FLOWERING LOCUS T/TERMINAL FLOWER1-Like Genes Affect Growth Rhythm and Bud Set in Norway Spruce[1][W][OPEN]

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The timing of bud set, as one determinant of the annual growth rhythm, is critical for local adaptation of the conifer Norway spruce (*Picea abies*). Previous gene expression and population genetic studies have suggested a role for *P. abies FLOWERING LOCUS T/TERMINAL FLOWER1-Like2* (*PaFTL2*) in the control of growth cessation and bud set in Norway spruce as well as in local adaptation resulting in clinal variation for timing of bud set. Using transgenic plants with *PaFTL2* driven by an inducible promoter, we found that *PaFTL2* indeed induces bud set and most probably also growth cessation. *PaFTL2* shows high expression around the procambium and vascular tissue and in the crown region in buds of both seedlings and older trees. Furthermore, *PaFTL2* expression is induced in vegetative shoots and all bud types in late summer, when growth cessation occurs. This supports the notion that *PaFTL2* is involved in growth cessation. A close paralog to *PaFTL2*, *PaFTL1*, is strongly expressed in meristems during the summer, possibly to repress meristem activity and the formation of needle primordia during this period. The temporal and spatial expression of *PaFTL1* and *PaFTL2* largely complement each other, which suggests that they act in concert to control perennial growth in Norway spruce.

Since plants are sessile, there is strong pressure to adapt to local abiotic and biotic environments. For perennials, an important aspect is the matching of the growing period to the seasonal changes in the environment, which often vary locally. In the gymnosperm Norway spruce (*Picea abies*) and other temperate trees, photoperiod is an important regulator of the annual growth cycle (Ekberg et al., 1979). Each year, when the days are shortened in the autumn, trees initiate growth cessation, bud set, and dormancy in order to achieve full cold hardiness before the winter (Rohde and Bhalerao, 2007). As early bud set reduces growth and late bud set increases the risk for frost damage, there is a tradeoff between the risk of frost damage and increased growth. Consequently, the timing of growth cessation and bud set is often strongly adapted to the local environment, despite high levels of gene flow. As a result, strong clinal variation is often observed for the timing of bud set in trees. Such clinal variation of a heritable trait is considered adaptive as a result of the action of natural selection. In Norway spruce, the clinal variation in growth cessation and bud set is coupled to a variation in critical night length, which steadily increases from about 2 h in the north to 6 to 7 h in the more southern populations (Ekberg et al., 1979).

In the angiosperm plant model species Arabidopsis (*Arabidopsis thaliana*), the photoperiodic control of flowering has been extensively studied. An important mediator of environmental signals (including photoperiod) in floral transition is *FLOWERING LOCUS T* (*FT*; Kobayashi et al., 1999). *FT* is expressed in the vascular tissue of cotyledons and young leaves but not in the shoot apical meristem (SAM), where the floral transition occurs (Takada and Goto, 2003). The FT protein is transported to the SAM via the phloem (Corbesier et al., 2007; Jaeger and Wigge, 2007). In the SAM, FT interacts with the basic leucine zipper transcription factor FLOWERING LOCUS D (FD), most likely to promote flowering and the activation of floral meristem identity genes (Abe et al., 2005; Wigge et al., 2005). By ectopic and/or overexpression of *FT* homologs in different species, the flowering-promoting FT function has been shown to be conserved in the angiosperm lineage in both monocot and dicot species (for review, see Pin and Nilsson, 2012). These species include day-neutral species as well as plants induced to flower by long days or short days, showing that *FT* genes can function as a universal florigenic signal. Furthermore, the function of FT homologs has been reported to extend beyond flowering and also affect growth cessation and bud set in poplar (*Populus* spp.; Böhlenius et al., 2006; Hsu et al., 2011), growth termination in tomato (*Solanum*

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lycopersicum; Lifschitz et al., 2006), and tuberization in potato (*Solanum tuberosum*; Navarro et al., 2011). Recent experiments also suggest that functional diversification of FT paralogs may be quite common. In sugar beet (*Beta vulgaris*), *BvFT1* and *BvFT2* have antagonistic functions in the control of flowering (Pin et al., 2010), and in poplar, *FT1* regulates reproductive onset in response to winter temperatures, whereas vegetative growth and the inhibition of bud set are promoted by *FT2* in response to warm temperatures and long days in the growing season (Hsu et al., 2011).

A homolog to *FT* is the floral inhibitor *TERMINAL FLOWER1* (*TFL1*). *TFL1* acts as a repressor of flowering and extends the vegetative growth state while maintaining the indeterminate state of inflorescences (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1997; Ratcliffe et al., 1998). TFL1 is expressed in the nucleus and cytoplasm but interacts with FD solely in the nucleus and represses genes activated by FT (Hanano and Goto, 2011). The antagonistic function of FT and TFL1 is partly controlled by a single amino acid exchange, even though the remaining protein sequence is also important for full protein function (Hanzawa et al., 2005). *TFL1* mRNA is expressed in the central part of both lateral and main shoot meristems, but the TFL1 protein moves and spreads out over the whole meristem, allowing the repression of floral identity genes (Conti and Bradley, 2007). As for FT, TFL1 function appears to be at least partially conserved in angiosperms (Pnueli et al., 1998; Nakagawa et al., 2002; Carmona et al., 2007; Hou and Yang, 2009; Danilevskaya et al., 2010; Mohamed et al., 2010; Repinski et al., 2012; Tsaftaris et al., 2012). In the perennial Arabidopsis relative *Arabis alpina*, the *TFL1* homolog *AaTFL1* prevents flowering in young vernalized plants and prolongs the required vernalization period in older plants (Wang et al., 2011). Furthermore, *AaTFL1* expression in axillary meristems ensures that the vegetative branches are preserved, to maintain a perennial growth habit (Wang et al., 2011). Besides a function through interaction with FD, TFL1 has been reported to be involved in the trafficking of proteins to the protein storage vacuoles (Sohn et al., 2007).

Angiosperms and gymnosperms diverged about 300 million years ago (Bowe et al., 2000), and the degree of conservation in pathways controlling the induction of flowering or bud set is so far unclear. We have previously shown that the antagonistically functioning paralogs FT and TFL1 likely arose after duplication in the angiosperm lineage (Karlgren et al., 2011). In the conifer Norway spruce, two FT/TFL1-like genes were identified (*PaFTL1* and *PaFTL2*), with roughly equal similarity to FT and TFL1 (Supplemental Fig. S1). With the recent publication of the Norway spruce genome, four additional copies of FT/TFL1-like genes were identified that cluster close to *PaFTL1* and *PaFTL2* (Nystedt et al., 2013). Whether these newly identified genes are expressed and functional is, to our knowledge, unknown at present. When *PaFTL1* and *PaFTL2* were ectopically expressed in Arabidopsis, flowering time was delayed and flower morphology showed similarities with *TFL1* overexpressors (Karlgren et al., 2011; Klintenas et al., 2012). Expressing *PaFTL1* and *PaFTL2* in the *tfl1* mutant further showed that both genes can substitute for *TFL1* (Klintenas et al., 2012). These data suggest that the flowering-promoting function of FT evolved after the split between angiosperms and gymnosperms (Karlgren et al., 2011).

