CO/FT Regulatory Module Controls Timing of Flowering and Seasonal Growth Cessation in Trees

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suggests that its precursors may have evolved before 14 Ma in the great apes. Together with recent evidence from scrub jays (*8*, *9*, *21*), our results suggest that future planning is not a uniquely human ability, thus contradicting the notion that it emerged in hominids only within the past 2.5 to 1.6 million years (*10*, *11*, *22*). Indeed, its presence in distantly related taxa such as corvids and apes reinforces the hypothesis that these taxa may have undergone convergent cognitive evolution (*23*). Future studies should investigate whether apes, like corvids, will not only transport tools for future use but also protect them from conspecifics that may steal them.

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Supporting Online Material

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CO/FT Regulatory Module Controls Timing of Flowering and Seasonal Growth Cessation in Trees

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Forest trees display a perennial growth behavior characterized by a multiple-year delay in flowering and, in temperate regions, an annual cycling between growth and dormancy. We show here that the *CO/FT* regulatory module, which controls flowering time in response to variations in daylength in annual plants, controls flowering in aspen trees. Unexpectedly, however, it also controls the short-day–induced growth cessation and bud set occurring in the fall. This regulatory mechanism can explain the ecogenetic variation in a highly adaptive trait: the critical daylength for growth cessation displayed by aspen trees sampled across a latitudinal gradient spanning northern Europe.

Trees have extended juvenile phases that can last for decades before the first flower is formed. Trees can also cycle between periods of growth and dormancy. In temperate regions, this involves a short-day–induced growth cessation and bud set in the fall, after which the tree enters a dormant state characterized by an enhanced cold tolerance. Tree populations (provenances) from northern latitudes typically display growth cessation at a longer critical daylength, leading to earlier bud set during fall compared with southern populations (*1*). This is a highly adaptive trait because it ensures that bud set and dormancy have been induced well before the risk of frost damage. This response is under strong genetic control and is maintained when trees are moved between latitudes (*2*, *3*). The molecular mechanism that controls growth cessation at different critical daylengths is not known; neither is the mechanism controlling the multiple-year delay in flowering.

In the annual plant *Arabidopsis*, the genes *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) are necessary for the daylength regulation of flowering, inducing flowering as a response to long days (*4*). *CO* displays a diurnal regulation in which the mRNA accumulation peaks at the end of the day in long days and during the night in short days (*5*). Furthermore, the CO protein is extremely labile in darkness, leading to an accumulation of CO protein only in long days (*6*). CO then induces transcription of the gene *FT* in the leaf, and the *FT* mRNA moves from leaf to shoot apex (*7*, *8*), where the translated FT protein induces the formation of flowers (*8*, *9*). The *FT* mRNA fulfills many of the criteria characterizing the elusive flower-inducing molecule "florigen" described in the 1930s (*7*).

To determine whether a tree *FT* ortholog is also involved in the regulation of flowering time in trees, a process that is not obviously regulated by daylength because of their long juvenile phase, or whether it is involved in the daylength regulation of perennial growth and dormancy, we have investigated the role of the *FT* ortholog in *Populus* trees (poplars, aspens, and cottonwoods).

We isolated the *Populus trichocarpa FT* ortholog, which we call *PtFT1* (fig. S1) and showed that its function in inducing early flowering is conserved in transgenic *Arabidopsis* (fig. S2) (*10*). *Populus trichocarpa* is difficult to transform, but all *Populus* species are closely related, and the sequence identity between homologous genes in different *Populus* species is often 99% (*11*). Male *Populus tremula x tremuloides* transformed with *35S::PtFT1* initiated flowerlike structures directly from the Agrobacterium-infected stem segments within 4 weeks (Fig. 1, A and B), compared with the normal flowering time of 8 to 20 years (*12*). This shows that *PtFT1* is a powerful inducer of flowering in *Populus*. Weaker expressing lines could be regenerated and planted in the greenhouse. These trees produced inflorescences (catkins) (Fig. 1, C to E, H, and I; and fig. S3, A to C) containing phenotypically normal male flowers (Fig. 1, F and J) with an apparently normal pollen development (Fig. 1, G and K). We also generated early-flowering female *Populus tremula* with normal inflorescence development (fig. S3D). This is the first report of juvenile transgenic trees producing inflorescences. In contrast, early-flowering *Populus* ectopically expressing

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the *Arabidopsis* flower meristem-identity gene *LEAFY* (*LFY*) produced shoots containing single flowers instead of catkins (*12*), reflecting the role of *LFY* as a flower meristem identity gene, while *PtFT1*, just as *FT*, appears to act as a flowering time gene, inducing more developmentally normal flowering.

