Circadian Clock Components Regulate Entry and Affect Exit of Seasonal Dormancy as Well as Winter Hardiness in *Populus* Trees

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This study addresses the role of the circadian clock in the seasonal growth cycle of trees: growth cessation, bud set, freezing tolerance, and bud burst. *Populus tremula* × *Populus tremuloides* (Pt) LATE ELONGATED HYOCOTYL1 (PtLHY1), PtLHY2, and TIMING OF CAB EXPRESSION1 constitute regulatory clock components because down-regulation by RNA interference of these genes leads to altered phase and period of clock-controlled gene expression as compared to the wild type. Also, both RNA interference lines show about 1-h-shorter critical daylength for growth cessation as compared to the wild type, extending their period of growth. During winter dormancy, when the diurnal variation in clock gene expression stops altogether, down-regulation of PtLHY1 and PtLHY2 expression compromises freezing tolerance and the expression of 6-C-REPEAT BINDING FACTOR1, suggesting a role of these genes in cold hardiness. Moreover, down-regulation of PtLHY1 and PtLHY2 causes a delay in bud burst. This evidence shows that in addition to a role in daylength-controlled processes, PtLHY plays a role in the temperature-dependent processes of dormancy in *Populus* such as cold hardiness and bud burst.

In plants, timekeeping of daily and seasonal processes relies on a circadian clock that uses negative-feedback loops at the transcript/protein level to sustain a rhythmicity of approximately 24 h in the absence of time-giving cues. Daylength is a reliable environmental cue that numerous organisms use to control growth and reproduction cycles in relation to seasonal change. Prominent examples are flowering in many plants, seasonal growth in trees, and diapauses in insects (Carré, 2001; Tauber and Kyrilacou, 2001; Rohde and Bhalerao, 2007). Temperate tree species, including *Populus* spp., cease growing and set buds when daylength is shortened below a critical value, the critical daylength (CDL; Junttila, 1982; Howe et al., 2003; Böhlenius et al., 2006). Daylength is one environmental cue that resets the clock to local time. Perception of light via photoreceptors entrains the circadian clock and allows the plant to adjust itself to the 24-h cycle. The central clock homologs in the genome of the sequenced deciduous tree *Populus trichocarpa* (Pt) comprise LATE ELONGATED HYOCOTYL1 (PtLHY1), PtLHY2, and TIMING OF CAB EXPRESSION1 (PtTOC1; Ramírez-Carvajal et al., 2008; Takata et al., 2009).

Also, light received during the day modulates photoperiod-dependent processes downstream of the circadian clock such as flowering. In the presence of blue light during daytime, the circadian clock-controlled genes FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1), and GIANTANEA (GI) form a protein complex that degrades the transcriptional repressor CYCLING OF DOF FACTOR1 and results in CONSTANS (CO) expression (Fowler et al., 1999; Park et al., 1999; Nelson et al., 2000; Imaizumi et al., 2003; Sawà et al., 2007). Then, CO activates the expression of FLOWERING LOCUS T (FT) that promotes flowering (Putterill et al., 1995; Samach et al., 2000; Suárez-López et al., 2001; Corbesier et al., 2007; Turck et al., 2008). Thus, when CO expression coincides with the daily light period, it...
accelerates flowering in Arabidopsis (Arabidopsis thaliana); when its expression falls into the dark period under short days (SDs), flowering is inhibited (Roden et al., 2002; Yanovsky and Kay, 2002). Both CO and FT have been implicated in seasonal regulation of growth cessation and bud set, since overexpression of either delays growth cessation under SDs in Populus trees (Böhlenius et al., 2006). Also, the phases of CO and FT expression correlate with bud set behavior of photo-periodic ecotypes (Böhlenius et al., 2006).

The seasonal growth control is central to the perennial habit of deciduous trees and determines the time points of growth cessation, bud set, dormancy entry, cold hardening, and bud burst. Despite the fact that the circadian clock has been implied as being central to the regulation of growth in Arabidopsis (Dodd et al., 2005) and in Populus tremula × Populus tremuloides (Pt) (Kozarewa et al., 2010), the molecular function of the circadian clock in perennial plants has not been addressed thoroughly with respect to seasonal growth patterns. To this end, we set out to investigate the role of the circadian clock in seasonal responses using Populus.

In this article, we report characterization of plants with modified levels of Populus clock component homologs PtllHY1, PtllHY2, and PtTOC1. We demonstrate that reducing their expression by RNA interference (RNAi) leads to a shortened internal period of clock-controlled gene expression rhythms and results in an approximately 1-h-shorter daylength requirement for growth cessation in these lines. Importantly, we also show that PtllHY1 and PtllHY2 mediate the clock’s response to cold temperature as well as promote freezing tolerance and bud burst. These findings demonstrate that in deciduous species, the timing of seasonal traits, including cold hardiness, depends on the performance of these circadian clock components.

