cm long).

Long day grown plants were adapted to DD for 4 days. When given an hour FR pulse of ~20μmol m^{-2} s^{-1} total irradiation from LEDs with peak wavelength at 730 nm (MD Electronics), leaf blades were harvested from 8 to 9th internode from three different plants per genotype.

Statistical analysis

The plants were grown following completely randomized design. All physiological data were subjected to analysis of variance (ANOVA). Balanced or unbalanced ANOVAs were performed depending on the number of replications in the experiments. Post hoc tests were performed typically using Dunnett's or Tukey. Means were separated using Fisher's protected least significant difference at 1–5% level as indicated.

Leaf movement analysis

Leaf movement rhythms were measured in trimmed cuttings from WT, *oatPHYAox* line 22, and *aPttPHYA-1* shoots that had been cultivated in vitro on sucrose-free half strength MS medium and 1.5% agar. The cuttings were entrained to a SD photoperiod in a Percival chamber (Percival Inc, IA, USA) at 100 μmol m^{-2} s^{-1} (22°C for 7 days). Then they were transferred to LL in chamber (KBK/LS 4330, EHRET, Labor- u. Pharmatechnik, Germany) at 40 μmol m^{-2} s^{-1}, 22°C, where images were captured every 20 min using Dinion 1/3 color cameras (LTC0455, Bosch Security Systems, Germany). Digital photographs were collected by means of the Video gala framegrabber, PixelSmart, (http://www.pixelsmart.com) using an adapted image acquisition plug-in for the ImageJ package (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/). The captured pictures were analyzed by Metamorph 6.3 image-analysis software (Molecular Devices, PA, USA). Individual period estimates were generated by importing data into BRASS (available

from www.amillar.org) and using BRASS to run fast Fourier transform nonlinear least-squares analysis programs to estimate rhythmic cycles and period lengths (Millar et al. 1995; Plautz et al. 1997; Locke et al. 2005). The two experiments each with two to three replicated plates were considered as replications of one experiment over time and analyzed together.

Luminescence assays

Populus WT, *aPttPHYA-1*, and *aPttPHYA-5* plants were transformed by *Agrobacterium tumefaciens* carrying the heterologous *Arabidopsis* promoters fused to *LUCIFERASE (LUC)* as indicated. Sterile in vitro generated shoots from independent transformation events were grown on MS medium with 2% sucrose and supplemented with 5 mM luciferin 24 h before the assay and imaged by ORCA-II-ERG 1024 cooled camera (Hamamatsu Photonics, Japan) at 22°C. Image acquisition and light control were driven by WASABI imaging software (Hamamatsu Photonics, Japan). Shoots were entrained to LDs under white or red (660 nm) and blue (470 nm) light emitting diodes with 15 μmol m^{-2} s^{-1} and 20 μmol m^{-2} s^{-1} (MD Electronics). Expression of *AtCCA1pro:LUC* and *AtCCR2pro:LUC* under diurnal or constant conditions was imaged by ORCA-II-ERG 1024 camera cooled to −60°C (Hamamatsu Photonics, Japan) at 22°C. Image acquisition and light control were driven by WASABI imaging software (Hamamatsu Photonics). Images were processed by Metamorph (Molecular Devices) and individual period estimates were generated as described above.

Acknowledgments We are grateful to Ingabritt Carlsson, Gunilla Malmberg, Marie Nygren, Kjell Olofsson, Medisa Hasić, Isabella Pekkari and Sofia Österberg for their excellent technical assistance and to the Wallenberg greenhouse staff for their valuable help. Luis Muniz and Harriet G. McWatters are acknowledged for helpful scientific discussions. We thank Andrew J. Millar for the gift of the *promoter:LUC* constructs and BRASS software. We are also grateful for valuable help from Yury Shatz regarding the PixelSmart plug-in for ImageJ. This work was supported by grants to MEE from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), the Swedish Research Council (VR), *the Knut and Alice Wallenberg Foundation* and Nils och Dorthi Troëdssons forskningsfond. The research was also supported by the Swedish Natural Science Research Council (NFR), Swedish Research Council for Agricultural Sciences (SJFR), the Swedish Foundation for Strategic Research (SSF), the Kempe Foundation and a Human Frontier Science Program (RG0303) to TM.