The fact that *35S::PtFT1* expression leads to early flowering suggests that the level of *PtFT1* expression could be an important determinant of flowering time in *Populus* trees. To test this, we collected shoot tips from a *Populus* clone aged 2 to 6 years. This particular clone flowers first after 5 to 6 years, and floral initiation takes place between mid-May and mid-June (*13*). Samples collected in May and June displayed a gradual increase in *PtFT1* expression as the tree grew older (fig. S4), suggesting that a critical level of *PtFT1* expression is needed to initiate flowering. What kind of mechanism could be responsible for such a gradual increase over many years? In *Arabidopsis*, the gene *EARLY BOLTING IN SHORT DAYS* (*EBS*) acts as a repressor of *FT* transcription, probably through regulation of chromatin structure (*14*). It could be that the role of repressors such as *EBS* are much more pronounced in trees than in *Arabidopsis* and that each annual cycle of growth and dormancy leads to a gradual release of the chromatin structure–based *PtFT1* repression, making *PtFT1* more and more susceptible to transcriptional activation by *PtCO*.

In *Arabidopsis*, *FT*-dependent induction of flowering is the only daylength-regulated developmental transition. In contrast, shortening of the daylength induces growth cessation and bud set in *Populus* trees (*3*), and we postulated that *PtFT1* could also have a role in this daylength-regulated process. When wild-type *Populus tremula x tremuloides* plants are shifted from long days to short days, they respond by growth cessation and bud set after 32 days (Fig. 2, A and B; and fig. S5A). After another 31 days in short days and 5 days in darkness at 5°C, the tree has abscised its leaves and is dormant (Fig. 2C). In contrast, *35S::PtFT1*-expressing trees displayed no signs of growth cessation but continued growing for more than 60 days in short-day conditions (Fig. 2, E to G; and fig. S5A). This indicates that *PtFT1* expression is an efficient suppressor of short-day–induced growth cessation and bud set. We then analyzed the expression of *PtFT1* in response to a long-day to short-day shift. In long days, *PtFT1* displayed an expression pattern very similar to that of *Arabidopsis FT*, with a diurnal regulation showing a peak of expression in the beginning of the night (Fig. 2I). After transfer of plants to short days, down-regulation of *PtFT1* expression could be detected within 3 days (fig. S5B), and after one week *PtFT1* expression was severely reduced, with no signs of a diurnal variation (Fig. 2I). This indicates that the down-regulation of *PtFT1* is a very early response to a shift to short days and that this down-regulation is necessary for normal growth cessation and bud set to occur. To test this, we generated transgenic plants in which the level of *PtFT* was down-regulated through RNA interference (RNAi). These trees were more sensitive to shortening of the daylength, with nine of the *PtFT* RNAi lines setting vegetative buds 2 weeks after a shift from a 16-hour day to a 15-hour day (Fig. 2J), whereas wild-type plants required 10 weeks before buds were visible (Fig. 2J). Unlike wild-type trees, four of the RNAi lines even set buds at the 16-hour daylength (Fig. 2, D, H, and J). The increased sensitivity to the short-day signal was correlated to the level of *PtFT1* down-regulation (Fig. 2J).