RESULTS

The Clock Governs Timing of Growth Cessation and Bud Set

Studies of the Pt genome sequence suggest PtllHY1 and PtllHY2 result from a lineage-specific gene duplication event and show highest similarity to the LHY genes of chestnut (Castanea sativa) and Arabidopsis (Takata et al., 2009). Transcripts of both genes were detected in wild-type plants under 18-h long day (LD18 h; Fig. 1). Their expression peaked at Zeitgeber time 1 (ZT1; 1 h after dawn). PtllHY2 showed 5 to 10 times higher expression levels than PtllHY1 in wild-type plants under LD18 h conditions (Supplemental Figs. S1A and S2, A and B). Transcripts of both genes were targeted by one RNAi construct and are jointly referred to as PtllHY. RNAi down-regulation led to between 20% and 50% reduction in transcript levels of PtllHY in lhy lines at ZT4 under LD18 h (Supplemental Fig. S1B). When PtllHY1 and PtllHY2 were assayed separately at ZT0, expression of PtllHY1 varied between approximately 35% and 60%, and PtllHY2 showed approximately 60% to 80% of wild-type expression at this time point, with lhy-3 being the most strongly down-regulated line (Supplemental Fig. S1A).

The single PtTOC1 homolog in the Populus genome (Ramirez-Carvajal et al., 2008) has a peak expression at ZT9 under LD18 h (Fig. 1). PtllHY1 was successfully targeted by our RNAi construct. Down-regulation of PtllTOC1 led to between 20% and 65% reduction in expression levels to ZT16 (Supplemental Fig. S1C).

Because of their strong down-regulation toc1 lines 1, 4, and 5 and lhy lines 3, 8, and 10 were selected for investigation of the physiological consequences of PtllHY and PtllTOC1 down-regulation on growth cessation and bud set in Populus. The wild-type hybrid clone used in the study has a CDL of 15.5 h (Olsen et al., 1997). Consequently, we investigated the growth response of the RNAi lines when exposed to 15-h SD (SD15 h), just below the CDL of the wild type. As shown in Figure 2, A and B, growth cessation was completed in 100% of wild-type plants and bud set was completed in 80% of them after 6 weeks (42 d). At the same time, less than 30% of lhy and toc1 lines showed signs of growth cessation, suggesting the transgenic lines had not yet sensed their CDL under these conditions (Fig. 2, A and B). The photoperiod was subsequently shortened by an additional hour to SD14 h, which prompted growth cessation and bud set 20 to 33 d after the daylength shift in the remaining lhy and toc1 plants (Fig. 2, A and C). This response suggested that the CDL for growth cessation in the RNAi lines is about 1 h shorter than for the wild type.

The response of wild-type and lhy lines to stronger daylength shifts was addressed by transfer from LD18 h to SD12 h. All lhy lines showed a tendency to delay growth cessation and bud set, although only lhy-3 was statistically different from the wild type (Supplemental Table S1). Also, when wild-type, lhy, and toc1 plants were shifted to SD8 h, growth cessation was delayed in lhy-3, but the timing of bud set was not statistically different from the wild type for any line (Supplemental Table S1). These results showed that RNAi lines have no overall response deficiency, but are altered in daylength sensing specifically near the CDL.

Circadian-Controlled Rhythms Show Early Phase and Shortened Period in lhy and toc1 RNAi Lines

To address the effect of RNAi down-regulation on clock-controlled gene expression, the heterologous reporter gene AtCCR2pro:LUC (for Arabidopsis COLD CIRCADIAN RHYTHM RNA BINDING2 promoter fused to firefly LUCIFERASE) was introduced and monitored using independent transformed lines of the wild type and the two representative down-regulated lines lhy-10 and toc1-5.

AtCCR2 encodes a putative slave oscillator protein and is used as reporter for clock performance under...
various conditions (Heintzen et al., 1997; Kreps and Simon, 1997; Strayer et al., 2000). AtCCR2pro:LUC-driven luminescence cycled robustly with a broad peak in all genotypes under LD18 h and with the highest amplitude in toc1-5 (Supplemental Fig. S3). The estimated peak time of expression under LD18 h (as calculated with Biological Rhythms Analysis Software System [BRASS]) occurred at ZT14 in the wild type and was approximately 4 h earlier (advanced) in lhy-10 and at approximately wild-type peak time in toc1-5 RNAi lines (Supplemental Fig. S3).

Upon transfer from LD18 h to constant light (LL) at dawn, the wild type showed a robust rhythmicity and lhy-10 showed weak rhythmicity and toc1-5 was rhythmic with an amplitude almost 3-fold higher than the wild type (Fig. 3A).

The mean peak time for the first peak of AtCCR2pro:LUC expression following transfer to LL was earlier...