References

Alabadí D, Oyama T, Yanovsky MJ, Harmon FG, Más P, Kay SA (2001) Reciprocal regulation between TOC1 and LHY/CCA1 within the *Arabidopsis* circadian clock. Science 293:880–883

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

Bünning E (1936) Die endogene Tagesrhythmik als Grudlage der photoperiodischen Reaktion. Ber Deut Bot Ges 54:590–607

Clack T, Mathews S, Sharrock RA (1994) The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: The sequences and expression of *PHYD* and *PHYE*. Plant Mol Biol 25:413–427

Corbesier L, Vincent C, Jang SH, Fornara F, Fan QZ, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science 316:1030–1033

Czechowski T, Bari RP, Stitt M, Scheible WR, Udvardi MK (2004) Real-time RT-PCR profiling of over 1400 *Arabidopsis* transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J 38:366–379

Devlin PF (2002) Signs of the time: environmental input to the circadian clock. J Exp Bot 53:1535–1550

Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271–290

Eriksson ME, Millar AJ (2003) The circadian clock: a plant's best friend in a spinning world. Plant Physiol 13:732–738

Eriksson M, Moritz T (1997) Isolation of a cDNA encoding a phytochrome A (Accession No. AJ001318) from *Populus tremula* × *tremuloides*. Plant Gene Register # PGR97-186. Plant Physiol 115:1731

Eriksson ME, Moritz T (2002) Daylength and spatial expression of a gibberellin 20-oxidase isolated from hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.). Planta 214:920–930

Eriksson ME, Israelsson M, Olsson O, Moritz T (2000) Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. Nat Biotechnol 18:784–788

Flint HL, Boyce BR, Beattie DJ (1967) Index of injury—a useful expression of freezing injury to plant tissues as determined by electrolytic method. Can J Plant Sci 47:229–230

Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290:2110–2113

Howe GT, Bucciagalia PA, Hackett WP, Furnier GR, Cordonnier-Pratt M-M, Gardner G (1998) Evidence that the phytochrome gene family in black cottonwood has one *PHYA* locus and two *PHYB* loci but lacks members of the *PHYC/F* and *PHYE* family. Mol Biol Evol 15:160–175

Imaizumi T, Tran HG, Swartz T, Briggs WR, Kay SA (2003) FKF1 is essential for photoperiodic-specific light signaling in *Arabidopsis*. Nature 426:302–306

Imaizumi T, Schultz TF, Harmon FG, Ho LA, Kay SA (2005) FKF1 F-BOX protein mediates cyclic degradation of a repressor of CONSTANS in *Arabidopsis*. Science 309:293–297

Johnson E, Bradley M, Harberd NP, Whitelam GC (1994) Photoresponses of light-grown phyA mutants of *Arabidopsis*—phytochrome A is required for the perception of daylength extensions. Plant Physiol 105:141–149

Koncz C, Schell J (1986) The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of binary vector. Mol Gen Genet 204:383–396

Kreps JA, Simon AE (1997) Environmental and genetic effects on circadian clock-regulated gene expression in *Arabidopsis*. Plant Cell 9:297–304

Lin C, Ahmad M, Cashmore AR (1996) Arabidopsis cryptochrome 1 is a soluble protein mediating blue light-dependent regulation of plant growth and development. Plant J 10:893–902

Lin C, Yang H, Guo H, Mockler T, Chen J, Cashmore AR (1998) Enhancement of blue-light sensitivity of Arabidopsis seedlings by a blue light receptor cryptochrome 2. Proc Natl Acad Sci USA 95:2686–2690

Locke JC, Millar AJ, Turner MS (2005) Modelling genetic networks with noisy and varied experimental data: the circadian clock in *Arabidopsis thaliana*. J Theor Biol 234:383–393

McWatters HG, Bastow RM, Hall A, Millar AJ (2000) The ELF3 zeitnehmer regulates light signalling to the circadian clock. Nature 408:716–720

Millar AJ, Carré IA, Strayer CA, Chua N-H, Kay SA (1995) Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. Science 267:1161–1163

Møhlmann JA, Asante DKA, Jensen JB, Krane MN, Ernstsen A, Junttila O, Olsen JE (2005) Low night temperature and inhibition of gibberellin biosynthesis override phytochrome action and induce bud set and cold acclimation, but not dormancy in PHYA overexpressors and wild-type of hybrid aspen. Plant Cell Environ 28:1579–1588

Olsen JE, Junttila O (2002) Far red end-of-day treatment restores wild type-like plant length in hybrid aspen overexpressing phytochrome A. Physiol Plant 115:448–457