The expression of *PaFTL2* is induced by long nights, and its expression is strongly correlated with bud set under various photoperiodic treatments (Gyllenstrand et al., 2007). Furthermore, the expression of *PaFTL2* in plants from Scandinavian natural populations shows a latitudinal cline again associated with the clinal variation in bud set (Chen et al., 2012). Chen et al. (2012) also identified single-nucleotide polymorphisms in the promoter of *PaFTL2* with a clinal variation in allele frequency indicative of divergent selection in populations from northern and southern latitudes. These data suggest that *PaFTL2* is important for the control of bud set and that the gene might be involved in local adaptation conferring clinal variation.

By controlled induction of *PaFTL2* in transgenic Norway spruce, we show here that the gene does indeed regulate bud set. We also present a detailed localization of *PaFTL2* expression in buds under inductive conditions and show that *PaFTL1* and *PaFTL2* appear to have complementary expression patterns indicating that they may act in concert to control the growth cycle in Norway spruce.

RESULTS

PaFTL2 Induces Bud Set in Transgenic Spruce

In order to test the role of *PaFTL2* in growth cessation and bud set, we first aimed to constitutively overexpress the gene in Norway spruce. A construct with the strong cauliflower mosaic virus 35S promoter driving the expression of the coding region of *PaFTL2* was cotransformed into embryogenic cell cultures together with the *bar* gene using particle bombardment (Clapham et al., 2000). Three independent experiments with approximately 40 bombardments each were performed, but all of them failed to produce viable callus. Similar results were also reported by Klintenas et al. (2012). Since it appeared impossible to produce viable transgenic Norway spruce lines constitutively expressing *PaFTL2*, we instead tested to activate *PaFTL2* from an inducible heat shock promoter (hsp::*PaFTL2*). This promoter is activated when subjected to a fast and drastic increase in temperature (Baumann et al., 1987; Saidi et al., 2005). Several individually transformed calli were obtained, and the presence of the hsp::*PaFTL2* transcript was confirmed by PCR. Plantlets of several different transgenic lines were tested with heat shock treatment (HS). Three lines showed a good response to a first HS and were selected for further studies.

To obtain sufficient induction of *PaFTL2*, we optimized the treatment. High expression was induced by

a 1-h HS at 40°C. Unfortunately, the induced expression of *PaFTL2* declined quite quickly, and the *PaFTL2* mRNA levels were almost back to normal after 24 h. If HS was given once every 24 h, one of the three selected transgenic lines, *PaFTL2-ox3*, responded only to the first two HS and the other two lines showed varying results. Successive HS given every 24 h did not result in a consistent increase in bud set in the *PaFTL2-ox* lines as compared with the controls. Since several nights, each inducing high *PaFTL2* expression, are needed to induce bud set in the genotype used for transformation, we hypothesized that a certain amount of *PaFTL2* over a period of several days is required for bud set. Therefore, we decided to use a treatment where HS was alternated with short nights (7 h) to partially replace the poor sequent HS induction with elevated endogenous expression of *PaFTL2*. The first 2 d (days 1 and 2), the plants were subjected to a 1-h heat shock at 40°C, followed by three short nights (days 2–4), and the treatment was ended with 2 d of heat shocks (days 5 and 6). The induced *PaFTL2* expression for the first of three independent experiments is shown in Figure 1. *PaFTL2-ox1* responded to all four HS, while *PaFTL2-ox3* responded only to the first two HS. The control plants displayed a slight increase in endogenous *PaFTL2* expression as a result of the short night treatment, but this was not sufficient to induce bud set.

Plant height and the frequency of bud set were measured before HS and during the following weeks. Although the bud set induction frequency differed between experiments, it is clear that HS-induced expression of *PaFTL2* leads to a significant increase in bud set in the transgenic lines compared with control plants (Table I). However, the effect of hsp::*PaFTL2* induction on growth cessation was weak, and only *PaFTL2-ox1* showed a clear growth retardation (Supplemental Fig. S2). Still, the two most responsive transgenic lines (*PaFTL2-ox1* and *PaFTL2-ox2*) displayed a generally reduced growth rate when grown in ambient temperatures, possibly as an effect of background activity from the hsp promoter (Wang et al., 2005). In conclusion, our data strongly support our hypothesis that *PaFTL2* plays an important role in the control of bud set and growth arrest in Norway spruce.

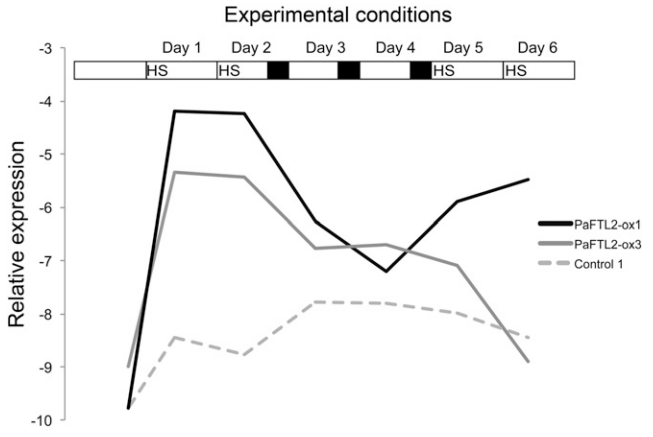

Figure 1. Expression of *PaFTL2* in transgenic Norway spruce hsp::*PaFTL2* and control plants after HS. The bar at top illustrates the experimental conditions. During a 6-d period, HS of 1 h (40°C) was given four times. After the second HS, plants were transferred to a photoperiod of 19 h of light/7 h of darkness for 3 d, after which they were returned to constant light before administration of the last two HS. The graph shows daily averages of expression levels of *PaFTL2* (endogenous expression plus that produced by hsp::*PaFTL2*). Samples for qRT-PCR were taken just before HS or immediately after the dark period and 5 h later each day. In the graph, the two daily measurements are averaged to reveal general trends in expression.

PaFTL2 mRNA Is Localized beneath the Meristem and Procambium after Induction in Short Days

Previous studies have shown that *PaFTL2* is expressed in needles, where the expression is induced by dark periods exceeding the critical night length for the genotype in question (Gyllenstrand et al., 2007). Expression was also detected in developing buds (Karlgren et al., 2011). To determine the temporal and spatial mRNA expression during induction of bud set, stem pieces approximately 5 mm long including the SAM were sampled for mRNA in situ hybridization. The material was obtained from seedlings that were raised under constant light and transferred to short-day (SD) conditions (8 h of light and 16 h of dark). This treatment is known to induce bud set in all genotypes of Norway spruce (Ekberg et al., 1979). Two sources of material were used: seeds originating from latitude 67° (SE-67) and seeds from latitude 47° (RO-47).

No *PaFTL2* expression could be detected at day 0 (Fig. 2A; Supplemental Fig. S3A), but a strong signal below the meristem and around the procambium and vascular tissue could be observed already after 10 h in darkness (Fig. 2B; Supplemental Fig. S3B). A similar pattern was observed in all samples until day 4 (Fig. 2, B–F; Supplemental Fig. S3, B–F). At day 7, *PaFTL2* expression became confined to the area underneath the developing bud, and at day 14, expression was detected in a region delineating the newly formed bud and the shoot (the crown region or colenchymatous plate) and extended into the pith of the bud (Fig. 2G; Supplemental Fig. S3G). Cross sections of RO-47 at day 2 confirmed that no expression was detectable at the tip of the meristem (Fig. 3A) and that the strongest signal was found below the meristem in the center of the shoot (Fig. 3B). Farther down the stem, a defined expression around the procambium and vascular bundles could be observed (Fig. 3C). A very similar expression pattern to that in top shoots was also observed in axillary shoots: no expression was observable at day 0, whereas *PaFTL2* mRNA was detected around the procambium at days 4 and 7 and at the crown region and the pith of the bud at day 14 (Supplemental Fig. S4).