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Figure 1. LD expression of wild-type PttLHY and PttTOC1. Gene expression in wild-type leaves was assessed using qPCR every 4 h during 48 h of LD18 h, starting 3 h before dawn. Mean of expression was obtained from two biological pools, each consisting of two leaves, harvested from independent plants and run with three technical replicates. Variation is shown as mean ± se. Gene expression level was standardized with respect to 18S rRNA levels.

Figure 2. PttLHY and PttTOC1 define CDL in Populus. Bud set in plants shifted from LD to below CDL (15 h, black arrow) and again to 14 h to capture the difference between genotypes. Mean ± se of timing of bud set was measured for lhy-3 (n = 9), lhy-8 (n = 9), lhy-10 (n = 6), toc1-1 (n = 9), toc1-4 (n = 9), toc1-5 (n = 6), and the wild type (WT; n = 5). A, Mean days to bud set after onset of daylength shortening. B, Performance of wild-type, lhy, and toc-1 RNAi lines at 6 weeks (42 d) after the onset of a 15-h day. y axis represents percentage of plants that reached growth cessation and bud set at this time. Asterisks (*) indicate means that were significantly different from the wild type with a probability level of 0.05 (*), 0.01 (**), and 0.001 (**). C, Performance of wild-type, lhy, and toc-1 RNAi lines after the onset of 14-h day. Asterisks (*) represent means that are significantly different from the wild type, P < 0.05. Circles represent outliers.
for lhy-10 lines (6.4 ± 0.1 h and 6.4 ± 0.1 h) as well as for toc1-5 lines (10.2 ± 0.1 h and 10.6 ± 0.2 h) as compared to wild-type lines (11.4 ± 0.1 h and 11.0 ± 0.2 h; Fig. 3A; Table I). This result showed that both lhy-10 and toc1-5 had an advanced phase of peak expression of clock-controlled gene expression relative to the wild type.

Upon transfer from LD18 h to constant dark (DD) at dusk, the wild type was clearly rhythmic, whereas the lhy-10 and toc1-5 showed arrhythmia at the first anticipated dawn and dusk (Fig. 3B). The phase difference between the wild type and lhy-10 was consistently about approximately 5 h and approximately 1 to 2 h for toc1-5 following transfer to LL and DD (Table I).

Estimates of period of AtCCR2pro:LUC expression in LL and DD during 24 to 144 h showed that both lhy-10 and toc1-5 lines had a clearly reduced period robustness and a reduction of internal period by approximately 2.5 and approximately 1.5 h compared to the wild type under LL (Table I). Period estimates under DD were similar to LL for the wild-type lines, but no reliable estimates of lhy-10 were obtained because most plants lacked rhythms (Table I). Rhythms in toc1-5 were initially slightly shorter than the wild type, and subsequently became arrhythmic in DD (Fig. 3B). Therefore, both PttLHY and PttTOC1 gene expression are needed to support AtCCR2pro:LUC rhythmicity in Populus under constant conditions.

To also test circadian clock-controlled expression of genes implicated in photoperiodic response, we concentrated on the lhy lines since they showed most clear-cut phase and period defects (Fig. 3; Table I). Gene expression was analyzed in leaves of wild-type and lhy lines after 6 d under SD12 h, a daylength that delayed growth cessation and bud set most notably in the lhy-3 line (Supplemental Table S1). PttLHY expression in the wild type peaked at ZT0 (dawn; Fig. 4A), and the remaining PttLHY expression in lhy lines was advanced by approximately 4 h, closely matching the phase advance detected for AtCCR2pro:LUC.

Populus homologs of FKF1 and GI, both acting in photoperiod-controlled processes in Arabidopsis (Fowler et al., 1999; Park et al., 1999; Nelson et al., 2000; Imaizumi et al., 2003), were assessed under SD12 h (Fig. 4, B and C). The peak times of PttFKF1 and PttGI occurred at ZT8 to ZT12 in the wild type and at ZT4 to ZT8 in the lhy lines. Together, the advanced phase of peak expression of PttLHY, PttFKF1, and PttGI under SD12 h in the lhy lines suggests that they are controlled by PttLHY.

In addition, since overexpression of PttCO2 was shown to postpone growth cessation in Populus (Böhlenius et al., 2006), its expression was analyzed in wild-type and lhy lines. (Fig. 4D). PttCO2 showed a biphasic pattern of expression under SD12 h, peaking at ZT24/0 and ZT8 to ZT12 in the wild type. In lhy lines, the peaks of PttCO2 expression occurred at approximately ZT16 to ZT20 with high amplitude during the dark period. Hence, as a consequence of downregulation of PttLHY, there was an advance of 4 to 8 h in the phase and increase of level of PttCO2 expression in lhy lines relative to the wild type, a difference that could explain the delay in growth cessation and bud set detected under SD12 h and near the CDL (Supplemental Table S1; Fig. 2A).

