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ELF3 coordinates temperature and photoperiodic control of seasonal growth in hybrid aspen

Highlights

- The tree ELF3 protein undergoes temperature-induced phase separation
- ELF3 mediates low-temperature-induced growth cessation
- ELF3 acts upstream of the AIL1 and hormonal pathways
- ELF3 mediates photoperiodic control of *FT2* expression

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In brief

Nair et al. show that tree ELF3 ortholog, a protein with a prion-like domain, undergoes temperature-sensitive phase separation and plays a key role in regulating the timing of growth cessation in response to temperature and photoperiodic signals in hybrid aspen.

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ELF3 coordinates temperature and photoperiodic control of seasonal growth in hybrid aspen

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SUMMARY

Timely growth cessation before winter is crucial for the survival of perennial plants in temperate and boreal regions. Short photoperiod (SP) and low temperature (LT) are major seasonal cues regulating growth cessation. SP, sensed in the leaves, initiates growth cessation by downregulating *FLOWERING LOCUS T 2 (FT2)* expression, but how LT regulates seasonal growth is unclear. Genetic and cell biological approaches identified a hybrid aspen *EARLY FLOWERING 3*(*ELF3*) ortholog with a prion-like domain (PrLD) that undergoes LT-responsive phase separation as a key mediator of LT-induced growth cessation. In contrast with SP, LT acts independently of *FT2* downregulation and targets the AIL1-BRC1 transcription factor network and hormonal pathways via ELF3 to induce growth cessation. Intriguingly, ELF3 also functions in SP-mediated growth cessation by downregulating *FT2* in leaves. Our work thus reveals a previously unrecognized role of ELF3 in growth cessation and in coordinating temperature and photoperiodic pathways to enable robust adaptation to seasonal change.

INTRODUCTION

Autumnal growth cessation is a crucial adaptation for winter survival in perennial plants in boreal and temperate regions.[1] Shortening of the photoperiod (SP) is a well-characterized environmental cue inducing growth cessation in trees. SP is sensed in the leaves and causes growth cessation by downregulating the expression of *FLOWERING LOCUS T* 2 (*FT2*), a mobile growth promoter.[2–4] This, in turn, suppresses the formation of new leaf primordia and induces morphogenetic transformation to form a bud enclosing the shoot apical meristem and arrested leaf primordia at the shoot apex.[4] However, SP is not the only growth-cessation signal: field and growth chamber studies have identified low temperature (LT) as another important signal[5,6] and have shown that it is the dominant cue regulating growth cessation in trees such as Rosaceae.[5] Despite the critical role of LT in seasonal control of tree growth, the molecular mechanisms by which it acts remain largely unknown. Here, we address this major gap in our understanding of seasonal adaptation in perennials by uncovering mechanisms underlying LT-mediated control of growth cessation in the experimental model tree hybrid aspen. We show that a prion-like domain (PrLD)-containing protein orthologous to Arabidopsis EARLY FLOWERING 3 (ELF3) plays a key role in LT-mediated control of growth cessation in this model species. Interestingly, our results show that *ELF3* also functions as a repressor in SP-mediated growth cessation, acting upstream of *FT2*, the key factor in the photoperiodic pathway. *ELF3* thus coordinates responses to the two primary cues that enable robust temporal control of autumnal phenology and adaptation to seasonal changes.

RESULTS

LT induces growth cessation in hybrid aspen

To understand the molecular basis of LT-mediated control of seasonal growth cessation, we first investigated whether LT could induce growth cessation and bud set independently of SP in hybrid aspen by growing plants at 12°C (LT) and 20°C under growth-permissive long days (thereby avoiding any photoperiodic input). Upon sensing the growth-cessation-inducing signal, the formation of new leaves is terminated, the growth of the leaf primordia is arrested, and the leaf primordia are enclosed in the apical bud.[7] Thus, the number of leaves formed up to bud set after exposure to the growth-cessation signal is a sensitive marker for analyzing growth-cessation responses.[7–9] Hybrid aspen plants cultivated at 12°C ceased growth earlier, forming significantly fewer leaves up to bud set than those cultivated at 20°C, which continued growing (Figures 1A and B). These results indicate that LT alone is sufficient to induce growth cessation and bud set in hybrid aspen.

LT targets expression of growth-cessation regulators expressed in the shoot apex

Having established that LT can induce growth cessation, we next investigated the potential targets of the LT pathway. FT2 and the

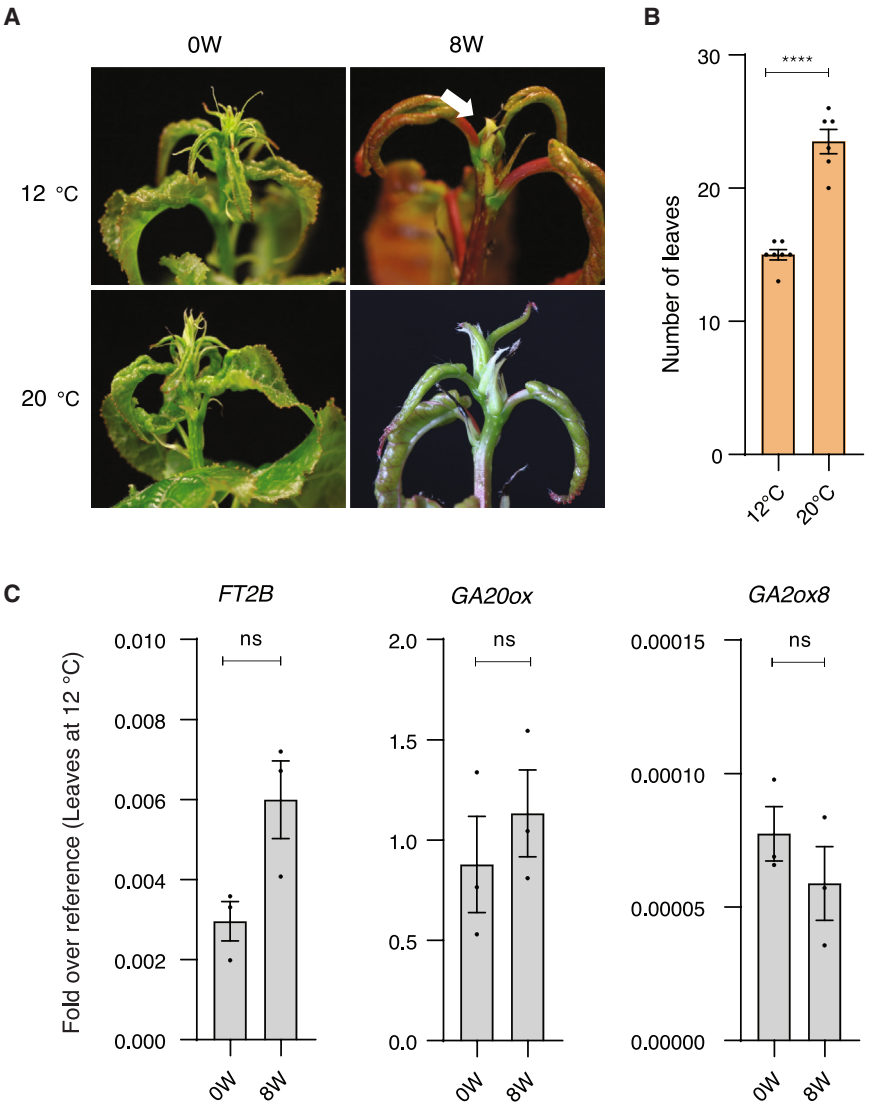

Figure 1. LT is sufficient to induce growth cessation

(A) Representative pictures of shoot apices of wild-type (WT) T89 plants grown at 12°C and 20°C under LD (18 h day/6 h night) conditions taken at 0 and 8 weeks.

(B) Average numbers of leaves (±SEM) produced by WT plants at 12°C and 20°C under LD conditions ($n \geq 6$). Note that plants grown at 12°C had ceased growth and produced buds by the end of the experiment, unlike those grown at 20°C.