Table I. *Assessment of bud formation 25 d (experiments 1 and 2) or 32 d (experiment 3) after first HS*

Bud set was assessed on a scale from 0 to 3, where 0 = no sign of bud set, 1 = beginning of bud formation (needle extension reduced, but no white bud scales evident), 2 = bud scales formed, and 3 = bud burst and resumption of growth.

Experiment	Transgenic Line	Bud Stage 0	Bud Stage 1	Bud Stage 2	Bud Stage 3	Percentage Bud Stage 1 to 3
1	*PaFTL2-ox1*	0	8	1	0	100
	PaFTL2-ox3	10	2	1	0	23
	Control 1	15	0	0	0	0
2	*PaFTL2-ox1*	0	7	4	1	100
	PaFTL2-ox2	1	8	1	7	94
	Control 1	17	0	0	0	0
	Control 2	11	1	0	0	8
3	*PaFTL2-ox1*	14	2	20	1	62
	PaFTL2-ox2	9	4	3	0	44
	Control 1	59	0	0	0	0

PaFTL2 Expression Differs between Genotypes from Different Latitudes

Recent data suggest that the expression level of *PaFTL2* in needles displays a latitudinal cline and that single-nucleotide polymorphisms in the promoter might be responsible for part of the clinal variation (Chen et al., 2012). Our new in situ data indicated that *PaFTL2* might display a stronger expression also in the shoot tip of the high-latitude genotype SE-67, compared with the low-latitude genotype RO-47, early after transfer to SD conditions (Fig. 2; Supplemental Fig. S3). To quantify the expression in the shoot tip in genotypes from different latitudes, seedlings from three populations, SE-67, SE-62, and RO-47, were sampled and subjected to quantitative reverse transcription (qRT)-PCR. Shoot tips containing the SAM and 1 to 2 mm of stem were obtained from all three populations, while needles closest to the SAM were available only for RO-47 and SE-67, and shoot tips from lateral branches were available only from RO-47.

PaFTL2 expression was very low in all tissues in constant light, but already after 10 h of darkness, a strong increase in expression could be observed in all tissues (Fig. 4). In needles, the expression levels steadily increased until day 7 but had declined at day 14 (Fig. 4). Expression levels were consistently lower in samples from RO-47. In shoot tips, a strong and largely stable expression was observed already from day 1 in SE-62 and SE-67, while samples from RO-47 reached a similar level around day 4 (Fig. 4). A conspicuously high expression was observed for SE-67 at day 7 in shoot tips. Shoot tips from lateral branches showed a similar increase in *PaFTL2* expression as the top shoots, supporting that PaFTL2 function is not limited to the SAM (data not shown).

PaFTL2 Is Highly Expressed in the Autumn

To study the expression of *PaFTL2* in older trees under natural conditions, shoot tips from basal branches were collected on several occasions during one growing season. Each sample contained the meristem and approximately 5 mm of stem. qRT-PCR data indicated a slow increase in *PaFTL2* expression in June and early July and a sharp increase in expression in late July and early August (Fig. 5A). The sharp increase coincides with the time when the night length exceeds the critical night length for growth cessation at the study site in late July.

To investigate the expression of *PaFTL2* in subsequent stages of developing buds, samples of vegetative, male, and female buds were collected from bud set until bud burst (August until April). qRT-PCR data showed that *PaFTL2* displayed similar expression levels in all three bud types (Fig. 5A). Expression peaked at the time of cessation of shoot elongation and decreased steadily until late December, coincident with the transition from endodormancy to ecodormancy (Qamaruddin et al., 1993). A further decrease was observed at bud burst, in particular in vegetative and female buds, consistent with data from vegetative bud flush in forcing experiments (Gyllenstrand et al., 2007).

Data from shoot tips and buds were compared with the yearly *PaFTL2* expression pattern in needles. Needles were collected monthly over 1 year, and in contrast to the decline in *PaFTL2* expression in buds during the transition from endodormancy to ecodormancy, expression remained high in needles until March (Fig. 5C). Expression then rapidly declined in March and remained at a relatively steady level until the increase in August coinciding with the advent of the critical night length, as observed for shoot tips (Fig. 5, A and C). This suggests that the regulation of *PaFTL2* expression in needles seems to be largely controlled by photoperiod (Gyllenstrand et al., 2007), while additional factors seem important for its expression in buds. To test if *PaFTL2* showed a diurnal expression pattern in adult trees under natural conditions, as observed for seedlings grown under controlled conditions, needles were sampled from an adult tree every 4 h during 2 d in August. *PaFTL2* showed a rhythmic expression with a peak in the middle of the day (Fig. 5D).

The spatial expression of *PaFTL2* in female, male, and vegetative buds was investigated with in situ hybridization. In young developing male cones (collected in August), *PaFTL2* expression was detected in the pith and the procambial region (Fig. 6A). One month later, *PaFTL2* expression was concentrated around the procambium and the resin ducts, but some expression also remained