**PttLHY Down-Regulation Reduces Acclimation to Freezing Temperatures**

Processes associated with growth cessation and bud set may affect intertwined and downstream processes, such as cold hardening and dormancy release. To explore whether PttLHY has any impact on freezing
Table 1. Peak time and period estimates of circadian-controlled AtCCR2pro:LUC expression in LL and DD for wild-type, lhy-10, and toc1-5 lines

<table>
<thead>
<tr>
<th>Condition</th>
<th>Genotype</th>
<th>Line</th>
<th>Period ± SE h</th>
<th>N Traces</th>
<th>Rhythmic N</th>
<th>Peak Time ± SE h</th>
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<td>LL</td>
<td>Wild type</td>
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<td>22.8 ± 0.1</td>
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<td>11.4 ± 0.1</td>
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<td>9</td>
<td>22.8 ± 0.1</td>
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<td>11.0 ± 0.2</td>
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<td></td>
<td>lhy-10</td>
<td>2</td>
<td>20.4 ± 0.1</td>
<td>9</td>
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<td></td>
<td></td>
<td>3^b</td>
<td>20.5 ± 0.5</td>
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<td>6.4 ± 0.1</td>
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<td></td>
<td>toc1-5</td>
<td>5</td>
<td>21.5 ± 0.2</td>
<td>9</td>
<td>8</td>
<td>10.2 ± 0.2</td>
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<td></td>
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<td>7^b</td>
<td>21.2 ± 0.2</td>
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<td>10.6 ± 0.2</td>
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<tr>
<td>DD</td>
<td>Wild type</td>
<td>5^b</td>
<td>22.1 ± 0.1</td>
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<td></td>
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<td>20.9 ± 0.9</td>
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<td>toc1-5</td>
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To entrain plants in LD18 h and shift them to DD explained by an aberrant clock response to cold, so we selected the wild type and the representative lhy-10 line for studies.

Plants were exposed to SD8 h, which prompted simultaneous growth cessation in the wild type and the lhy-10 line (Supplemental Table S1). Plants were subsequently cold hardened in SD6 h at 6°C for 6 weeks. At the time of the freezing-tolerance tests, the stem anatomy was examined and the plants were found to be clearly dormant (Supplemental Fig. S4A). The wild type was resisting freezing without injury as assessed by electrolyte leakage and tissue discoloration (Fig. 5, A and B), while the lhy-10 line consistently showed freezing injuries. The stem parenchyma of the lhy-10 line was visibly injured already at −18°C and severely injured at −51°C, whereas the wild-type line was largely unaffected over this range (Fig. 5C). Impaired freezing tolerance remained in older lhy-10 trees after additional cycles of growth and dormancy (Supplemental Fig. S4C). By contrast, the toc1-5 line showed increased freezing tolerance as compared to the wild-type line when tested in a separate, but identical experiment (P < 0.05; see Supplemental Fig. S4B).

The impaired freezing tolerance in lhy lines may be explained by an aberrant clock response to cold, so we entrained plants in LD18 h and shifted them to DD and cold (4°C) from lights off at ZT18. As shown in Figure 6, A to D, we found that the wild type responded with high and constant expression levels of PttLHY1, PttLHY2, PttTOC1, and Populus PSEUDO RESPONSE REGULATOR5 (Ramírez-Carvajal et al., 2008; PttPRR5). In contrast, lhy-10 plants were unable to up-regulate PttLHY1 and PttLHY2 expression and kept a low and constant expression pattern of these genes (Fig. 6, A and B). PttTOC1 and PttPRR5 showed a reduced expression in lhy-10 as compared to the wild type (Fig. 6, C and D).

A gene specifically implicated in cold hardening in Populus is the C-REPEAT BINDING FACTOR1 (CBF1). Ectopic expression of Arabidopsis CBF1 causes increased cold hardiness in leaves and stem of Populus (Benedict et al., 2006). We found that PttCBF1 was expressed in wild-type leaves with a peak occurring at ZT28 (Fig. 6E), whereas the PttCBF1 expression was abolished in lhy-10 (Fig. 6E). By contrast, toc1-5, the line being more freezing tolerant than the wild type, showed increased PttLHY1, PttLHY2, and PttCBF1 expression (Supplemental Fig. S5, A–C). This provides further evidence that PttLHY1 and PttLHY2 expression is required for cold hardening (Fig. 5; Supplemental Fig. S4), and for regulating the response of circadian clock genes and PttCBF1 in cold (Fig. 6; Supplemental Fig. S5).

The Timing of Bud Burst Is Promoted by PttLHY Expression

Growth from buds can resume when plants have received sufficient chilling but plants stay ecodormant until temperatures become favorable for growth (Rohde and Bhalerao, 2007).

Bud burst was investigated in plants that had set bud under CDL conditions and that were subsequently cold treated for 18 weeks (Fig. 7, A and B). As shown in Figure 7A, the wild type showed initial bud burst after about 11 d and growing shoots after 18 d (scores 2 and 5, respectively). In contrast, the same stages were delayed to 15 and 20 d in the lhy-10 plants (scores 2 and 5, respectively). toc1-5 was not significantly different from the wild type (Fig. 7A). Hence, low levels of...
PttLHY led to a statistically significant delay of reaction of transgenic trees that had set bud at their CDL.