(C) Expression of growth regulators in the leaves of plants exposed to LT (12°C). All values are means (±SEM) of three biological replicates and are normalized against *UBQ*. Asterisks indicate significant differences (****$p < 0.0001$, ns, not significant; t test analysis).

gibberellic acid (GA) pathways are major targets of growth-cessation-inducing seasonal cues such as SP in the leaves. SP downregulates the expression of *FT2* and *GIBBERELLIN 20 OXIDASE* (*GA20ox*, a key enzyme in the biosynthesis of the phytohormone GA) while upregulating that of *GIBBERELLIN 2 OXIDASE* (*GA2ox*, a key enzyme in GA degradation).[3,10,11] We therefore investigated the effects of LT on *FT2B*, *GA20ox*, and *GA2ox* expression in the leaves. Intriguingly, unlike SP,[3,11] LT treatment did not downregulate *FT2B* and *GA20ox* or upregulate *GA2ox* in wild-type (WT) hybrid aspen leaves (Figure 1C).

Because LT had no effect on the transcription of key SP targets in the leaves, we hypothesized that it might instead target genes expressed in the shoot apex that regulate growth cessation, such as *BRANCHED 1 (BRC1)* and the transcription factors *LAP1* and *AIL1* (orthologous to the Arabidopsis genes APETALA1 and AINTEGUMENTA, respectively).[9,12] This revealed that LT downregulated *LAP1* and *AIL1* expression in the shoot apex while upregulating *BRC1* (Figures 2A–2C). Additionally, *GA2ox8* expression increased 10-fold in the apex of plants exposed to 8 weeks of LT compared with plants without LT exposure (0 weeks) (Figure 2D). The phytohormone abscisic acid (ABA) is a well-known mediator of LT responses, hence we also analyzed the effect of LT on the expression of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*), which encodes the rate-limiting enzyme in ABA biosynthesis. *NCED3* expression in the shoot apex was significantly upregulated in LT-treated plants (Figure 2E). LT thus acts independently of transcriptional downregulation of canonical SP targets, such as FT2 or the GA pathway in leaves, instead targeting key regulators of growth cessation in the apex.

We next used a genetic approach to functionally investigate the role of the LAP1-AIL1-BRC1 module and hormonal pathways in LT-mediated growth cessation. Hybrid aspen plants with reduced *LAP1* expression (LAP1-RNAi) displayed faster response to LT than WT, ceasing growth earlier and forming significantly fewer leaves up to bud set (Figure 2F). Conversely, LAP1ox plants with enhanced *LAP1* expression were insensitive to LT and did not stop growing at 12°C (Figure 2F). *AIL1*, a downstream target of LAP1,[8] was also downregulated by LT (Figure 2B), prompting us to investigate its role in LT-mediated growth cessation. Hybrid aspen has four highly similar, redundantly acting *AIL* genes (*AIL1–4*). To overcome this redundancy, we expressed *AIL1* fused with SRDX (SUPERMAN repressor domain X - a synthetic dominant repressor domain) in hybrid aspen. AIL1-SRDX functions as a dominant-negative transcriptional repressor and has been shown to suppress the function of the *AIL* family.[12,13] AIL1-SRDX plants responded faster to LTs, undergoing earlier growth cessation and forming significantly fewer leaves up to bud set at 12°C compared with the WT (Figure 2G). In contrast to its effects on *LAP1* and *AIL1*, LT induced the expression of *BRC1*. Accordingly, transgenic plants with high *BRC1* expression responded strongly to LT, undergoing earlier growth cessation and forming significantly fewer leaves up to bud set than the WT (Figure 2H).

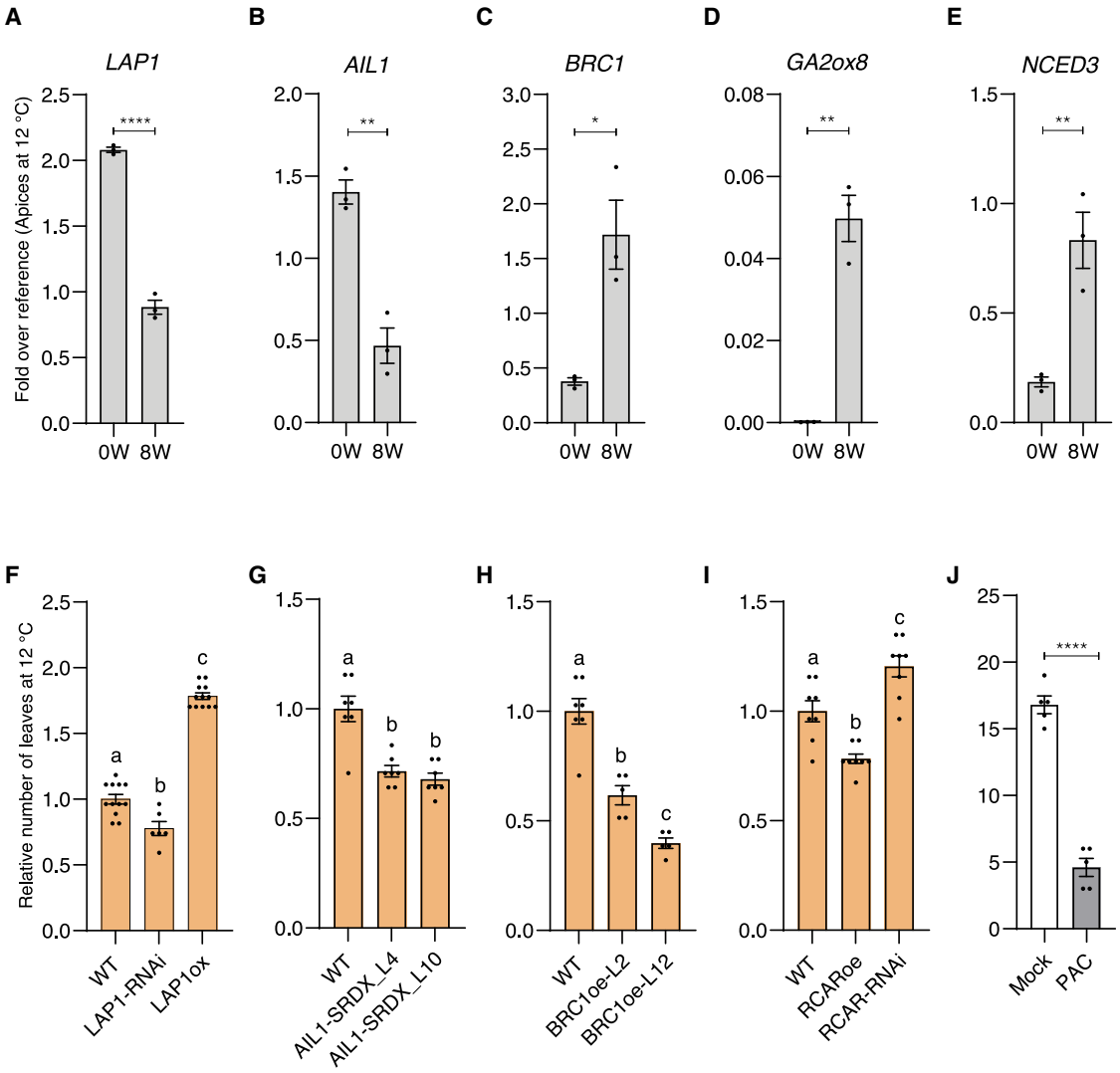

Figure 2. LT targets the LAP1-AIL1-BRC1 and hormonal pathways to induce growth cessation

(A–E) Quantitative real-time PCR data showing relative levels of *LAP1*, *AIL1*, *BRC1*, *GA2ox8*, and *NCED3* transcripts in apices of WT plants grown under LT conditions (12°C) for the indicated number of weeks.