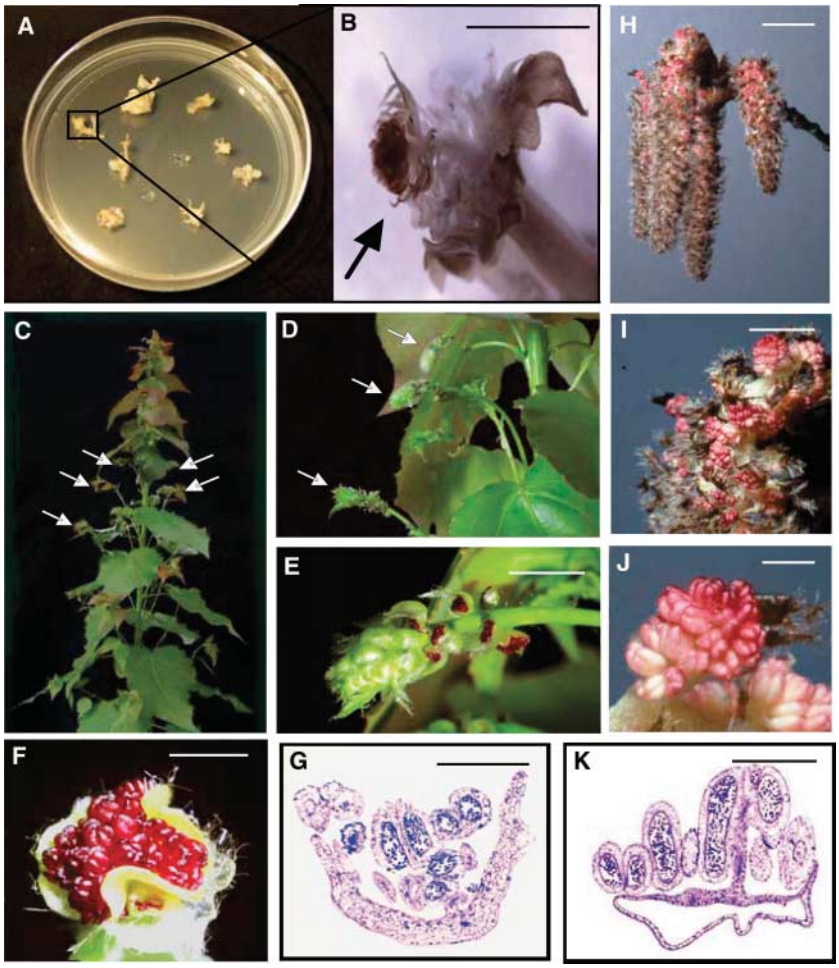


Fig. 1. *PtFT1* expression induces early flowering in *Populus* trees. (**A** and **B**) Four-week-old stem segments of hybrid aspen (*Populus tremula x tremuloides)* grown in tissue culture. (A) Overview of a tissue culture plate with stem segments transformed with *35S::PtFT1*. (B) Close-up view of a developing flower (black arrow). (**C** to **G**) Six-month-old greenhouse-grown *35S::PtFT1* plants. (C) Overview of a *35S::PtFT1* tree. (D) Developing male catkins (white arrows). (E) Close-up view of a developing male catkin. (F) Male flower removed from catkin. (G) Section of a male flower. (**H** to **K**) *Populus tremula* wild-type flowers. (H) Developing male catkins. (I) Close-up view of a developing male catkin. (J) Male flower. (K) Section of a male flower. Scale bars, 1 mm [(B), (F), (G), (J), and (K)]; 1 cm [(E), (H), and (I)].

To determine whether the regulation of *PtFT1* correlated with the critical daylengths for growth cessation displayed by European aspen (*Populus tremula*) trees originating from different latitudes (provenances), we compared trees from four different latitudes stretching from 51°N (the middle of Germany) to 63°N (Northern Sweden) (Fig. 3A). We established these plants in controlled growth conditions and determined the critical daylengths for growth cessation and bud set. They varied from 21 hours for the provenance from northern Sweden to 15 hours for the German provenance (Fig. 3B). When we grew these plants under a 19-hour photoperiod, which is above the critical daylength for the two southernmost provenances, *PtFT1* displayed a clear peak of expression at the end of the day in the southern provenances but was only very weakly expressed in the two