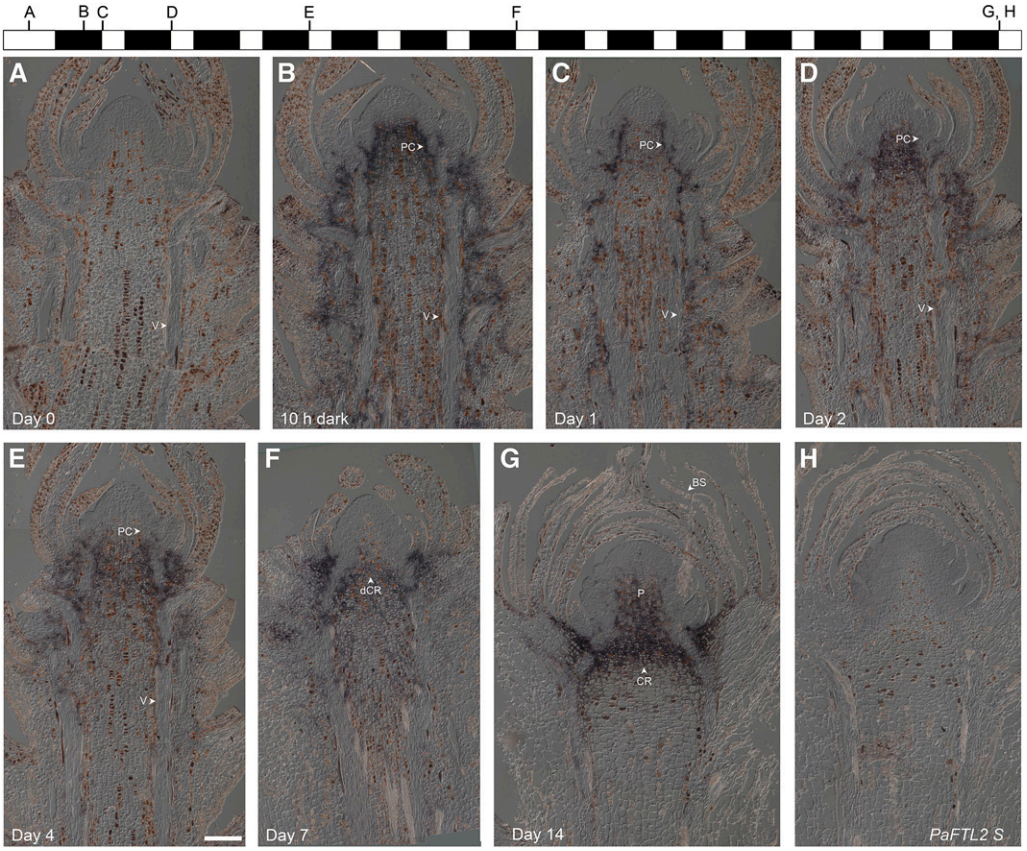

Figure 2. In situ localization of *PaFTL2* mRNA in top shoots from SE-67 Norway spruce seedlings. A to G, Antisense probe. H, Sense probe. Seedlings were grown in constant light until day 0, followed by transfer to short days with 8 h of light/16 h of dark. Shoots were collected at day 0 (A), after 10 h in darkness (B), and then directly before the lights were turned on at day 1 (C), day 2 (D), day 4 (E), day 7 (F), and day 14 (G and H). Before transfer to darkness, no *PaFTL2* expression could be detected (A), but a strong induction of *PaFTL2* mRNA was visible below the meristem and around the vascular tissue (V) and procambium (PC) after transfer to darkness (B–E). After approximately 7 d (F), *PaFTL2* expression began to concentrate in the developing crown region (dCR) when the bud started to form needle primordia. At day 14 (G), buds with bud scales (BS) had formed and the expression was concentrated to the crown region (CR) and the pith (P) of the bud. Bar = 200 μm.

in the pith (Fig. 6, B and C). In developing female cones, a similar expression pattern was observed (Fig. 6, D–F). In young vegetative buds, the expression was localized in the crown region delineating the actual bud and the stem (Fig. 6G), which is consistent with the expression observed in the shoots from seedlings (Fig. 2G). In September, when the vegetative buds had developed several leaf primordia, *PaFTL2* expression had moved up into the pith and the procambial region as for the early reproductive cones (Fig. 6H). Vegetative buds develop later than the reproductive cones (Sundström, 2001), which may explain the delay of expression in the actual bud.

The Expression Pattern of *PaFTL1* Is Largely Complementary to That of *PaFTL2*

As the protein sequence of *PaFTL1* shows high similarity to *PaFTL2* and both genes generate similar phenotypes when expressed in Arabidopsis (Karlgren et al., 2011), both genes might possess related functions in Norway spruce. Previous studies have revealed a high expression of *PaFTL1* during male cone development (Karlgren et al., 2011), but its expression pattern in other tissues was not known.

In shoot tips and needles, expression was relatively high during active shoot elongation in the summer, but it was low during winter in needles and vegetative and female buds (Fig. 5, B and C). Concordant with previous data, expression increased to very high levels in male buds from late November until bud burst (Fig. 5B; Karlgren et al., 2011). In needles sampled in August, *PaFTL1*, like *PaFTL2*, showed a rhythmic expression with a peak in the middle of the day, but the expression level of *PaFTL1* was lower than for *PaFTL2* (Fig. 5D). In situ localization of *PaFTL1* mRNA suggested a high expression during the growing season at the top and flanks of the inactive SAM (Fig. 7). Thus, in many respects, the temporal and spatial expression pattern of *PaFTL1* was complementary to that of *PaFTL2*.

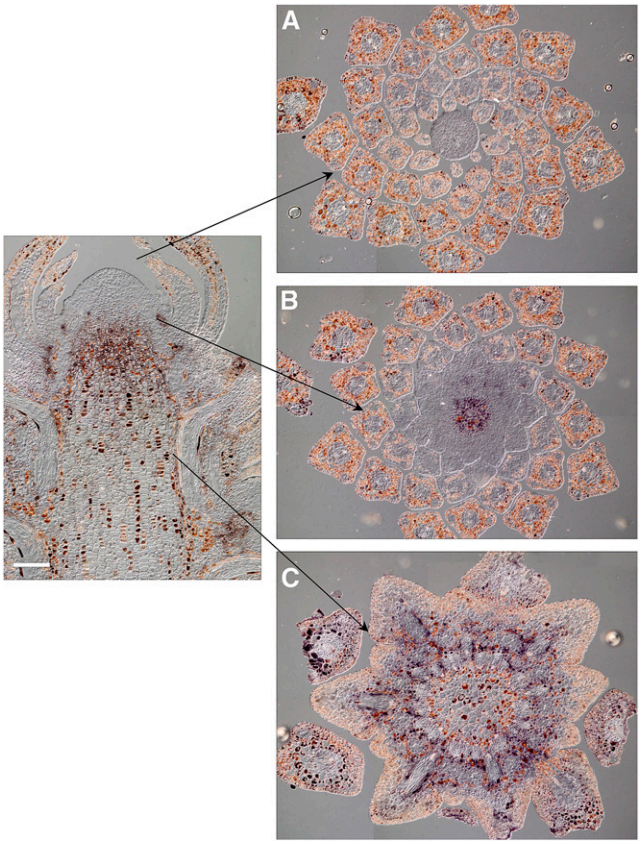

Figure 3. In situ localization of *PaFTL2* mRNA in transverse sections of a top shoot from a RO-47 Norway spruce seedling subjected to two short days of 8 h of light/16 h of dark (day 2). A, In the tip of the meristem, no signal could be observed. B, Just below the meristem, a strong signal could be observed in the center of the shoot. C, Farther down the stem, the signal was concentrated around the procambium and vascular tissue. Bar = 200 μm.

DISCUSSION

PaFTL2 Induces Bud Set and Likely Functions as a Growth Repressor

Previous expression data and population genetic analyses have suggested a role for *PaFTL2* in the control of growth cessation and bud set in Norway spruce as well as in local adaptation resulting in clinal variation for the timing of bud set (Gyllenstrand et al., 2007; Chen et al., 2012). In this study, we confirm, by manipulation of the expression of *PaFTL2* in transgenic Norway spruce seedlings, that the gene does indeed have a role in the control of bud set. Analysis of *PaFTL2* expression domains and temporal patterns further supported that role. Our data further suggest that *PaFTL2* has a function in the repression of growth. We were unable to produce transgenic Norway spruce plants constitutively expressing *PaFTL2*. Likewise, Klintenas et al. (2012) reported that embryogenic spruce cultures expressing *FTL2* stopped growing and died after a few months of tissue culture. Furthermore, an obvious phenotype of our hsp::*PaFTL2* plant was a retarded growth, also when grown under ambient temperatures. The latter suggests increased background levels of *PaFTL2* expression, and collectively, these results support that *PaFTL2* functions as a growth repressor.

In angiosperm poplar trees, one *FT* homolog, *FT2*, has an important role in the control of annual growth (Hsu et al., 2011). *FT2* is controlled by photoperiod and temperature and shows a high expression during the growing season. Transgenic experiments manipulating the expression levels of *FT2* further showed that high expression prevents growth cessation (Hsu et al., 2011). Available data suggest that the protein function of PaFTL2 is more similar to TFL1 than to FT (Karlgren et al., 2011). Thus, the observation that a high expression of *FT2* is associated with growth while a high expression of *PaFTL2* is associated with growth cessation is compatible with the presumed opposite functions of the two corresponding proteins.