To probe gene expression at bud burst, the expression of PttLHY, PttTOC1, and PttPRR5 was monitored in a developmental series of buds in the wild type and lhy-10 lines. PttLHY1 and PttLHY2 are expressed in a similar manner throughout bud burst (Supplemental Fig. S2, C and D). As shown in Figure 7, C and D, PttLHY and PttTOC1 expression levels in the wild type were highest in the morning at ZT4 and in the evening at ZT12, respectively, and increased with progression of bud burst. PttLHY expression in lhy-10 buds was decreased as compared to the wild type (Fig. 7C). PttTOC1 expression varied, but showed lower expression before bud burst (score 1; Fig. 7D). The expression of PttPRR5 in the wild type was low and increased slightly after leaves were developed (score 4; Fig. 7E). In contrast, the level of PttPRR5 in the lhy-10 plants was high at all times, it normally peaks at ZT12 in LD18 h, suggesting that the lower PttLHY expression led to a continuous high PttPRR5 expression similar to leaves or dormant buds in the wild type in cold (Fig. 6D; Supplemental Fig. S6). Together these data show that PttLHY expression was needed for proper clock gene expression during bud burst and for the speed of growth resumption.

DISCUSSION

The Circadian Clock Regulates Growth by Setting the CDL for Growth Cessation in Populus

The major function of the circadian clock is to track accurately the external light/dark and high/low temperature conditions.
temperature cycles and time internal processes accordingly to optimize growth (Dodd et al., 2005). In fact, it affects most of the gene expression induced by cycling conditions of light and temperature (Harmer et al., 2000; Michael et al., 2008).

To assess the role of the circadian clock in the control of seasonal and diurnal gene expression in *Populus*, we created transgenic trees with down-regulated levels of the putative key clock components *PttLHY1*, *PttLHY2*, and *PttTOC1* by RNAi. Their peak times of gene expression occur in the morning and afternoon, respectively, 8 h apart, while in Arabidopsis *LHY* and TOC1 peak with approximately 12-h difference (Locke et al., 2005). Thus, *PttTOC1* expression in LD is phased earlier in *Populus*. However, clock-driven rhythms of the introduced *AtCCR2pro:LUC* construct peaked at 4 h before dusk in the wild type, similar to the timing in Arabidopsis under LD^{16 h} (Kreps and Simon, 1997). Both *lhy* and *toc1* RNAi lines in *Populus* showed an earlier phase of *AtCCR2pro:LUC*, apparent following shifts to constant conditions.

Although not a complete knockdown, the *lhy-10* line with approximately 60% *PttLHY* expression initially showed short period, but failed to sustain *AtCCR2pro:LUC* expression and presumably other clock functions under constant conditions beyond 48 to 72 h (Fig. 3; Table I). The advanced phase of approximately 5 h of the *AtCCR2pro:LUC* expression in *lhy-10* and the rapid dampening of rhythms under constant conditions were similar to the approximately 6-h-earlier phase and pattern of expression in the Arabidopsis *lhy12cca1-1* mutant (Mizoguchi et al., 2002), indicating that the *Populus* clock’s function is sensitive to *PttLHY* gene’s expression levels and/or regulation. Similarly, the approximately 30% *PttTOC1* expression in *toc1-5* showed short period and eventually led to arrhythmia under DD (Fig. 3; Table I). The *toc1-5* line showed less phase advance (approximately 1 h) and period difference (approximately 2 h) in LL, but was similar to the detected difference between the Arabidopsis wild type and *toc1-1* mutant (Kreps and Simon, 1997). In conclusion, the proper clock function in *Populus* was shown to highly depend on level and period of transcript levels of *PttLHY* and *PttTOC1*.

Circadian clock mutants with shorter internal periods generally show an advanced phase of clock-regulated gene expression (Carré, 2001). Following the external coincidence model, such a phase advance, may in turn affect photoperiodic time keeping.

The importance of the circadian clock was most clear at moderate changes in daylength near the CDL. Near CDL, a significantly delayed, but otherwise comparable, growth cessation of all tested *lhy* and *toc1* lines was observed. When *lhy* plants were shifted from LD^{18 h} to either SD^{9 h} or SD^{12 h}, the delay in growth cessation found under CDL conditions was almost lost. In fact, large daylength shifts from LD^{18 h} to SD^{9 h} probably led to an instant disruption between inner clock rhythms and external light conditions, resulting in little or no difference in the timing of growth cessation and bud set between the wild type and any of the RNAi lines.