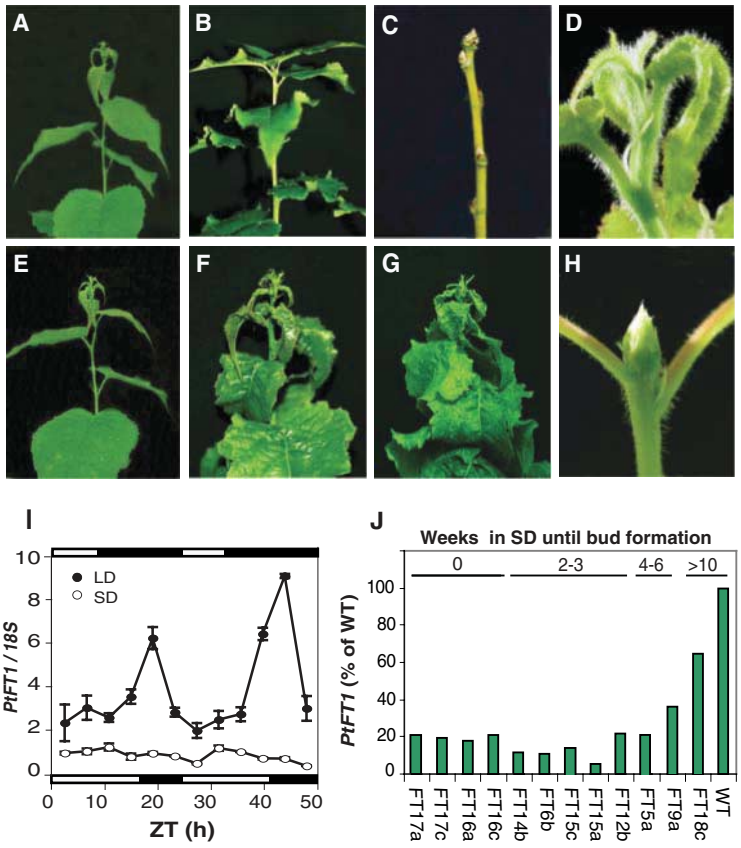


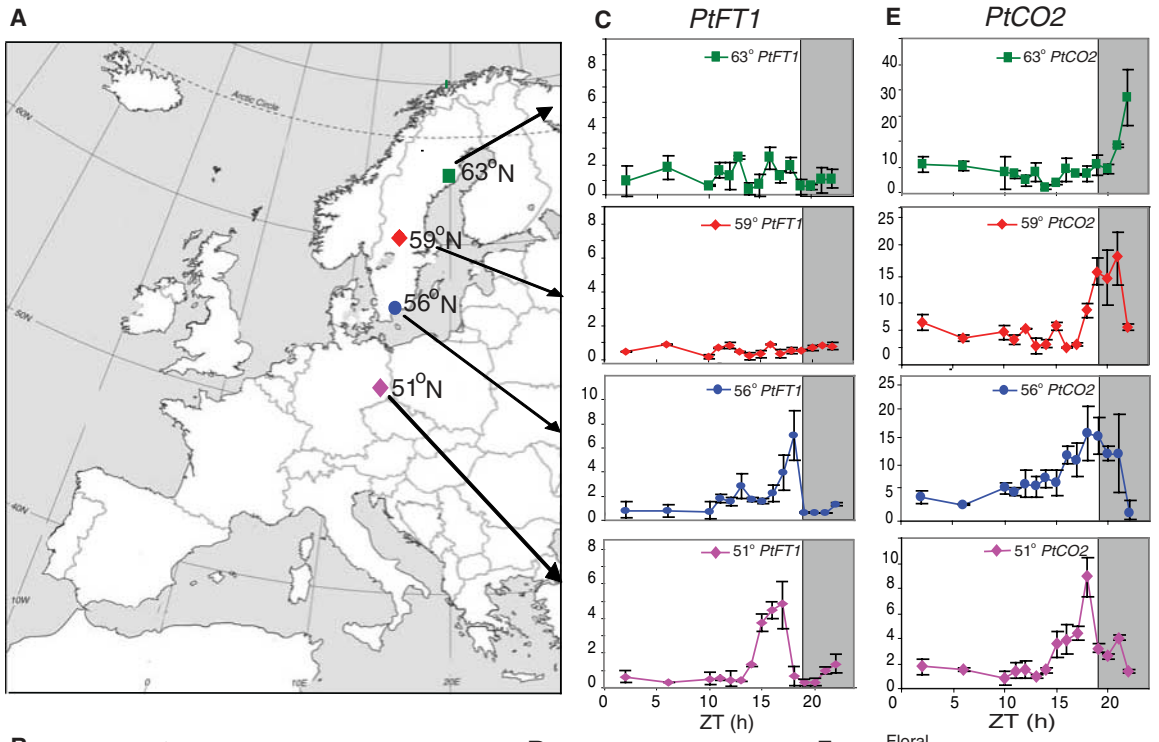
Fig. 2. *PtFT1* expression affects short-day–induced growth cessation and bud set in *Populus* trees. (**A** to **C**) Wild-type plants and (**E** to **G**) *35S::PtFT1 Populus tremula x tremuloides* in long days [(A) and (E)], 32 short days [(B) and (F)], and 63 short days and 5 days in darkness at 5°C [(C) and (G)]. (**D**) Wild-type and (**H**) *PtFT* (*PtFT1* and *PtFT2*) RNAi plants grown in 16-hour-long days. (**I**) Diurnal expression pattern of *PtFT1* in long days (closed circles) and 1 week after transfer to short days (open circles). Three mature leaves were collected at the different time points from three independent *Populus tremula x tremuloides* (T89) ramets. Black boxes indicate darkness and white boxes light. Error bars, ± SD. ZT, zeitgeber time. (**J**) *PtFT1* expression in 12 independent transgenic *PtFT* (*PtFT1* and *PtFT2*) RNAi lines setting bud 0 to 10 weeks after a shift from 16-hour to 15-hour days (5 ramets per clone). All ramets of the same clone set bud within the indicated time intervals. Three mature leaves were collected and pooled in the evening from three ramets of each clone growing in 16-hour days. Details of these experiments are described in (*10*).