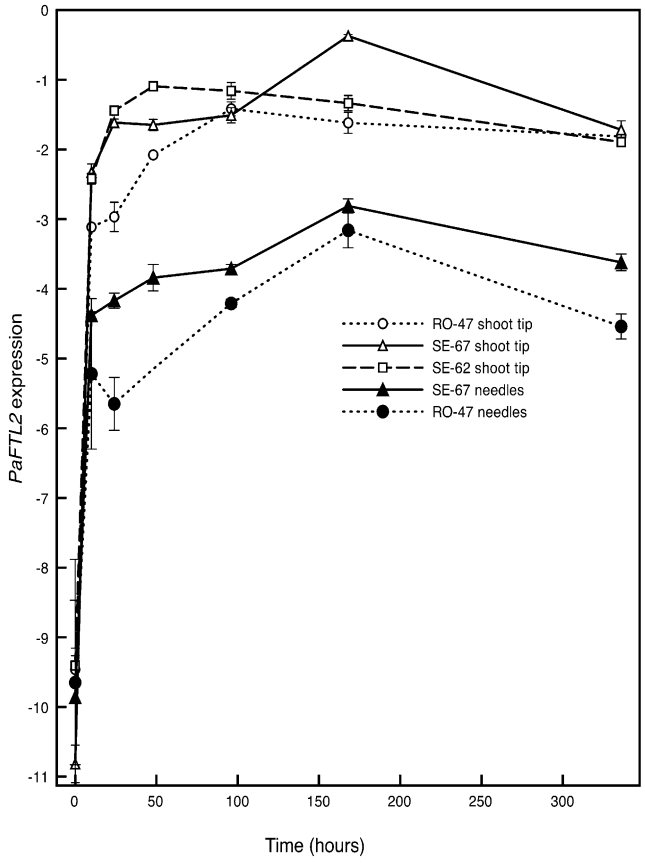

Figure 4. Quantification of *PaFTL2* expression in shoot tips and needles of Norway spruce seedlings. Norway spruce seedlings from two populations, SE-67 and RO-47, were raised in constant light and transferred to short days (8 h of light/16 h of dark). The expression of *PaFTL2* was determined with qRT-PCR in needles closest to the SAM and in the shoot tip including the SAM and 1 to 2 mm of the stem. Relative expression values (*y* axis) were calculated as delta cycle threshold values ($Ct_{PaUBQ} - Ct_{PaFTL2}$). Error bars indicate SE values of technical replicates. The *x* axis indicates hours after transfer to short days.

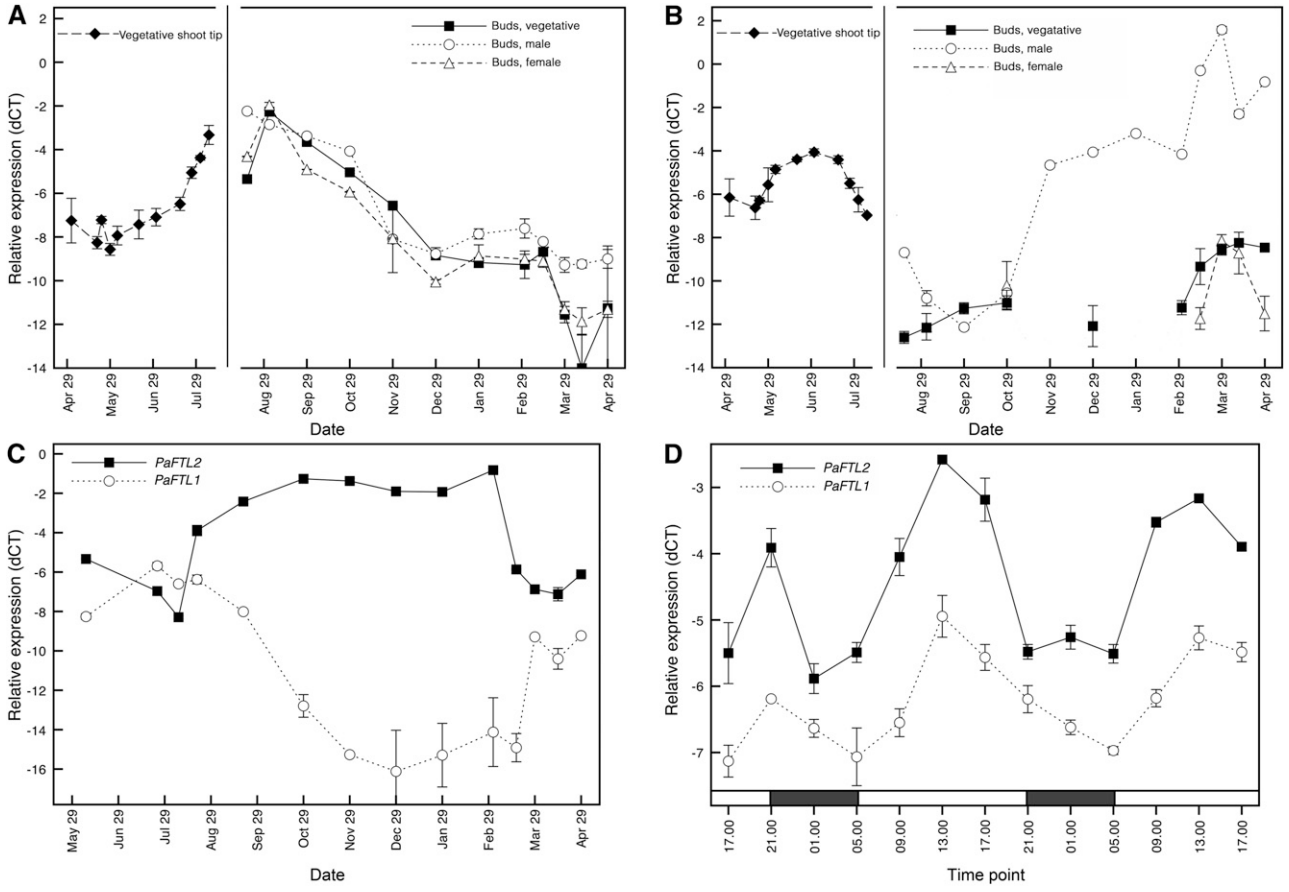

Figure 5. The expression of *PaFTL1* and *PaFTL2* in trees with predetermined growth was determined with qRT-PCR. A, Expression of *PaFTL2* in vegetative shoot tips before growth cessation (left) and in vegetative, male, and female buds after growth cessation (right). B, Expression of *PaFTL1* in vegetative shoot tips before growth cessation (left) and in vegetative, male, and female buds after growth cessation (right). C, Expression of *PaFTL1* and *PaFTL2* in needles as determined over 1 year. D, Diurnal expression of *PaFTL1* and *PaFTL2* in needles as estimated during 2 d in August. Relative expression refers to delta cycle threshold (dCT) values ($Ct_{PaUBQ} - Ct_{PaFTL}$). Vegetative shoots were sampled from three different trees, and error bars derive from biological replicates. Buds and needles were collected from a single tree, and error bars derive from technical replicates.

Genotypes from northern latitudes (SE-62 and SE-67) require only a single short day to set bud when raised in constant light, while those from lower latitudes (RO-47) need four short days (Qamaruddin et al., 1995). Still, similar spatial *PaFTL2* expression was evident at the shoot tip 10 h after transfer to darkness in all genotypes. The rapid induction of *PaFTL2* expression in shoot apices already 10 h into the first long night suggests that the transport of PaFTL2 protein from needles is not required for the induction of bud set. The different SD requirement for bud set in the studied genotypes is paralleled by the expression levels of *PaFTL2* in shoot tips, which reached a high steady level already after 1 d in SE-62 and SE-67, in contrast to the gradual increase in RO-47, where expression reached a similar high level after 4 d. The similar spatial patterns of expression observed from 10 h until 4 d after induction in both southern and northern genotypes suggest that the accumulation of a threshold level of protein is required to induce bud set.