All values are means (± SEM) of three biological replicates and are normalized against *UBQ*. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$; t test analysis).

(F–I) Average numbers of leaves (±SEM) to bud set in LT for the LAP1ox, LAP1-RNAi, AIL1SRDX, BRC1ox, RCARox, and RCAR-RNAi transgenics relative to the parental WT plants ($n \geq 8$). (G) and (H) show the same values for WT, as the experiment was conducted together. Lowercase letters denote significant difference (*$p < 0.05$) based on ordinary one-way ANOVA followed by Kruskal-Wallis test. See also Figure S1.

(J) Average numbers of leaves (±SEM) to bud set produced at LT by WT plants treated with 100 μM paclobutrazol (PAC) ($n = 5$). Asterisks indicate significant differences (****$p < 0.0001$; t test analysis).

In addition to its effects on the LAP1-AIL1-BRC1 module, the induction of *GA2ox* and *NCED3* indicates that LT targets GA-ABA hormonal pathways. To address the role of ABA in LT-induced growth cessation, we used two transgenic hybrid aspen lines, one with an enhanced ABA response due to overexpression of the ABA receptor REGULATORY COMPONENTS OF ABA RECEPTOR (RCAR1oe)[14] and another with a weakened ABA response due to downregulated RCAR expression (RCAR-RNAi).[14] RCAR1oe plants displayed early growth cessation at LT, forming significantly fewer leaves up to bud set, whereas RCAR-RNAi plants ceased growth later, forming more leaves up to bud set than the WT (Figure 2I). Because LT also induces *GA2ox* expression, we studied its effects on growth suppression by using paclobutrazol (PAC),[15] a GA biosynthesis inhibitor, to block GA production in the shoot apex. PAC-treated apices exhibited significantly earlier growth cessation than mock-treated negative controls (Figure 2J), forming significantly fewer leaves. The effects of this pharmacological treatment, together with the observation that LT upregulates *GA2ox* expression, suggest that LT-induced growth cessation involves negative regulation of the GA biosynthesis pathway. Importantly, in contrast with the response to LT, the transgenic lines grown in warm condition

did not cease growth and produced similar numbers of leaves to WT plants at the end of the experiment (Figures S1A–S1D). These results suggest that LT targets the LAP1-AIL1-BRC1 and hormonal pathways to induce growth cessation.

The PrLD-containing protein ELF3 is required for LT-mediated growth cessation

Our results (Figure 2) indicated that LT alters the expression of several genes crucial for growth cessation, and experiments using transgenic hybrid aspen supported the involvement of these genes in LT-induced growth cessation. However, LT did not downregulate *FT2*, the key upstream component whose downregulation by SP is a crucial early event in the photoperiodic regulation of these growth-cessation regulators. These results suggest that, presumably, a different component mediates the LT response of the growth-cessation pathway. PrLD-containing proteins such as ELF3 have been shown to mediate temperature sensing in the hypocotyl elongation response and flowering in Arabidopsis.[16,17] Moreover, previous studies have shown that ELF3 undergoes a reversible PrLD-mediated shift from an active diffuse phase to an inactive condensate phase.[16,18] We therefore screened the hybrid aspen genome for ELF3-like genes and investigated their involvement in LT-induced growth cessation. Our phylogenetic analysis indicates that *Populus tremula* and *Populus trichocarpa* each possess one ELF3-like protein as well as an EEC (ESSENCE OF ELF3 CONSENSUS)-like protein. By contrast, hybrid aspen contains only the ELF3-like protein (Figure S2A). A key feature of the Arabidopsis ELF3 protein is that it contains a PrLD. We, therefore, sought to determine whether such a domain also exists in the Populus ELF3-like protein. Using the prion-like amino acid composition (PLAAC) prediction tool,[19] we observed that *Populus trichocarpa* and hybrid aspen ELF3 had a log-likelihood ratio (LLR) of 3.12 and 1.12, respectively, indicating the presence of a potential prion-like sequence. By contrast, the ECC proteins from Arabidopsis and *Populus trichocarpa* showed negative LLR scores of −3.48 and −3.06, suggesting an absence of prion-like composition in these proteins (Figures S2B and S2C). Moreover, when we applied the PrionW prediction tool,[20] setting a Q+N richness threshold of 18 and a pWALTZ cutoff of 50, we found that Arabidopsis ELF3 achieved a pWALTZ score of 68.70, whereas *Populus trichocarpa* (and hybrid aspen) ELF3 had a score of 64.59 (Figure S2D). Our results therefore suggest that, despite lacking a polyglutamine stretch found in Arabidopsis ELF3 protein, hybrid aspen protein does contain a PrLD domain, albeit one that appears weaker than that of Arabidopsis.

To characterize the function of the hybrid aspen ELF3 ortholog, we first sought to determine whether it undergoes a temperature-responsive phase transition. To this end, we transiently expressed the hybrid aspen ELF3 protein with a C-terminal YFP (yellow fluorescent protein) under the control of the 35S constitutive promoter by agroinfiltration in *N. benthamiana* leaves. Two days after infiltration, the plants were maintained at room temperature or shifted to LT (12°C) and imaged the next day. The hybrid Aspen ELF3 formed nuclear condensates in both cases, but the proportion of condensates formed in plants kept at 12°C overnight was significantly lower than in those held at room temperature (Figure 3A). Moreover, the intensity of diffuse ELF3-YFP was higher under LTs than at warm temperatures (Figure 3B), indicating that diffuse YFP (potentially corresponding to active ELF3) was more abundant in the nucleus under LT conditions, whereas condensates (corresponding to inactive ELF3) were more abundant under the warm conditions (Figure 3C). These results indicate that the hybrid aspen ELF3 displays LT-responsive phase separation, as has been reported for Arabidopsis ELF3.

The sequence similarity of hybrid aspen ELF3 to Arabidopsis ELF3 and LT-responsive phase separation prompted us to investigate its involvement in LT-induced growth cessation in hybrid aspen trees. To dissect the function of ELF3 in LT-induced growth cessation, we generated two independent hybrid aspen ELF3 loss-of-function mutant lines using CRISPR-Cas9 (*elf3*-9 and *elf3*-16) (Figures S3A–S3D) and studied their LT-mediated growth cessation. Both *elf3* mutants were insensitive to LT and, unlike WT controls, failed to undergo growth cessation and form buds (Figures 3D and 3E). By the end of the LT treatment, the *elf3* mutants had not stopped growth and produced ten more leaves than WT plants on average (Figure 3E). ELF3 thus appears to be crucial for LT to induce growth cessation. Importantly, in contrast with LT-induced phase separation of the ELF3 protein, *ELF3* transcript levels did not respond to LT and remained unchanged after 8 weeks of LT exposure (Figure S3E). Our results thus show that ELF3 is crucial for LT to induce growth cessation.

ELF3 mediates in the LT response of growth-cessation modulators

The results presented above identify ELF3 as a crucial mediator of the LT response. We, therefore, investigated its role in mediating the LT regulation of the LAP1-AIL1-BRC1 module and the hormonal pathways involved in LT-induced growth cessation. To this end, we examined the effect of LT on the expression of these growth regulators in the *elf3* mutant and the WT. This revealed that the LT-mediated induction of *GA2ox8*, *NCED3*, and *BRC1* was severely reduced in the *elf3* mutant shoot apex (Figures 4A–4C). Moreover, the repression of *AIL1* seen in WT plants was attenuated in the *elf3* mutant (Figure S4A). Intriguingly, *LAP1* was repressed by LT in both the WT and the *elf3* mutant (Figure S4B), suggesting that LT-induced *LAP1* downregulation is independent of ELF3. These results indicate that (with the exception of *LAP1*), ELF3 mediates in the response of key growth-cessation regulators to LT.