Olsen JE, Junttila O, Moritz T (1995) A localised decrease of GA1 in shoot tips of Salix pentandra seedlings preceds cessation of shoot elongation under short photoperiod. Physiol Plant 95:627–632

Olsen JE, Junttila O, Nilsen J, Eriksson ME, Martinussen I, Olsson O, Sandberg G, Moritz T (1997) Ectopic expression of oat phytochrome A in hybrid aspen changes critical day length for growth and prevents cold acclimation. Plant J 12:1339–1350

Perales M, Más P (2007) A functional link between rhythmic changes in chromatin structure and the *Arabidopsis* biological clock. Plant Cell 19:2111–2123

Plautz JD, Straume M, Stanewsky R, Jamison CF, Brandes C, Dowse HB, Hall JC, Kay SA (1997) Quantitative analysis of *Drosophila* period gene transcription in living animals. J Biol Rhythms 12:204–217

Putterill J, Robson F, Lee K, Simon R, Coupland G (1995) The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to Zinc-finger transcription factors. Cell 80:847–857

Ramírez-Carvajal GA, Morse AM, Davis JM (2008) Transcript profiles of the cytokinin response regulator gene family in *Populus* imply diverse roles in plant development. New Phytol 177:77–89

Ramos A, Pérez-Solís E, Ibáñez C, Casado R, Collada C, Gómez L, Aragoncillo C, Allona I (2005) Winter disruption of the circadian clock in chestnut. Proc Natl Acad Sci USA 102:7037–7042

Roden LC, Song H-R, Jackson S, Morris K, Carre IA (2002) Floral responses to photoperiod are correlated with the timing of rhythmic expression relative to dawn and dusk in *Arabidopsis*. Proc Natl Acad Sci USA 99:13313–13318

Rohde A, Bhalerao RP (2007) Plant dormancy in the perennial context. Trends Plant Sci 12:217–223

Ruonala R, Rinne PLH, Kangasjärvi J, Schoot C (2008) CENL1 expression in the rib meristem affects stem elongation and the transition to dormancy in *Populus*. Plant Cell 20:59–74

Ruttink T, Arend M, Morreel K, Storme V, Rombauts S, Fromm J, Bhalerao RP, Boerjan W, Rohde A (2007) A molecular timetable for apical bud formation and dormancy induction in poplar. Plant Cell 19:2370–2390

Salazar JD, Saithong T, Brown PE, Foreman J, Locke JCW, Halliday KJ, Carré IA, Rand DA, Millar AJ (2009) Prediction of photoperiodic regulators from quantitative gene circuit models. Cell 139:1170–1179

Salome PA, McClung CR (2005) What makes the *Arabidopsis* clock tick on time? A review on entrainment. Plant Cell Environ 28:21–38

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G (2000) Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis*. Science 288:1613–1616

Sawa M, Nusinow DA, Kay SA, Imaizumi T (2007) FKF1 and GIGANTEA complex formation is required for day-length measurement in *Arabidopsis*. Science 318:261–265

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carre IA, Coupland G (1998) The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. Cell 93:1219–1229

Somers DE, Devlin PF, Kay SA (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. Science 282:1482–1490

Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Más P, Panda S, Kreps JA, Kay SA (2000) Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. Science 289:768–771

Suárez-López P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G (2001) *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. Nature 410:1116–1120

Swarup K, Alonso-Blanco C, Lynn JR, Michaels SD, Amasino RM, Koorneef M, Millar AJ (1999) Natural allelic variation identifies new genes in the *Arabidobsis* circadian system. Plant J 20:67–77

Takata N, Saito S, Saito CT, Nanjo T, Shinohara K, Uemura M (2009) Molecular phylogeny and expression of poplar circadian clock genes, *LHY1* and *LHY2*. New Phytol 181:808–819

Tepperman JM, Zhu T, Chang HS, Wang X, Quail PH (2001) Multiple transcription-factor genes are early targets of phytochrome A signaling. Proc Natl Acad Sci USA 98:9437–9442

Thomas B, Vince-Prue D (1997) Photoperiodism in plants, 2nd edn. Academic Press, London

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science 303:1003–1006

Wang ZY, Tobin EM (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. Cell 93:1207–1217

Wareing PF (1956) Photoperiodism in woody plants. Annu Rev Plant Physiol Plant Mol Biol 7:191–214

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

Yanovsky MJ, Kay SA (2002) Molecular basis of seasonal time measurement in *Arabidopsis*. Nature 419:308–312