The strong expression of *PaFTL2* evident at the shoot tip already after 10 h in darkness was concentrated around the vascular tissue and procambium. When buds were formed after 2 weeks, the spatial expression pattern of *PaFTL2* had clearly changed. A strong expression was concentrated in the crown region (colenchymatous plate) at the base of the bud. The crown region is, in effect, a plate across the base of the bud, consisting of cells with thickened cell walls (Krasowski and Owens, 1989). The role of the crown region is unclear, but it is thought to physiologically isolate the bud from the shoot, although the plate is penetrated by procambial tissue (Krasowski and Owens, 1989). The plate also separates the region forming bud scales from the one forming needle primordia (Banasiak and Zagorska-Marek, 2006). When buds were formed and new needle primordia developed, the expression of *PaFTL2* also extended into the pith of the bud. Studies of later stages of bud development from adult trees showed that expression was

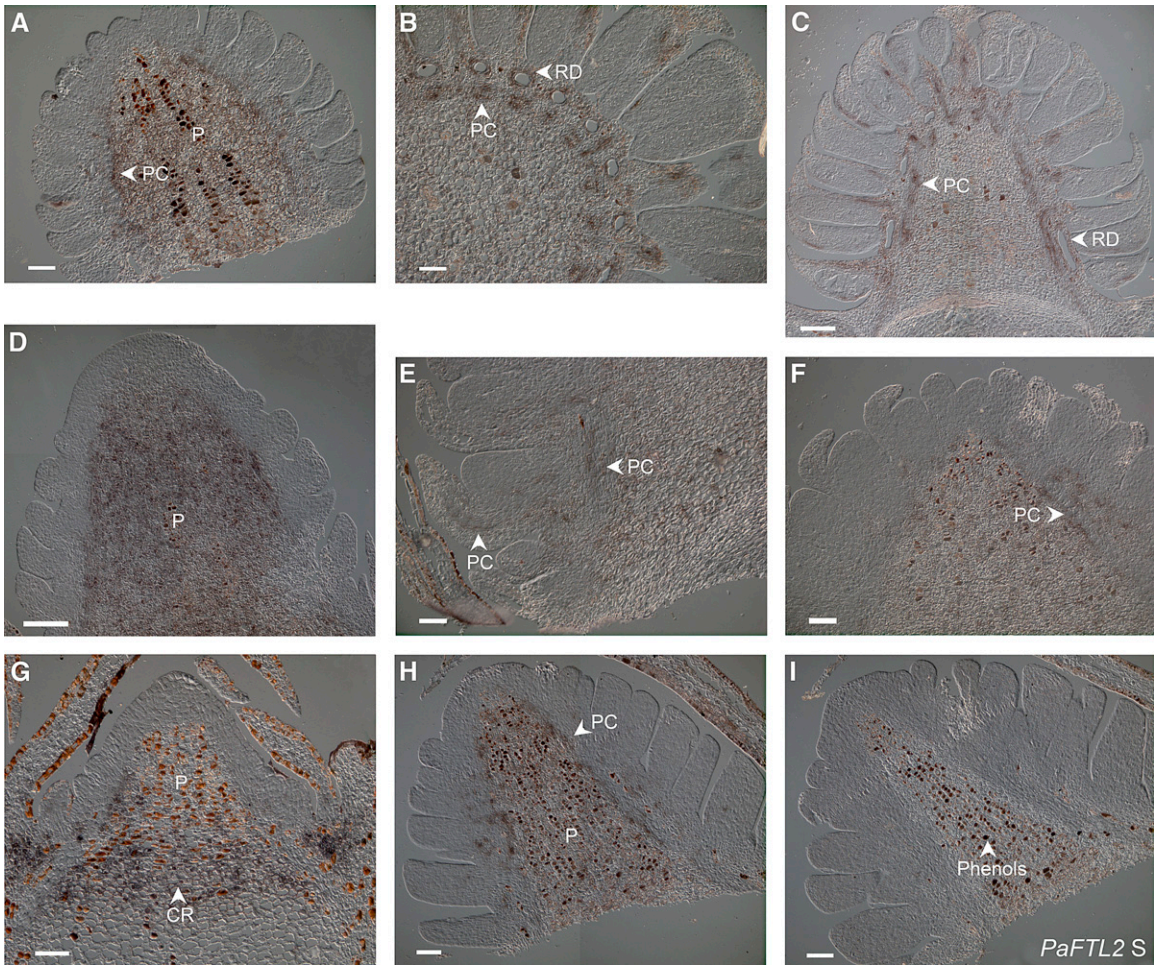

Figure 6. In situ localization of *PaFTL2* mRNA in vegetative and reproductive buds from an adult Norway spruce tree. A, In young male cones sampled in August, *PaFTL2* mRNA were localized in the pith (P) and around the procambium (PC). B and C, When the male bud had developed all microsporophylls in late September (transverse section [B] and longitudinal section [C]), the expression was centered around the resin ducts (RD) and procambium, although some expression remained in the pith. D to F, A similar pattern can be observed in female cones, with high expression in the pith early in the development (August; D) and a more focused expression around the procambium later in September (E and F). G, Vegetative buds in August have *PaFTL2* expression in the crown region (CR) and the pith. H, When more needle primordia have developed in September, the expression, as in male and female buds, is more concentrated around the procambium. I, Sections treated with sense probes showed no visible signal. Bars = 100 μm (A, B, and E–I) and 200 μm (C and D).

gradually focused to the procambial regions surrounding the pith.

The strong expression in the crown region and the pith of the bud is consistent with the hypothesis that *PaFTL2* represses cell division or expansion in these regions. The crown region consists of layers of compact cells, where cell expansion seems to be conspicuously retarded. Likewise, elongation of the cells in pith of the bud must be repressed during the active formation of new needle primordia, within the bud, for the next growing season. As the crown region where *PaFTL2* is strongly expressed coincides with the region where bud scales are formed instead of needles, the gene could potentially also be involved in the specification of identity of primordia formed at the flanks of the meristem.

The conspicuous expression of *PaFTL2* around procambial tissue, both during the induction of bud set and later in developing buds, would, in analogy with the movement of FT in the phloem, suggest that PaFTL2 protein is transported in vascular tissue. Alternatively, PaFTL2 might repress the development or maturation of vascular tissue in order to prevent transport to the meristem. Isolation of the bud from growth-promoting substances seems to be important for developing and maintaining a dormant state of the bud (Rinne et al., 2001). Such a function would be consistent with a general function for the protein in repressing cell proliferation.

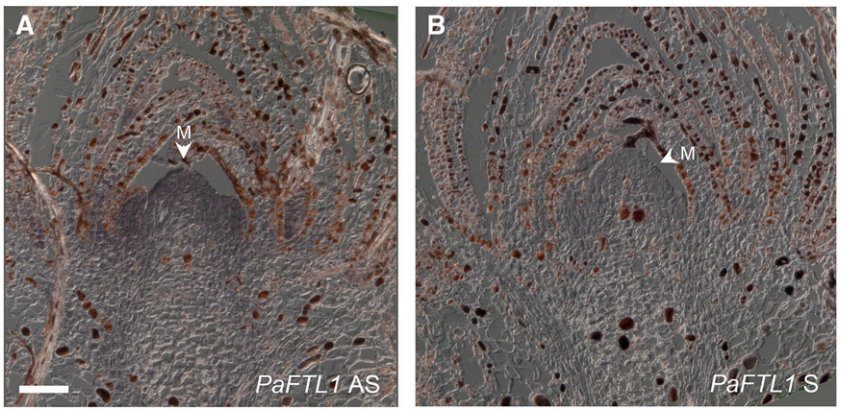

Figure 7. In situ localization of *PaFTL1* mRNA in a vegetative shoot from a lateral branch of a Norway spruce tree sampled outdoor in the beginning of July. A, *PaFTL1* was expressed in the meristem (M). B, No signal was visible with the sense probe. Bar = 100 μm.