Still, under SD^{12 h}, the *lhy* lines showed a delayed growth cessation and bud set as compared to the wild type, and profiles of circadian gene expression consequently were advanced by 4 to 8 h (Fig. 4). Based on these expression profiles, it is possible that *PttFKF1* and *PttGI* control the *PttCO2* expression similar to the situation in Arabidopsis (Sawa et al., 2007). The *PttCO2* expression peaked at least 4 h earlier in the *lhy* lines and were higher as compared to the wild type and likely contributed to the delayed growth cessation in these lines. *PttLHY1*, *PttFKF1*, and *PttFT* were previously shown to be linked to growth cessation in antisense phytochrome A *Populus* (Kozarewa et al., 2010).
In nature, *Populus* responds to a gradually shortening of photoperiod, when the circadian clock entrains on differences of minutes each day. The approximately 1-h CDL difference found between wild-type and transgenic lines will translate to a longer growing season, e.g. of about 3 weeks at 50°C/176°C and of approximately 10 d at 63°C/176°C. This projected but significant delay of the growth cessation process highlights the role of the circadian clock in the setting of CDL in *Populus*.

### PttLHY Is Promoting Cold Hardiness and Bud Burst in *Populus*

We found that during winter dormancy, *lhy-10* was less tolerant to freezing temperatures (Fig. 5). Accordingly, we studied the expression of the central clock components *PttLHY1*, *PttLHY2*, *PttTOC1*, and *PttPRR5* in the wild type and the *lhy-10* line in response to cold (Fig. 6). This study confirmed high and constant expression levels of central *Populus* clock components expression in cold conditions in a manner identical to what was described as clock disruption in chestnut (Ramos et al., 2005; Ibáñez et al., 2008).

In dormant but less freezing-tolerant *lhy-10*, *PttLHY1* and *PttLHY2* expression was low, whereas in increased freezing-tolerant *toc1-5*, *PttLHY1* and *PttLHY2* expression were higher than in the wild type (Fig. 6; Supplemental Fig. S5). Thus, a high and sustained level of *PttLHY* expression is essential for the clock’s adjustment to cold in *Populus*. The lack of clock response to cold and impaired hardiness in *lhy* lines may alter expression patterns of genes that act downstream of the clock involved in cold acclimation such as *PttCBF1* (Benedict et al., 2006). We found that *PttCBF1* expression in the wild type occurred at 4 h after subjective dawn (Fig. 6), which is in accordance with previous data for *Populus* and Arabidopsis (Benedict et al., 2006; Hotta et al., 2007). *PttLHY1*, *PttLHY2*, and *PttCBF1* expression was reduced in *lhy-10*, but was induced in the cold-tolerant *toc1-5* (Fig. 6; Supplemental Fig. S5). Together, the lack of expression of these clock components and of *PttCBF1*

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*Figure 7.* Levels of *PttLHY* expression affect timing of bud burst in response to warm temperature. Wild-type (WT), *lhy*, and *toc1* RNA lines plants were treated as shown in A. Following shift from SD 8 h (6°C) to LD 18 h (18°C), black arrow, apical growth reactivation was monitored. A, Mean bud burst scores ± se were measured for the wild type (n = 5), *lhy-3* (n = 9), *lhy-10* (n = 6), *toc1-1* (n = 9), and *toc1-5* (n = 6). B, Bud burst in representative wild-type, *lhy*, and *toc1* RNAi plants after 13 d in LD 18 h conditions. Expression in buds of *PttLHY* (C), *PttTOC1* (D), and *PttPRR5* (E) from the wild type and the *lhy-10* line sampled separately for each developmental score of bud burst. Each bar represents the mean ± se of two groups of plants per genotype, each consisting of three buds sampled from three independent plants at 4 h (ZT4) and 12 h after dawn (ZT12) using two to three technical repeats. Asterisks (*) represent means that are significantly different from the wild type, *P* < 0.05.
in response to cold in lhy-10 could explain the reduction of freezing tolerance in lhy lines.

Recent studies in Arabidopsis suggest that the central clock components CCA1 and LHY are important for coping with cold and other stressfull conditions (Kant et al., 2008). Interestingly, the arrhythmic triple prr9prr7prr5 mutant showed enhanced resistance to cold as well as high levels of CCA1:LUC expression (Nakamichi et al., 2009). Likewise, in response to cold we find a correlation between the level of expressed PttLHY1, PttLHY2, and PttCBF1 and cold hardening in Populus. Thus, sustained and high levels of Populus PttLHY genes and Arabidopsis CCA1 seem to be required for proper cold hardening. In Populus PttTOC1 is likely inhibitory to this process, since the toc1-5 RNAi line had increased levels of these genes as well as an increased freezing tolerance. However, the response of the clock to cold appears complex and will need further research.

In winter, trees require a period of chilling to break bud endodormancy but growth in spring leading to bud burst proceeds in response to warming temperatures. We found that bud burst depends on PttLHY expression (Fig. 7), suggesting that PttLHY expression is essential not only for coping with freezing temperatures in winter, but also for timing of bud burst, a process essentially driven by temperature.