Reducing GA levels rescues the growth-cessation defect of the *elf3* mutant

GA downregulation plays a crucial role in growth cessation. Accordingly, LT significantly upregulates the expression of *GA2ox8*, which encodes an enzyme vital for GA deactivation[21] (Figure 2D). However, LT induction of *GA2ox8* is severely reduced in the *elf3* mutant (Figure 4A). Therefore, to determine whether misregulation of the GA pathway (i.e., failure to induce GA degradation) contributes to the *elf3* mutants' defects in LT-induced growth cessation, we treated WT and *elf3* mutant plants with 100 μM PAC (a GA biosynthesis inhibitor) or DMSO as a control and subjected them to LT. WT plants treated with PAC produced fewer leaves and ceased growth earlier than mock-treated plants. As discussed above, the mock-treated *elf3* mutant was insensitive to LT and did not undergo growth cessation. However, the PAC-treated *elf3* mutant stopped growth, albeit with a delayed response compared with the WT plants

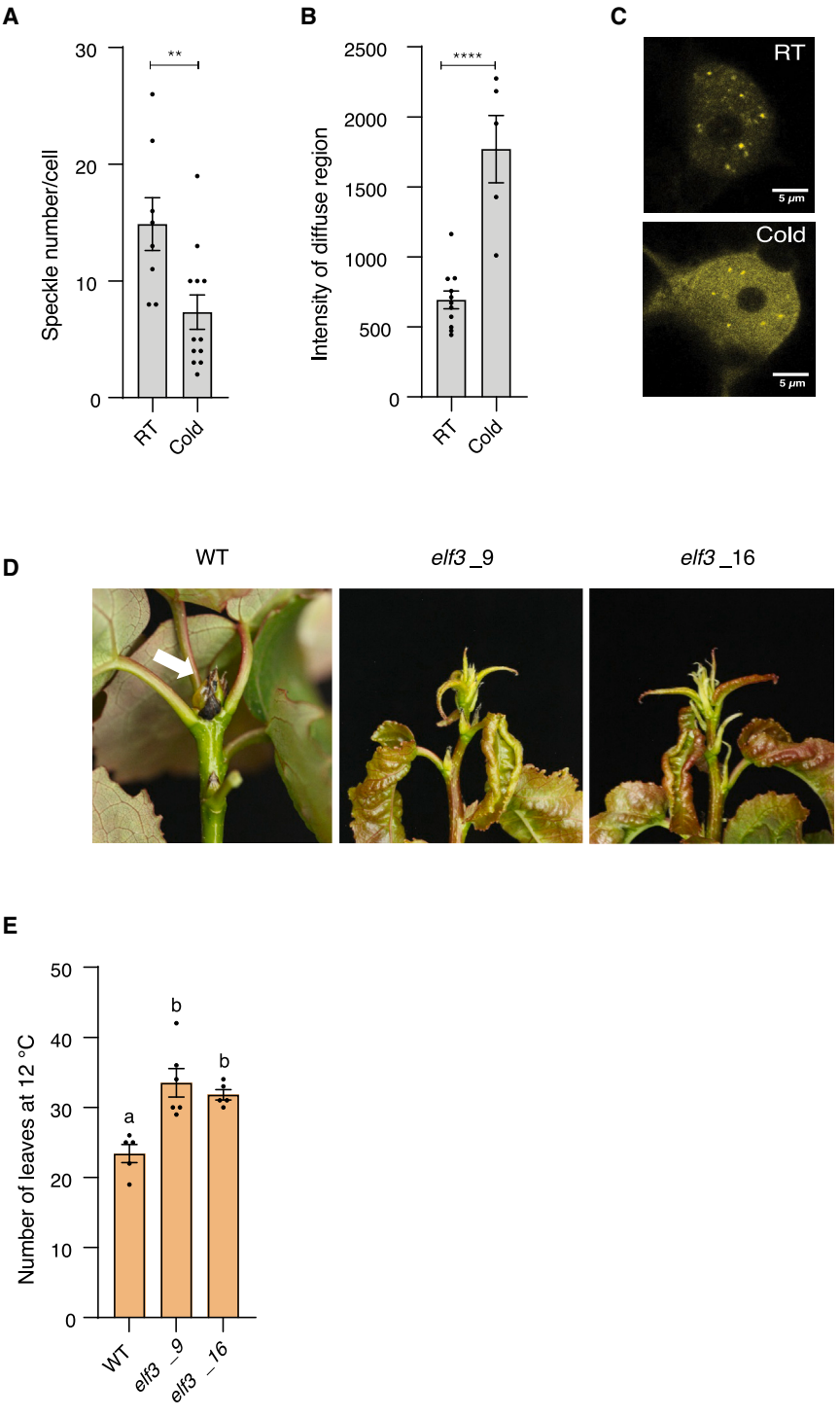

Figure 3. ELF3 is required for LT-mediated growth cessation

(A and B) (A) Speckle number/cell and (B) intensity of diffuse ELF3-YFP in the nucleus of *Nicotiana benthamiana* plants transiently expressing 35S:ELF3-YFP ($n \geq 5$). Plants were kept at room temperature (RT) or 12°C overnight (cold) before imaging. Asterisks indicate significant differences (**$p < 0.01$, ****$p < 0.0001$; t test analysis)

(C) Representative confocal images used in (A) and (B).

(D) Representative images of shoot apices of WT and *elf3* plants grown at 12°C under LD (18 h day/6 h night) conditions for 8 weeks. The white arrow points to bud formation in WT.

(E) Average numbers of leaves (±SEM) produced until bud set by the WT and *elf3* plants under LT conditions ($n \geq 5$). The *elf3* plants did not produce buds. Lowercase letters denote significant difference (*$p < 0.05$) based on ordinary one-way ANOVA followed by Kruskal-Wallis test.

See also Figures S2, S3, and S6.

(Figures 4D and 4E). Together with the results of our gene expression studies, these data strongly suggest that the GA biosynthesis pathway is a target of LT, downstream of ELF3 in the induction of growth cessation.

ELF3 mediates photoperiod control of growth

In nature, both LTs and the shortening of photoperiod are crucial for controlling growth-cessation timing.[22] ELF3 is a component of the evening complex (EC),[23] and, in addition to thermosensing, ELF3 also controls photoperiodic responses such as flowering time.[24] Because flowering in Arabidopsis shares regulatory similarities with photoperiodic control of growth cessation,[1] we hypothesized that ELF3 might also play a role in SP-induced growth cessation. To address this, we measured *ELF3* expression in hybrid aspen leaves under long-day (LD) conditions and after 4 weeks' growth under short-day (4WSD) conditions. This revealed that ELF3 was strongly expressed in the leaves and that its expression increased 5-fold in the short-day (SD) plants (Figure 5A).

Next, we compared growth cessation in the WT and *elf3* mutant lines by shifting the plants from LD to SD conditions and measuring their height weekly until bud formation. The WT plants ceased growth and formed buds within 6 weeks, whereas the *elf3* mutants showed no sign of growth cessation and continued growing, forming twice as many leaves as the WT plants without producing buds (Figures 5B and 5C). Thus, ELF3 is required for SP-induced growth cessation in hybrid aspen.