Annual Patterns of *PaFTL2* Expression in Adult Trees

Expression patterns of *PaFTL2* in older trees support its role in the control of bud set and bud burst also in adult trees. In contrast to seedlings, new bud scales in adult Norway spruce are formed already when the buds from the previous year are flushing (predetermined growth). The shoot then elongates the needle primordia and intervening internodes of last year's bud. The meristem in the newly formed bud is then largely inactive until growth cessation in the autumn. At that time, the meristem is reactivated and starts to form needle primordia for the next growing season in vegetative meristems. In reproductive meristems, the reactivation of the meristem and the formation of primordia start somewhat earlier.

Expression data from needles and vegetative shoot tips of adult trees confirmed previous observations that *PaFTL2* expression increases in the autumn, around the time of growth cessation and vegetative meristem reactivation. In needles, expression increases until late October, after which high expression is retained until spring, when it drops to an intermediate level concurrent with increasing daylength and temperature. In vegetative buds, a peak of expression is reached in late August, and the gene is expressed in the crown region and the pith of the expanding bud, when new needle primordia are observed. A steady decrease in *PaFTL2* expression was then observed from September to January. Bud burst in Norway spruce requires a chilling period (several weeks of low subzero temperature, typically October to January) followed by an accumulation of high-temperature days and occurs well before the scotoperiod reaches the critical night length. As our data suggest that *PaFTL2* represses growth, they support that bud burst occurs as a result of low *PaFTL2* expression in the buds. The decline in *PaFTL2* expression during winter coincides with the chilling period, and a further reduction occurs concurrently with bud burst. Chromatin modification is important for the regulation of *FT* expression, directly on *FT* chromatin as well as on its upstream regulators (He, 2012). It is tempting to speculate that the reduction in *PaFTL2* expression, specifically in buds, during autumn and early winter is important for the transition from endodormancy to ecodormacy and that it is controlled in a similar way to the regulation of *FT* expression by vernalization.

PaFTL1 Is Highly Expressed in Inactive Meristems

Available data suggest that *PaFTL1* and *PaFTL2* proteins may confer similar functions (Karlgren et al., 2011), but their expression patterns seem to be largely complementary both spatially and temporally. We still lack access to Norway spruce plants with manipulated expression levels of *PaFTL1*, although expression data may give some hints on its putative function. The high expression during male cone development suggests a specific function in this process (Karlgren et al., 2011). An interesting observation from this study was that the gene displays a high expression in shoot tips during the growing season (during shoot elongation of previous year buds), where expression seems to be focused to the top and flanks of the SAM. During this time, the meristems are seemingly inactive, and when the meristems are reactivated and start producing needle primordia, *PaFTL1* expression declines. These data are consistent with a hypothesis in which *PaFTL1* represses meristem activity and the formation of needle primordia.

Concurrently with growth cessation (repression of stem elongation), reactivation of the meristem, and the decline in *PaFTL1* expression, *PaFTL2* expression increases at the base of the meristem. *PaFTL2* expression then spreads into the pith and procambium of the newly formed buds. Thus, *PaFTL1* and *PaFTL2* both seem to be expressed in regions of repressed growth, but their spatial and temporal patterns of expression largely complement each other.

Potential Molecular Functions of *PaFTL1* and *PaFTL2*

Besides its involvement in the induction of flowering, *FT* has been implicated in various developmental processes, such as tuberization, stomatal control, and vegetative growth (Gyllenstrand et al., 2007; Hsu et al.,

2011; Kinoshita et al., 2011; Navarro et al., 2011). In angiosperm flower induction, both FT and TFL1 are hypothesized to interact with the transcription factor FD, and the resulting protein complex either promotes (FT/FD) or represses (TFL1/FD) the transcription of floral meristem identity genes, primarily through the induction of *SOC1* and *FUL* (Hanano and Goto, 2011; Torti et al., 2012). No FD orthologs have been identified so far in gymnosperms (Tsuji et al., 2013), suggesting that interaction with other proteins might be important for PaFTL1 and PaFTL2 function.

The molecular functions of *FT*-like genes in other developmental processes are poorly known. In stomatal control, *FT* has been suggested to regulate proton-pumping ATPases (Kinoshita et al., 2011) and thus might act as a general growth regulator. Recent work in poplar suggests that *AINTEGUMENTA-LIKE* (*AIL*) transcription factors of the *AP2* family act downstream of poplar *FT2* in SD-induced growth cessation (Karlberg et al., 2011). The proposed function of *AIL* in this process is in the control of cell cycle regulators. Direct targets of *FT2* in poplar are unknown, but available data support that the regulation of *AIL* genes by *FT2* is dependent on intermediate genes (Karlberg et al., 2011). In poplar, the expression domain of *AIL1* was mainly confined to the zone of dividing cells in the apex, provascular tissue, and leaf primordia (Karlberg et al., 2011), similar expression domains to those observed for *PaFTL2* in Norway spruce.

Available data do not favor any of these proposed molecular functions for *PaFTL1* or *PaFTL2*, and more data are clearly needed to unravel the molecular control of bud development and meristem activities during the annual growth cycle in conifers.

CONCLUSION

This study clearly supports a role for *PaFTL2* in the control of growth cessation and bud set in first-year seedlings with free growth. Expression data from adult trees with predetermined growth further suggest a function during growth cessation and the development of new needle primordia for the coming season. Both these roles and a potential role of *PaFTL1* in the inactivation of meristems are compatible with a function that represses cell proliferation. An attractive hypothesis is that *PaFTL1* and *PaFTL2* act in concert to control perennial growth in Norway spruce. According to this hypothesis, *PaFTL1*, which is expressed in the meristem, prevents proliferation of the meristem during active extension growth in the summer, while *PaFTL2* attenuates extension growth during bud development in the autumn.

MATERIALS AND METHODS

Plasmids and Constructs for Transformation

Based on previous results (Gyllenstrand et al., 2007; Karlgren et al., 2011), *PaFTL2* was chosen for transformation as the most likely Norway spruce (*Picea abies*) PEBP gene candidate involved in the control of growth cessation and bud set. The hsp::*PaFTL2* construct was produced using Gateway technology (Invitrogen). The complete complementary DNA (cDNA) was amplified using gene-specific primers with *attB* sites as described (Karlgren et al., 2011), and the PCR product was cloned into the pDONR 221 vector (Invitrogen). The destination vector pMDC30 (Curtis and Grossniklaus, 2003), containing a heat-inducible promoter from soybean (*Glycine max*), was used to construct hsp::*PaFTL2* plasmids. A 35S::*PaFTL2* construct with the cauliflower mosaic virus 35S promoter generating constitutive expression was also created using the destination vector pMDC32 (Curtis and Grossniklaus, 2003).

The *bar* gene construct conferring resistance to phosphinothrycin and acting as a selectable marker for transformation was derived from plasmid pAHC25 (Christensen and Quail, 1996). The construct was partially cleaved from pAHC25 with *Eco*RI and cloned into pUC19 to give plasmid pUbi-bar. The *bar* gene was amplified from the plasmid pUbi-bar with the primers T3 (5′-AATTAACCCTCACTAAAG-3′) and T7 (5′-GTAATACGACTCACTATA-3′) using Phusion HF enzyme from Dynazyme. Conditions were 98°C for 30 s followed by 35 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 1 min.