northern provenances (Fig. 3C). Further experiments at 17- and 21-hour daylength confirmed that *PtFT1* only showed a peak in expression when the photoperiod was above the critical daylength for a particular provenance (fig. S6A), supporting the conclusion that the level of *PtFT1* expression is a critical determinant of the timing of growth cessation and bud set in the fall.

What, then, is the mechanism sensing these different critical daylengths? In *Arabidopsis*, *FT* transcription is under the control of the gene *CO* which, according to the external coincidence model, is responsible for *FT* regulation in response to long days (*15*, *16*). We therefore tested whether the function of the *CO*/*FT* regulon was conserved in *Populus* and whether variations in expression of the *Populus CO* ortholog could explain the differential regulation of *PtFT1* seen in the different provenances. We isolated a *Populus trichocarpa CO* ortholog, *PtCO2* (fig. S1) (*10*). In long days, *PtCO2* displays a diurnal expression pattern peaking at the end of the day. After a shift to short days, the expression peaks in the night, with very low expression in the light (Fig. 3D). This is very similar to the expression pattern of *CO* and corresponds to the *Arabidopsis* model of *FT* regulation by *CO*, in which *CO* can only activate *FT* transcription when *CO* transcription peaks at the end of the day (*15*, *16*). An attractive model explaining the different critical daylengths for growth cessation and bud set for the various provenances would be that the *PtCO2* expression starts increasing earlier after dawn in the more southern provenances. This would mean that the days must be shorter for the southern than for the northern provenances before *PtCO2* transcript abundance starts increasing in darkness and no *PtFT1* expression is induced. In a 19-hour photoperiod, this is indeed the case (Fig. 3E). In the southernmost provenance, the first detectable rise in *PtCO2* transcript accumulation occurs about 6 hours earlier than in the northernmost provenance (Fig. 3E); this is also the difference in critical daylength between these provenances (Fig. 3B). These changes in the phase of *PtCO2* mRNA oscillation are also seen at 17-hour and 21-hour daylengths (fig. S6B). The importance of the level of *PtCO* transcription for the timing of growth cessation is further corroborated by the fact that transgenic plants with a down-regulation of *PtCO* expression are more sensitive to the short-day signal, just like *PtFT* RNAi plants (fig. S7).

In *Populus*, phytochrome genes have been implicated in the regulation of short-day–induced bud set and growth cessation (*2*, *17*, *18*), and transgenic *Populus* trees expressing the oat *PHYTOCHROME A* (*PHYA*) gene fail to initiate growth cessation and bud set as a response to the short-day signal (*19*). Because PHYA is a known regulator of *FT* transcription through the regulation of *CO* (*16*), we tested whether one reason that *35S::PHYA* plants fail to respond to the short-day signal could be an inability to down-regulate *PtFT1*. In our conditions, the *35S::PHYA* plants continued growing after being transferred to short days, much in the same way as did *35S::PtFT1* plants (fig. S5A). Furthermore, the *35S::PHYA* plants displayed no signs of down-regulating *PtFT1* during short days (fig. S5B), and the amount of *PtCO2* mRNA did not decrease at the end of the day, suggesting a shift in the phase of *PtCO2* expression (fig. S5C). Taken together, these results suggest that the level of *PtFT1* transcription is an important regulator of short-day-induced growth cessation and bud set and that the phytochrome regulation of critical daylength could, at least partly, be mediated through a regulation of *PtCO2* and *PtFT1*.