Since we found that PttLHY and PttPRR5 are sensitive to temperature (Fig. 6), their expression was monitored throughout bud burst together with PttTOC1. Plants deficient in PttLHY expression showed decreased levels of PttLHY due to the RNAi downregulation, slightly lower PttTOC1 levels, and higher expression of PttPRR5 (Fig. 7). As plants were transferred back to warm temperatures, lhy-10 buds showed higher levels of PttPRR5 than the wild type. Since PttPRR5 expression appears high and constant in response to cold in the wild type (Fig. 6; Supplemental Fig. S6), it is possible that the decreased PttLHY expression impaired the ability to react to warming temperatures and that the high PttPRR5 expression in lhy-10 may indicate an impaired response that led to a delayed bud burst (Fig. 7). Hence, there is a significant dependence on PttLHY expression for adjusting the clock to changes in temperature also in the timing of bud burst when exiting dormancy.

CONCLUSION

The circadian clock regulation translates environmental information such as light and temperature into time cues and synchronizes the organism’s daily and seasonal metabolic and physiological response accordingly. We confirmed that the regulation of growth cessation in Populus depends on the circadian clock and particularly on a molecular circuitry including PttLHY1, PttLHY2, and PttTOC1. Importantly, we also demonstrated that during dormancy the Populus trees need sustained PttLHY expression levels to obtain full-freezing tolerance, a crucial trait especially at the higher latitudes. The dependence on PttLHY expression is extended throughout dormancy, and at bud burst it promotes reactivation of growth.

Therefore, it seems that the circadian clock and its components exert control on many aspect of annual growth and survival of temperate woody plants, and, hence, the elucidation of these mechanisms is of great interest for a wide range of disciplines and activities including tree breeding and global change studies.

MATERIALS AND METHODS

Constructs

The PttLHY and PttTOC1 fragments used in the construction of RNAi lines were obtained from Populus CDNA (Populus tremula × Populus tremuloides) by amplification with Platinum Pfx DNA polymerase (Invitrogen) and the primers including GATEWAY specific sequences: LHYF: 5’GGGACCAAGTTGTA-CAAAAACGCGCATGGAATATCCTCTCGGG3’; LHYR: 5’GGGAC-CACTTTCTGACAAAGAAGCCTGTTGCTCTAGGAACACCCT3’; TOCF: 5’GGGGACAAGTTGGAACCAAAAGGCCGATGTTTGTGTTGATTGA-GAA3’; TOCR: 5’GGGGACCACTCTTCTGACAAAGAAGCCTGTTGCTCTAGGAACACCCT3’.

Fragments were cloned into the Gateway entry vector pDONOR201 and then recombined into the plant binary destination vector pHILLSGATE8 (Hellwell et al., 2002) using Gateway BP Clonase enzyme mix (Invitrogen). The respective plasmids were transformed into the Agrobacterium tumefaciens C58 strain pMP90RK.

Generation of Transgenic Plants

P. tremula × P. tremuloides clone T89 was transformed with the RNAi constructs targeted toward PttLHY1/PttLHY2 and PttTOC1 and regenerated essentially as described (Eriksson et al., 2000). Per construct, 10 independent kanamycin-resistant lines were isolated and characterized. Additionally, the wild type and the RNAi lines lhy-10 and toc1-5 were retransformed with the heterologous AtCCR2::LUC construct (Strayer et al., 2000) with hygromycin as selectable marker. Seven independent lines were selected for each genotype and used in subsequent luminescence assays.

Luminescence Assay

Apices from in vitro-cultivated wild-type, lhy, and toc1 RNAi lines were cut and grown on Murashige and Skoog medium supplemented with 2% Suc, expression of ACCR2::LUC lines was conducted with seven independent lines in wild-type, lhy-10, and toc1-5 background under LD18 h (18 h light/6 h dark), and 20 μmol m−2 s−1 of red (660 nm) and blue (470 nm) light-emitting diodes (LEDs; MD Electronics).

In the following experiments, we selected two representative independent lines of each background, which were entrained to LD18 h under approximately 50 μmol m−2 s−1 of fluorescent light and used to study expression of ACCR2::LUC following transfer to constant conditions. For LL experiments, plants were transferred to 20 μmol m−2 s−1 of red and blue LEDs (MD Electronics) at dawn; for DD, they were grown for 2 d in LD with 15 μmol m−2 s−1 of white light LEDs during the days (MD Electronics), followed by the onset of darkness at dusk the second day.

Shoots were supplemented with 5 μmol luciferin 24 h before assay and imaged as described by Kozawa et al. (2010). Period estimates were generated in BRASS (available from www.amillar.org). A fast Fourier transform nonlinear least-squares analysis was used to estimate circadian parameters, such as rhythmicity, phase of peak expression, and period length (Millar et al., 1995; Pfaltz et al., 1997; Locke et al., 2005). Plants were considered rhythmic when the relative amplitude error was <0.6.