Plant Material for Transformation

The somatic embryogenic cell line 06:28:05 was induced by standard methods (von Arnold and Clapham, 2008) from seed resulting from a controlled cross made in 2006 between selected parents of Norway spruce as part of the Forestry Research Institute of Sweden's breeding program for southern Sweden.

Transgenic sublines of Norway spruce 06:28:05 were created by a particle acceleration method as described (von Arnold and Clapham, 2008). Embryogenic cultures were bombarded with gold particles double coated with hsp::*PaFTL2* and pAHC25 or 35S::*PaFTL2* and the *bar* cassette in the molecular proportion 1:2. Control bombardments contained only pAHC25 or the *bar* cassette. In the coating procedure, 20 μg of DNA was precipitated over 10-mg gold particles (1.5–3 μm in diameter). Callus cultures resistant to phosphinothrycin at 0.2 mg L^{-1}, supplied as the commercial preparation Basta, emerged 2 to 4 months after bombardment. Transformation was confirmed by PCR using the primers Forward (5′-TAATACGACTCACTATAGGGCCCCTGGTAGACCAATCCTA-3′) and Reverse (5′-TAATCCAAGGCCATTCATCTCT-3′).

Embryos were matured on BMI-SI (von Arnold and Clapham, 2008), and after collection, the embryos were left to dry at high humidity for at least 1 month before they were converted to plantlets on solid one-quarter-strength Schenk and Hildebrandt medium (von Arnold and Clapham, 2008). When the roots were 4 to 6 cm long, plantlets were transferred to pots of 8.0 × 8.0 × 6.5 cm containing mineral wool and watered with Ingestad's nutrient solution (Ingestad, 1979). They were raised in a controlled growth room under continuous light from metal halogen lamps (250–300 μmol m^{-2} s^{-1}) at 20°C and 75% relative humidity for 3 to 5 months depending on the rate of establishment of the sublines. At least 1 week before the HS experiments, the plants were removed to a growth cabinet with continuous light at 200 μmol m^{-2} s^{-1} at 20°C and 75% relative humidity in a randomized block design.

HS

The effectiveness of HS was tested by the isolation of total RNA from needles and PCR amplification of the *PaFTL2* gene transcript from reverse-transcribed mRNA as described (Karlgren et al., 2011). To induce optimal expression of the *PaFTL2* construct and find conditions that enabled repeated induction of expression, plants were placed in growth cabinets at 200 μmol m^{-2} s^{-1}, at 38°C or 40°C, for 1 or 3 h. The HS was repeated on subsequent days. The best procedure was 1 h at 40°C on consecutive days, followed by 2 d without heat shock. More frequent treatments were ineffective, apparently owing to side effects of the heat shock. Repeated HS was effective in two transformed sublines, *PaFTL2-ox1* and *PaFTL2-ox2*, whereas only the first HS was effective in *PaFTL2-ox3*.

The experimental regime adopted for the HS experiments (experiments 1–3) was as follows: days 1 and 2, continuous light, heat shock of 40°C from 9 to 10 AM; days 2 to 4, plants were transferred to a light regime of 17 h of light from 9 AM to 2 AM and 7 h of dark from 2 AM to 9 AM at 20°C and 75% relative humidity; days 5 and 6, HS as for days 1 and 2. After the treatment, plants were transferred to continuous light at 20°C and 75% humidity, and they were examined over the next month for growth cessation and bud set. The purpose of the 7-h nights on days 2 to 4 was to prevent a decline of *PaFTL2* expression

to background levels in the absence of heat shock in the *PaFTL2* transformants without inducing bud set in the controls; about seven 7-h nights are normally required to induce bud set in 3-month-old plants of the genotype utilized for transformation (data not shown).

To assess the effects of heat shock on *PaFTL2* expression, needles were sampled from the plants of each transformed subline at 9 AM and 3 PM on day 1 and at 3 PM on days 2 to 6. A total of eight to 12 needles were harvested from each transformed subline on each occasion. One needle was taken from each plant, randomly selected, where there were more than 12 plants per cell line.

Bud set was assessed on plants with the pot labels covered to allow unprejudiced judgment on a scale from 0 to 3, where 0 represents no sign of bud set; 1 represents beginning of bud formation, with needle extension reduced but no white bud scales evident; 2 represents bud scales formed; and 3 represents bud burst and resumption of growth.

Norway Spruce Plant Material

Female, male, and vegetative buds and needles were collected from adult trees of Norway spruce (more than 30 years old) growing at latitude 59°53′N (Uppsala, Sweden) during 2007, 2008, and 2009. Buds were collected on the first date after dissection on which they could be visually determined as female, male, or vegetative. Shoots from three several-year-old Norway spruce trees in different sizes were collected during May to August 2012. Seedlings from three populations were used to determine differences in expression levels of *PaFTL1* and *PaFTL2* in different tissues: a northern population from 66.68°N (Jock Valsjärv, Sweden) here named SE-67, another northern population from 61.57°N (Fulufjället, Sweden) named SE-62, and a southern population from 47.30°N (Frasin, Romania) named RO-47.

Quantitative Gene Expression Analysis

Total RNA was isolated with the Spectrum Plant Total RNA Kit (Sigma-Aldrich) using the On-Column DNase I Digestion Set (Sigma-Aldrich) according to the manufacturer's recommendations or following the protocol described by Azevedo et al. (2003) with minor modifications. cDNA was synthesized from 0.5 μg of total RNA using SuperScript III reverse transcriptase and random hexamer primers. Real-time PCR was performed according to Karlgren et al. (2011) or with the Eco Real-Time PCR System (Illumina). For Eco, each reaction was performed in duplicate samples containing 5 μL of DyNAmo Flash SYBRGreen (Thermo Scientific), 0.5 mM of each primer, and 4 μL of cDNA (diluted 1:100) in a total volume of 10 μL. Alphabot (Alphahelix Technologie) performed all pipetting prior to runs in the Eco. The thermal conditions used were 95°C for 7 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. For primers, see Gyllenstrand et al. (2007) or Karlgren et al. (2011).

In Situ Hybridization

In situ hybridization was performed according to Karlgren et al. (2009). For probe sequences, see Karlgren et al. (2011). After the experiment, the sections were photographed with Nomarski microscopy in 10× magnification. The photographs were merged in Adobe Photoshop CS4, and peripheral regions and background were, in some cases, modified to create a cohesive image. There has been no manipulation in regions containing signal or in crucial structures.

Phylogenetic Analyses

Amino acid sequences of Arabidopsis (*Arabidopsis thaliana*) and Norway spruce PEBP genes were analyzed using the Web service at www.Phylogeny.fr. This pipeline includes alignment with MUSCLE, curation with Gblocks, and phylogeny reconstruction with PhyML. The Web service was run with the "One Click" option and default settings.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Inferred phylogeny of Norway spruce and Arabidopsis PEBP genes.

Supplemental Figure S2. Height growth of transgenic Norway spruce with the hsp::*PaFTL2* construct.

Supplemental Figure S3. In situ localization of *PaFTL2* mRNA in top shoots of RO-47 Norway spruce seedlings.

Supplemental Figure S4. In situ localization of *PaFTL2* mRNA in longitudinal sections of axillary shoots from RO-47 Norway spruce seedlings.

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