We show in these experiments that *PtFT1*, the *Populus* ortholog of the *Arabidopsis* gene *FT*, is controlling two aspects of the perennial growth behavior. It is involved both in controlling the multiple-year delay in flowering time and in controlling growth cessation and bud set in the fall. In a reproductively mature tree, both these processes might be subjected to daylength control—flowering induced by long days in the spring and early summer, and growth cessation induced in the fall. Indeed, this is reflected in the *PtFT1* expression pattern over a season (Fig. 3F). Our results therefore suggest that *FT* orthologs can have a more



B	63°	59°	56°	51°
22 h	+	+	+	+
21 h	-	+	+	+
20 h	-	+	+	+
19 h	-	-	+	+
18 h	-	-	+	+
17 h	-	-	-	+
15 h	-	-	-	-

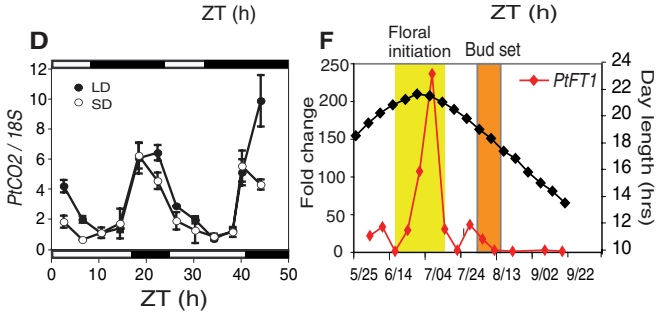


Fig. 3. The critical daylength for growth cessation in trees originating from different latitudes is controlled by *PtFT1* expression and the phase of *PtCO2* oscillation. (**A**) Map of Europe indicating the origin of the four different European aspen (*Populus tremula*) clones. Green squares, Umeå (63°N); red diamonds, Brunsberg (59°N); blue circles, Ronneby (56°N); lilac diamonds, Brauna (51°N). (**B**) Table showing the critical daylengths for growth cessation for the collected *Populus tremula* clones. Plus indicates growth, minus indicates bud set. (**C**) *PtFT1* and (**E**) *PtCO2* expression in the different *Populus tremula* clones in 19-hour daylength. Gray boxes indicate night, white boxes indicate light. (**D**) Diurnal expression pattern of *PtCO2* in *Populus tremula x tremuloides* (T89) under long days (filled circles) and 1 week after transfer to short days (open circles). Three mature leaves were collected at the different time points from three independent ramets of each clone. Black boxes indicate darkness and white boxes light. Error bars, ±SD. ZT, zeitgeber time. (**F**) Seasonal variation in *PtFT1* expression (red diamonds) in a 30-year-old *Populus tremula* tree growing in Umeå, Sweden (63°N). Black diamonds indicate daylength. Shaded areas indicate bud set and the likely period for floral initiation. Three mature leaves were collected and pooled in the middle of the day at the dates indicated. Details of these experiments are described in (*10*).

general role in regulating biological processes that are subjected to daylength control than previously anticipated from *Arabidopsis* work.

We also show a mechanism for how the *CO*/*FT* regulon is controlling a highly adaptive trait for forest trees: the variation in the critical daylength that induces growth cessation and bud set in tree populations originating from different latitudes. This response represents a critical ecological and evolutionary tradeoff between survival and growth in most forest trees (*20*–*22*) and is key to the adaptation to new geographical areas and to the current climate change. Knowledge about this mechanism will therefore be of importance for future tree breeding programs. The technique to induce early flowering also opens up the possibility to dramatically speed up tree breeding programs, which is greatly needed because the world's natural forests are declining partly as a result of an increasing demand for wood.

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Supporting Online Material

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References

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