ELF3 is required for SP-induced downregulation of FT2

Because SP-induced growth cessation is facilitated by the repression of *FT2* expression in the leaves,[2,25] we measured the expression of *FT2* in WT and *elf3* mutants. Hybrid aspen contains two copies of FT2 (FT2a and FT2b) with partially redundant

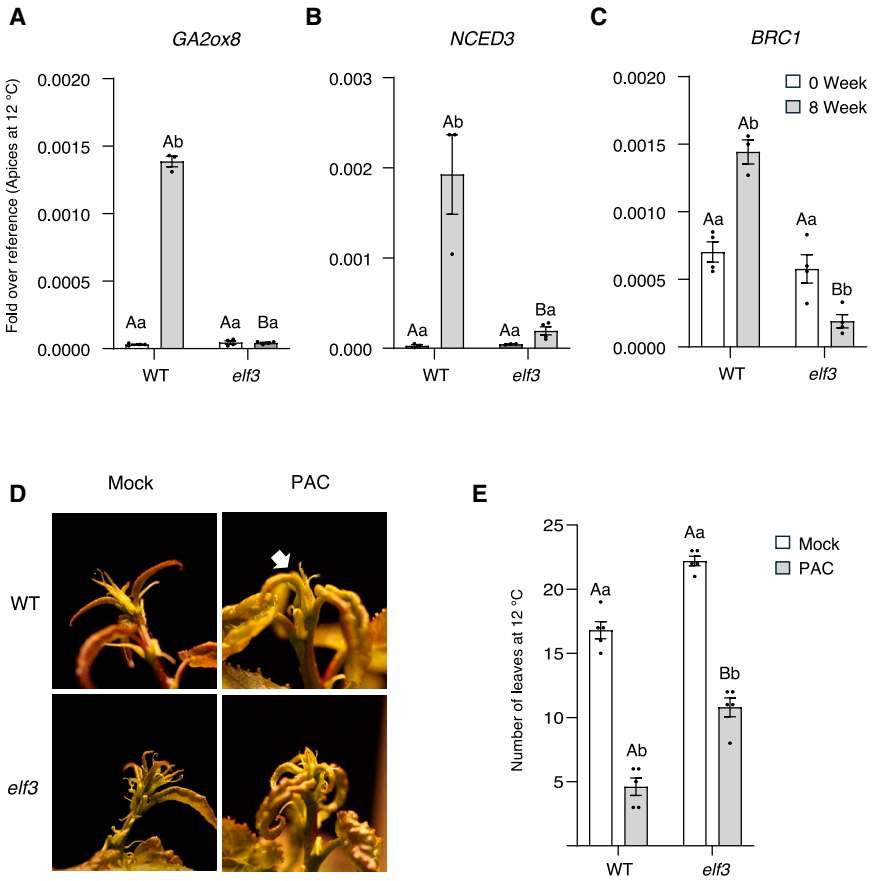

Figure 4. The LT response of growth-cessation modulators depends on ELF3

(A–C) *GA2ox8*, *NCED3*, and *BRC1* gene expression in the apices of WT and *elf3* plants grown under LT conditions (12°C) for the indicated number of weeks. Data represent the mean (±SEM) of ≥ three biological replicates and are normalized to *UBQ*. See also Figure S4.

(D) Representative images of WT and *elf3* shoot apices grown at LT treated with mock or PAC taken at 8 weeks. The white arrow points to bud formation in WT.

(E) Average number of leaves (±SEM) produced until bud set by WT and *elf3* plants treated with mock or 100 μM PAC in LT ($n = 5$). WT data shown here are taken from Figure 2J, as both experiments were performed together. Statistical analysis was performed using a two-way ANOVA followed by Sidak's test. Uppercase letters indicate significant differences ($p < 0.05$) between the WT and *elf3* CRISPR line subjected to the same treatment, and lowercase letters indicate significant differences ($p < 0.05$) between the treatments for each individual genotype.

roles. In WT plants, *FT2A* and *FT2B* expression in the leaves was repressed by a factor of 100 within 4 weeks of shifting to SD conditions. By contrast, *FT2* expression in *elf3* mutants was not repressed to WT levels, remaining significantly higher under SD conditions (Figures 6A and 6B). These results indicate that ELF3 negatively regulates *FT2* expression and that ELF3 function is required for downregulation of *FT2* by SP, a key early step in inducing growth cessation.

To further validate the genetic link between ELF3 and FT2, we used CRISPR to block FT2a and b expression in the *elf3* mutant background (Figure S5). The resulting *elf3*/*ft2* mutants showed a severe dwarf phenotype and produced buds even in the tissue culture jars, as previously observed for the *ft2* mutant[26] (Figure 6C). These results, together with expression data, suggest that ELF3 is essential for the SP-mediated suppression of FT2 for induction of growth cessation.

DISCUSSION

Precise timing of growth cessation is vital for the survival of perennial plants such as long-lived trees in temperate and boreal ecosystems. The role of photoperiod as a key signal regulating growth cessation has been studied thoroughly, and key components involved in sensing and responding to photoperiodic cues have been identified. Interestingly, although the inductive photoperiod for growth cessation for a genotype does not vary annually, field studies on poplars and Swedish aspen have revealed significant interannual variation in the timing of growth cessation.[27,28] This suggests that factors other than photoperiod also influence growth cessation, and modeling studies have implied that temperature is one such factor.[6] Moreover, in trees of rosacea species such as apples, LT is the main cue regulating growth cessation. However, despite the evidence that LT is another major environmental cue regulating growth cessation, the signaling mechanisms underpinning its effects have been relatively unexplored. Here, we address this knowledge gap by showing that a tree ortholog of the Arabidopsis thermosensor protein ELF3 plays a key role in mediating LT control of growth cessation.

LT response is distinct from photoperiodic pathway and targets growth regulators in the shoot apex

Photoperiodic cues regulating growth cessation are sensed in the leaves. As a result, SP control of growth cessation in the shoot apex is mediated systemically by transcriptional suppression of mobile FT2[10] in the leaves. This leads to downregulation of the *LAP1-AIL* pathway,[8,12] the downstream target of FT2, in the shoot apex, resulting in growth cessation and bud set. In contrast with SP, LT does not downregulate *FT2* expression in leaves and suppresses the expression of *LAP1-AIL1* in the shoot apex independently of FT2 downregulation. It also upregulates the expression of *BRC1* in the shoot apex, which is notable because BRC1 interacts with and inhibits FT2 function post transcriptionally.[29] Therefore, the suppression of *AIL* expression by LT (even in the absence of *FT2* downregulation in the leaves) is presumably a consequence of post-transcriptional inhibition of FT2 by upregulation of BRC1 in the shoot apex. Altogether, these data show that LT acts by inducing *BRC1* and suppressing *LAP1-AIL1* in the shoot apex, and although LT and SP share

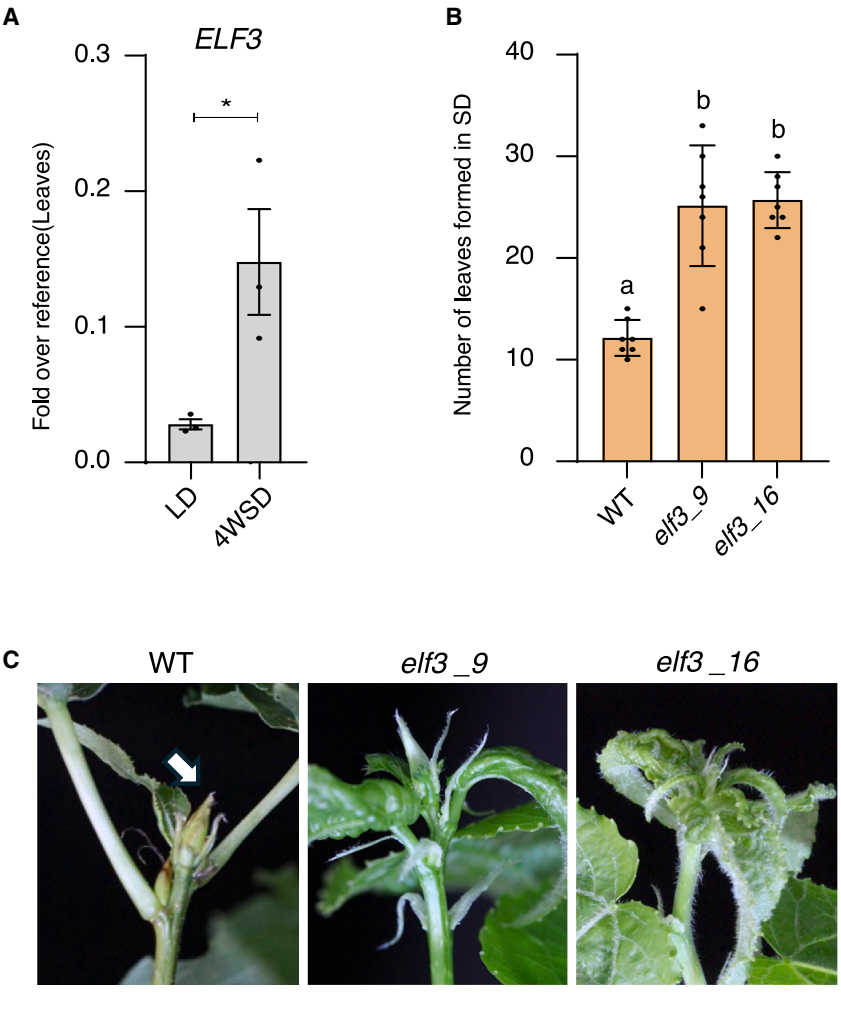

Figure 5. ELF3 mediates photoperiod control of growth

(A) Quantitative real-time PCR data showing relative levels *of ELF3* in WT leaves under LD and 4WSD conditions. All values are means (±SEM) of three biological replicates and are normalized against *UBQ*. Asterisks indicate significant differences (*$p < 0.05$; t test analysis).