Growth Conditions

Rooted cuttings of in vitro-cultivated wild-type, lhy, and toc1 RNAi lines were potted in a 3:1 mix of fertilized peat and perlite. Before any photoperiodic treatments, plants were grown under LD18 h of 200 μmol m−2 s−1 (Osram Powerstar HQI-T 400 W/D lamps, Osram), constant temperature (18°C), and
80% relative humidity for 4 weeks. The temperature, humidity, and irradiance conditions were the same during SD experiments. The shift from LD to SD was done by shortening the day from dusk, keeping dawn unchanged. Bud set scores were recorded to a predefined scale (Rohde et al., 2002; Fig. 2, left section), with 3 corresponding to vegetative growth and 0 to completed bud set.

For artificial winter conditions, dormant plants were kept under LD4h (8 h light/16 h dark) of about 20 μmol m⁻² s⁻¹ and constant low temperature (6°C). The regrowth was scored according to the six developmental stages of bud burst (stage 0 to stage 5) developed by UPOV (1981). Bud burst studies after dormancy induction and chilling were carried out in LD4h as above (Fig. 7). All phenological data were subject to ANOVA. Post-hoc tests were performed typically using Dunnnett’s or Tukey.

Real-Time Quantitative PCR
RNA was extracted from leaf samples collected every 4 h, DNase treated, and subjected to cDNA synthesis and real-time quantitative PCR as described (Kozarewa et al., 2010). Primers used for detection of PttCBF1 were according to Benedict et al. (2006); for detection of PttCO2, primers were according to Böhlenius et al. (2006), PttFKF1, PttLHY1, and PttLHY2 (combined); PttLHY1, PttLHY2, and PttTCO1 were described by Kozarewa et al. (2010). PttGI primers were according to Böhlenius (2007). PttPRR5a and PttPRR5b (combined) forward: CGCAGAATTCCGGCATTAAA; reverse: GCCCAATGCACCCGGTGAC; annealing at 55°C. For analysis of statistical difference in Figure 7, mean gene expression of the wild type and lhy-10 at bud burst 1 test was used following log2 transformation.

Freeze Test
Freeze tests were performed on dormant plants kept in cold (SD3h, 6°C) for 6 weeks. From each of seven replicate plants per genotype, a 20 to 60 cm stem section beneath the shoot tip was cut into segments of 5 cm and used for electrolyte leakage as described (Kozarewa et al., 2010) using the method by Flint et al. (1967) to calculate relative injury. The extent of leakage from unfrozen controls were similar for the wild-type and lhy-10 plants as indicated by values of pre- to post-heating conductivity ratios averaging 0.245 and 0.252, respectively (SE values < 0.01). The remains of segments after trimming for electrolyte analyses were assessed for visual injury after further storage at about 10°C and 100% relative humidity for 3 weeks. The photosynthetic parenchyma cells 5 mm away from the segment ends were examined for signs of discoloration under bright white light. Indices of injury of 0%, 25%, 50%, 75%, and 100% were assigned to no discoloration, initial and final discoloration of the inner parenchyma, and initial and final discoloration of the outer parenchyma, respectively (discoloration of inner parenchyma was completed before discoloration of outer parenchyma started). In a second experiment, electrolyte leakage was not assessed and segments remained intact until examined for visual injury. Also, the temperatures series was slightly different (41°C was represented) and six instead of seven replicate plants were used. The significance of difference between genotypes was tested using repetitive measures two-way ANOVA. Injury data, being expressed as proportions, were arc sine square-root transformed before analyses.

Associated Populus gene models: PtbCF1, grai5:0005054801; PICO2, estExt_Genewise1_v1.0_C_LG_X4235; PttFKF1, estExt_fgenesh1_pg.C笠V1131; PttLHY1, eugene3. 00021683; PttLHY2, estExt_Genewise1_v1.0_C_LG_X41950; PttPRR5a, gw1. XII.123131; PttPRR5b, eugene3.00150024; PttTCO1, fgenesh1 pg.C_scaffold_129000034.

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure S1. Transcript levels of core clock components observed in wild-type and RNAi transgenic lines grown under LD2h.
Supplemental Figure S2. Transcript levels of PttLHY1 and PttLHY2 in leaves during growth and in buds at reactivation of growth following dormancy.
Supplemental Figure S3. AtCCR2pro:LUC expression in wild-type and transgenic lines grown under LD2h. conditions.
Supplemental Figure S4. Inspection of dormancy and freeze injury as determined by visual examination.

Supplemental Figure S5. The freezing-tolerant toet-5 has increased levels of PttLHY1, PttLHY2, and PttCBF1 expression in response to cold.

Supplemental Figure S6. PttPRR5 expression in buds collected from trees under warm and cold SD conditions.

Supplemental Table S1. Growth cessation and bud set response of wild-type and selected lhy RNAi lines subjected to SD b and SD2h.

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LITERATURE CITED

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The Circadian Clock’s Role in Seasonal Regulation of Growth


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