(B) Average numbers of leaves (±SEM) to bud set for WT and *elf3* plants under SD conditions ($n \geq 6$). The *elf3* plants did not produce buds. Lowercase letters denote significant difference (*$p < 0.05$) based on ordinary one-way ANOVA followed by Kruskal-Wallis test.

(C) Representative images of shoot apices of WT and *elf3* plants taken after 5 weeks, grown under SD conditions (8 h light/16 h dark). The white arrow points to bud formation in WT.

See also Figure S6.

these downstream targets, the LT response pathway diverges itself significantly from the photoperiodic pathway by acting independently of *FT2* downregulation in the leaves.

The antagonistic GA-ABA pathways are targets of LT in growth-cessation responses

Photoperiodic growth regulation in trees has been linked to effects on hormonal pathways, particularly the suppression of GA biosynthesis.[21] We have shown that, like SP, LT also targets the GA biosynthesis pathway to regulate growth cessation. Gene expression analysis revealed that LT induces upregulation of *GA2ox*, a key enzyme in GA catabolism, in the shoot apex. Together with the more rapid growth cessation under LT conditions observed after inhibiting GA biosynthesis using PAC, this supports the hypothesis that LT negatively regulates the GA pathway to induce growth cessation. Our conclusion is further reinforced by previous reports showing that pharmacological inhibition of GA signaling can induce growth cessation in PHYB (Phytochrome B)-overexpressing plants.[15]

Unlike SP, LT not only targets the GA pathway but also the ABA pathway by upregulating *NCED3*, a key enzyme in ABA biosynthesis. Moreover, genetic evidence shows that downregulating the ABA response delays growth cessation, whereas upregulating the ABA response induces early growth cessation. These results are consistent with the hypothesis that ABA plays a role in LT-induced growth cessation. Interestingly, although ABA was shown to mediate bud dormancy regulation by SP,[30] its role in SP-induced growth cessation has not been observed in hybrid aspen. The LT- and SP-mediated growth-cessation mechanisms thus differ in this respect. The antagonistic action of GA and ABA regulates diverse physiological processes in plants, including seed germination and bud break in trees.[31,32] Our results indicate that LT similarly exploits antagonistic regulation of the ABA-GA hormonal pathways in the shoot apex by upregulating the ABA pathway while downregulating the GA pathway, which then contributes to LT-induced growth cessation.

An ELF3-like protein (ELF3) is crucial for LT-mediated growth cessation

SP induces growth cessation by downregulating *FT2* expression in leaves, whereas LT acts on targets of FT2 and hormonal pathways in the shoot apex. Our results indicate that LT-mediated induction of growth cessation depends on ELF3, a tree ortholog of the Arabidopsis ELF3 protein, which is thought to act as a thermosensor in Arabidopsis. Our gene expression analysis shows that ELF3 mediates in the LT response of genes such as *BRC1* and *AIL1*, which are crucial for growth cessation. Importantly, our results showing that *elf3* mutants do not cease growth in response to LT indicate that ELF3 is essential for LT-induced growth cessation.

However, ELF3 also mediates the LT regulation of hormonal pathways, and, in the hybrid aspen *elf3* mutant, the LT-induced expression of a key enzyme in GA inactivation, *GA2ox*, is significantly reduced. Moreover, although the *elf3* mutant did not respond to LT, upon treatment with the GA biosynthesis inhibitor PAC, the *elf3* mutant could undergo growth cessation and bud set in response to LT. Interestingly, ELF3 is known to control flowering in barley by regulating the GA pathway.[33] The GA

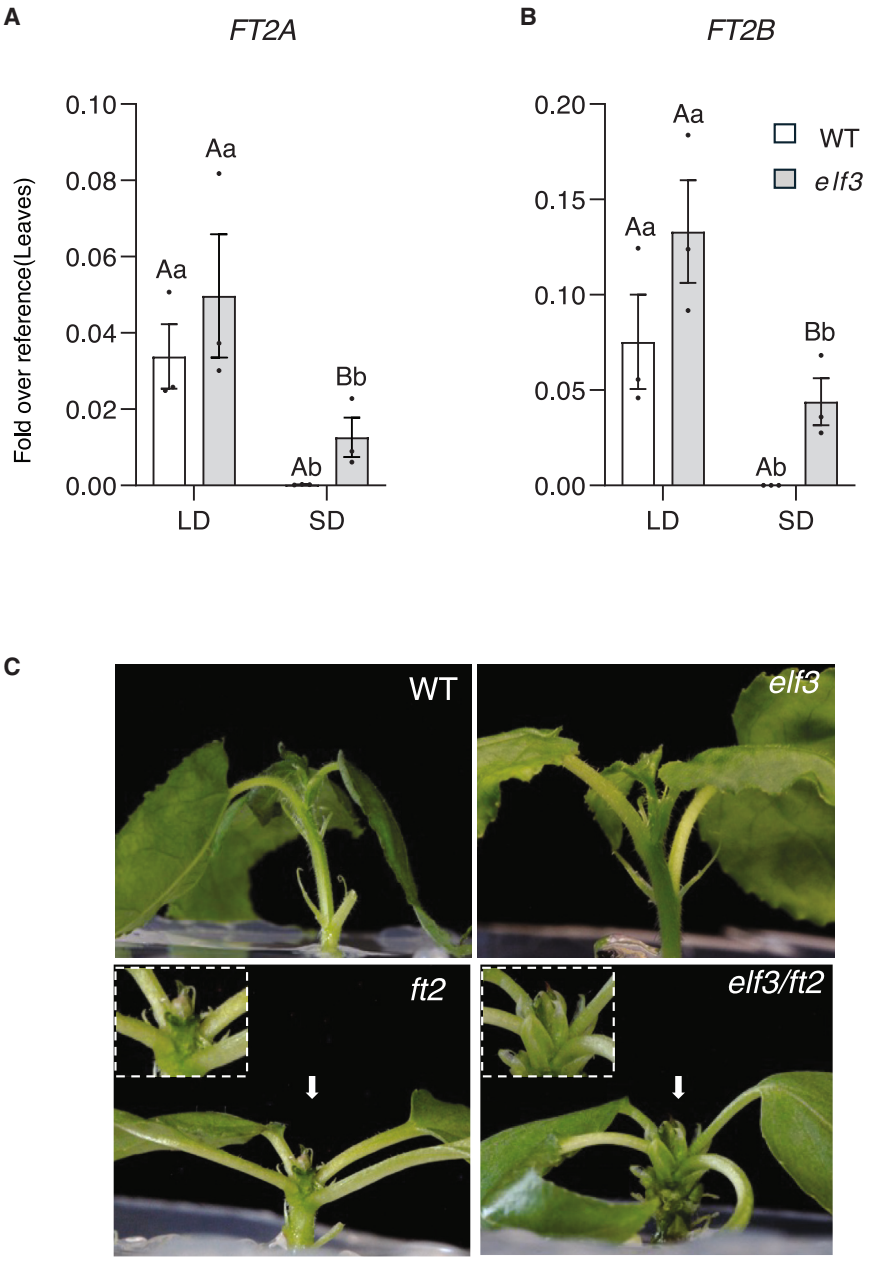

Figure 6. ELF3 is a negative regulator of FT2

(A and B) Quantitative real-time PCR data showing relative levels *of FT2A* and *FT2B* in leaves of WT and *elf3* plants under LD and 4WSD conditions. All values are means (±SEM) of three biological replicates and are normalized against *UBQ*. Statistical analysis was performed using a two-way ANOVA followed by Sidak's test. Uppercase letters indicate significant differences ($p < 0.05$) between the WT and *elf3* CRISPR line subjected to the same treatment, and lowercase letters indicate significant differences ($p < 0.05$) between the treatments for each individual genotype.

(C) Representative images of shoot apex of WT, *elf3, ft2*, and *elf3/ft2* plants, inset shows bud formation in *ft2* and *elf3/ft2*. The white arrow points to bud formation.

See also Figure S5.

pathway thus appears to be a conserved target of ELF3. These data thus highlight how conserved regulatory modules, for example, ELF3-GA, have been harnessed to regulate diverse downstream processes, such as flowering and seasonal growth in response to environmental cues.

Using an amino-acid-composition-based prediction model, PLAAC,[19] with a stringent cutoff, a recent analysis examining the presence of PrLDs across various plant species found that ELF3 protein with PrLDs was predominantly concentrated in *Brassicales*.[34] However, the study also highlighted that certain species outside the *Brassicales* order, including the bryophyte *Physcomitrium patens* and the monocot *Sorghum bicolor*, are also predicted to possess PrLDs. Additionally, using the PrionW[20] prediction tool (which integrates compositional and soft-amyloid models) to identify PrLDs and using default settings (Q+N richness of 25% and a pWALTZ cutoff of 73), only three species were found to contain PrLDs, with one notable hit being the EEC protein in *Populus trichocarpa*. However, by relaxing the parameters to a Q+N richness of 18% and a pWALTZ cutoff of 68, the PrionW tool predicted additional sequences with PrLDs.

In our analysis, the pWALTZ cutoff to 64 revealed that *Populus trichocarpa* (and hybrid aspen) ELF3 also contains a predicted PrLD. Moreover, using the PLAAC prediction tool, the *P. trichocarpa* and hybrid aspen ELF3 were assigned an LLR of 3.123 and 1.22, respectively, whereas the EEC protein received an LLR of −3.066. Overall, these findings suggest that the prediction tools may have somewhat different sensitivities and that the choice of tool and parameters can influence the results of PrLD prediction. Interestingly, experimental evidence shows that, like its Arabidopsis ortholog, hybrid aspen ELF3 undergoes temperature-responsive phase separation. In Arabidopsis, ELF3 interacts with LUX ARRYTHMO (LUX) and EARLY FLOWERING 4 (ELF4) to form the EC.[23] Recent studies have shown that it forms condensates at high temperatures, causing the EC to dissociate from the target DNA. At lower temperatures, the EC is stable and shows stronger binding affinity to DNA.[35] The temperature-dependent phase separation of ELF3 thus provides a mechanistic basis for thermo-responsive control of growth and flowering. Hybrid aspen ELF3 also exhibits phase-separation behavior like Arabidopsis ELF3: it forms significantly fewer condensates at LTs than at higher ones and a concomitant increase in the abundance of the soluble form at LT. This observation, together with the genetic data discussed above, strengthens the conclusion that ELF3 plays a central role in transducing temperature information that regulates growth cessation.

Interestingly, ELF3 does not regulate the expression of all growth regulators involved in LT-mediated growth cessation. Specifically, *LAP1* repression levels were comparable in both WT and *elf3* mutants subjected to LT treatment, suggesting the involvement of additional, ELF3-independent pathways in LT-mediated control of seasonal growth. Previous research in Arabidopsis has demonstrated that the photoreceptor PHYB acts as a thermosensor.[36] Moreover, *PIF7 (PHYTOCHROME INTERACTING FACTOR 7)* has been identified as an RNA thermoswitch.[37] It remains to be seen whether PHYB and PIFs function in ELF3-independent LT-mediated growth cessation. Arabidopsis ELF3 also exhibits EC-independent functions,[38,39] so it would be interesting to determine whether ELF3-mediated growth cessation in hybrid aspen is mediated via the EC or an independent mechanism.

ELF3 is a repressor of *FT2*

Arabidopsis ELF3 is a part of the EC that plays a crucial role in transducing photoperiodic cues.[23] Hybrid aspen ELF3 is also expressed in leaves, where photoperiod cues regulating growth cessation are sensed. We therefore investigated ELF3's role in the photoperiodic control of growth cessation. Our results show that the *elf3* mutant is insensitive to SP, indicating that ELF3 transduces photoperiodic cues and that it is essential for regulating growth cessation in response to SP. FT2 is a primary target of photoperiodic cues in growth-cessation responses and its downregulation by SP is a pivotal early event in inducing growth cessation. We found that although *FT2* expression in the *elf3* mutant is reduced after SP treatment, it is nevertheless significantly higher than in WT plants, which may explain the failure of the *elf3* mutant to undergo growth cessation. This is further supported by the early growth cessation of the *elf3*ft2* mutant, which behaves like the *ft2* mutant. Previous studies in *Populus* have shown that *GIGANTEA* (*GI*), *LATE ELONGATED HYPOCOTYL 1* (*LHY1*), and *TIMING OF CAB EXPRESSION 1* (*TOC1*) mediate photoperiodic control of *FT2* expression.[2,25] As in Arabidopsis, LHY1 and TOC1 act as repressors, whereas GI acts as an activator of *FT2* expression in Populus.[2,25] Our results show that ELF3 is a previously unrecognized component of the photoperiodic pathway in hybrid aspen that acts as a repressor of *FT2* in response to SP.

ELF3 integrates LT and SP responses

By subjecting hybrid aspen plants to growth-permissive long photoperiods at LT, we could investigate the LT-mediated control of growth cessation independently of photoperiodic cues and identify the molecular components that enable this control. Our data show that, unlike SP, LT targets key growth-cessation regulators in the shoot apex. Intriguingly, the LAP1-AIL1-BRC1 module and the GA pathway appear to form a shared core of signaling pathways controlling seasonal growth in response to SP and LT. The convergence of LT and SP on common downstream targets presumably reflects the fact that these cues act together in nature to regulate growth cessation.[6] Although LT can induce growth cessation independently of SP, both photoperiod and temperature decline in the autumn. The fact that they share downstream targets would thus facilitate the integration of signaling at the shoot apex to coordinate its response to these two major seasonal cues, enabling fine-tuning of growth cessation to align with seasonal changes. The finding that ELF3 plays dual roles in the photoperiodic and LT responses sheds further light on the means by which these two major cues are integrated (Figure S6).

Importantly, our data explain the observed interannual variation in the timing of growth cessation in field studies that cannot be explained by SP acting as the sole environmental cue regulating this process. For example, whereas autumnal senescence shows little interannual variation,[40] significant interannual variation in the timing of growth cessation was seen in field studies on Swedish aspen and other poplars.[27,28] Our discovery of shared core signaling components in growth-cessation pathways regulated by LT and SP strongly suggests that temperature is a key factor contributing to this interannual variation, as hypothesized by modeling studies and other studies as well.[41]

In summary, experimental and modeling studies indicate that photoperiod is unlikely to be the sole factor regulating growth cessation under natural conditions. Our results establish LT as another crucial factor in this process and identify ELF3 as a crucial, previously unrecognized mediator of its effects. Our elucidation of LT-mediated control of growth cessation at the molecular level will enable future genetic studies on other economically important trees in which LT significantly influences the seasonal control of growth, such as apples and pears.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Rishikesh P. Bhalerao (rishi.bhalerao@slu.se).

Materials availability

Transgenic lines and DNA constructs generated in this study are available upon request from the lead contact, Rishikesh P. Bhalerao.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

A.N., S.K.P., J.P.M., R.K.S., P.C.M., and B.A. performed the experiments. A.N., S.K.P., J.P.M., and R.P.B. designed the experiments and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Agrobacterium strain GV3101	Gold Biotechnology	CC-207
E. coli strain DH5a	Thermo Fischer Scientific	EC0112
Chemicals, peptides, and recombinant proteins		
Phusion high-fidelity DNA polymerase	Thermo Fischer Scientific	F-530XL
iScript™ cDNA Synthesis Kit	BIORAD	1708891
T4 DNA Ligase	NEB	M0202S
ECO31I	Thermo Fischer Scientific	FD0294
4'-Hydroxy-3',5'-dimethoxyacetophenone (Acetosyringone)	ThermoFisher Scientific	2478-38-8
Paclobutrazol	MCE	HY-B0853
Experimental models: Organisms/strains		
Hybrid aspen (*Populus tremula x tremuloides*) clone T89	Umeå, Sweden	N/A
Nicotiana benthamiana	Umeå, Sweden	N/A
Oligonucleotides		
Mentioned in Table S1		
Recombinant DNA		
pKSE401	Addgene	Plasmid #62202
pHSE401	Addgene	Plasmid #62201
PGWB541	Addgene	Plasmid #74875
pKSE401 ELF3 CRISPR	This study	N/A
pHSE401 FT2 CRISPR	This study	N/A
Software and algorithms		
MEGA X	https://www.megasoftware.net/mega4/index.php	N/A
PRISM	https://www.graphpad.com/features	N/A
ImageJ	https://imagej.net/software/imagej/	N/A
Other		
Gateway™ BP Clonase™ II Enzyme mix	Thermo Fischer Scientific	11789020
pENTR™ Directional TOPO®	Thermo Fischer Scientific	K240020
Gateway™ LR Clonase™ II Enzyme mix	Thermo Fischer Scientific	11791020
Spectrum™ Plant Total RNA Kit	MERK	STRN250

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Plant materials and growth conditions

WT hybrid aspen (*Populus tremula* × *tremuloides*, clone T89) and transgenic plants were grown on half-strength Murashige–Skoog medium (Duchefa) under sterile conditions for 4 weeks, then transferred to soil and cultivated with fertilisation for 5 weeks in a greenhouse (18h day/6h night, 22/18°C long days). To assess the effect of temperature on growth cessation, the plants were transferred to climate-controlled growth chambers and subjected to either LD (18h day/6h night, 22/18°C) or LD/low temperature (18h day/6h night and 12°C) conditions. Growth cessation responses were investigated as described previously[8,10] by counting number of leaves formed to bud set following transfer to low temperature. For paclobutrazol treatment, the apices of plants subjected to LD/low-temperature conditions were sprayed with 100 μM paclobutrazol in water; DMSO in water was used as a negative control. The stock solution of paclobutrazol used was made in DMSO. To study photoperiod-mediated growth cessation, plants were moved to SD (8 h day/16 h night, 20/15°C cycles) for up to 11 weeks. Tissue samples for gene expression analyses were collected from leaves or shoot apices. Each sample was immediately frozen in liquid nitrogen and stored at −80 °C until further use. Pictures of apices were taken using a Canon EOS digital camera to monitor bud formation.

METHOD DETAILS

Generation of transgenic lines

To generate *elf3* CRISPR lines, guide RNAs targeting the desired sites were identified using the online CRISPR-P designer tool (http://crispr.hzau.edu.cn/CRISPR2/). The CRISPR constructs were cloned following the protocols described by Xing et al.[42] A template for cloning was amplified from pCBC-DT1T2 using the oligos elf3 CRISPR F1 and R1 (Table S1). The PCR fragment was gel-purified and introduced into the pHSE401 binary vector using the Golden Gate assembly method. The reaction mixture for Golden Gate assembly consisted of 200 ng of vector, 200 ng of the PCR fragment, 1.5 μL of 10x FastDigest buffer, 1.5 μL of 30U/μL T4 ligase, 0.75 μL of 20 mM ATP, and 1 μL of Eco31I, in a total volume of 15 μL. The mixture was transformed into *Escherichia coli* strain DH5α for amplification, the *pHSE401-elf3 CRISPR* constructs were then sequence-verified and introduced into Agrobacterium strain GV3101pmp90RK.[43] Hybrid aspen transformation was done as previously described.[44] Transformed lines were screened for deletion mutations using the elf3 CRISPR sequencing primers (Table S1) and the deletions were confirmed by sequencing. To generate *elf3*ft2* lines, guide RNAs from André et al.[26] were used to create the *pKSE401-ft2 CRISPR* construct, which was then transformed into the *elf3* CRISPR background. The generation of the *LAP1oe*, *LAP1-RNAi*, *RCAR1oe*, *RCAR1-RNAi*, *BRC1oe* and *AIL-SRDX* lines were described previously.[8,9,13,45]

RNA isolation and qRT-PCR analysis

Total RNA was extracted from plant tissues (shoot apices or leaves) using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). RNA (10 μg) was treated with RNase-free DNaseI (Life Technologies, Ambion) and 1 μg was then utilized for cDNA synthesis using an iScript cDNA Synthesis Kit (BioRad). Ubiquitin was the reference gene in all experiments. Quantitative RT-PCR experiments were conducted using LightCycler 480 SYBR Green I Master mix and a LightCycler 480 II instrument (both supplied by Roche). The Δ-cq method was used to calculate relative expression values for genes of interest. Primer sequences used in the qPCR experiments are given in Table S1.

Cloning of *35Spro:ELF3-YFP* construct

To generate the 35Spro: ELF3-YFP, the coding region (CDS) of ELF3 without the stop codon was amplified from T89 cDNA and cloned into the TOPO entry vector using the pENTR/D-TOPO Cloning kit. An LR reaction was performed using the sequence-verified TOPO ELF3 vector and the PGWB541 destination vector (a Gateway-compatible binary vector for C-terminal fusion with eYFP) to create the final construct. The plasmids were amplified in Escherichia coli strain DH5α and sequence-verified plasmids were transformed into Agrobacterium strain GV3101pmp90RK. The primers used for cloning are listed in Table S1.

Transient expression in *Nicotiana benthamiana* and confocal imaging

Transient expression in *Nicotiana benthamiana* was carried out using agroinfiltration. Briefly, an overnight starter culture of *A. tumefaciens* carrying the *35Spro:ELF3-YFP* construct was prepared. The next day, 1.8 ml of LB containing 15 μM acetosyringone was inoculated with 200 μl of the starter culture and incubated at 28°C for 3-4 hrs. The culture was then centrifuged, washed twice with 1 ml MES buffer (10 mM MES, 10 mM MgCl2.6H2O., pH 5.6-NaOH), and dissolved in MES buffer containing 150 μM acetosyringone. The O.D was adjusted to 0.5 and mixed in a 1:1 ratio with *A. tumefaciens* expressing p19, a suppressor of posttranscriptional gene silencing.[46] Leaves of four-week-old *Nicotiana benthamiana* plants were infiltrated, and the plants were kept overnight at room temperature (22°C) in shade. Two days post infiltration, the plants were either shifted to 12°C or kept at 22°C overnight. Leaf discs were harvested, and YFP fluorescence was imaged using a confocal laser scanning microscope (LSM780, ZEISS) equipped with a 40× water immersion objective. YFP was excited with a 514 nm argon laser, with laser power set at 2.6%. Z-stack images were captured, and speckle counts were performed using FIJI (ImageJ) software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was done using Student's t-test or one-way ANOVA. Results are shown as means ± SEM.