Activity–dormancy transition in the cambial meristem involves stage-specific modulation of auxin response in hybrid aspen

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Edited* by Ronald R. Sederoff, North Carolina State University, Raleigh, NC, and approved January 11, 2011 (received for review August 11, 2010)

The molecular basis of short-day–induced growth cessation and dormancy in the meristems of perennial plants (e.g., forest trees growing in temperate and high-latitude regions) is poorly understood. Using global transcript profiling, we show distinct stage-specific alterations in auxin responsiveness of the transcriptome in the stem tissues during short-day–induced growth cessation and both the transition to and establishment of dormancy in the cambial meristem of hybrid aspen trees. This stage-specific modulation of auxin signaling appears to be controlled via distinct mechanisms. Whereas the induction of growth cessation in the cambium could involve induction of repressor auxin response factors (ARFs) and down-regulation of activator ARFs, dormancy is associated with perturbation of the activity of the SKP-Cullin-F-box^{TIR} (SCF^{TIR}) complex, leading to potential stabilization of repressor auxin (AUX)/indole-3-acetic acid (IAA) proteins. Although the role of hormones, such as abscisic acid (ABA) and gibberellic acid (GA), in growth cessation and dormancy is well established, our data now implicate auxin in this process. Importantly, in contrast to most developmental processes in which regulation by auxin involves changes in cellular auxin contents, day-length–regulated induction of cambial growth cessation and dormancy involves changes in auxin responses rather than auxin content.

short days | meristem identity

Perennial plants growing in temperate and high-latitude regions anticipate the approach of winter by sensing the associated reduction in day length (1), and when the day length becomes shorter than the critical length permitting growth [short-day signal (SD)], cell division terminates in the meristems (1). Immediately following cessation of cell division, exposure of plants to permissive conditions (e.g., long days) leads to the reversal of growth arrest (2), and this stage of growth arrest is referred to as ecodormancy. Continued exposure of ecodormant plants to short days brings about the transition from ecodormancy to endodormancy (2). The endodormant state is characterized by the inability of the meristems to respond to growth-promotive signals in contrast to the ecodormant state. Exposure to chilling temperatures is required to restore the ability of endodormant meristems to respond to growth-promotive signals and to reinitiate growth subsequently (3).

Recently, the photoreceptor *PHYA* (4) and homologs of the flowering time genes *CONSTANS* and *FT* (5, 6) have been shown to be early-acting components in SD-induced growth cessation in trees. The targets and signaling intermediates of the SD pathway downstream of these early-acting components in growth cessation and dormancy remain largely unexplored (7). Although ecodormant and endodormant states can be distinguished physiologically, the molecular mechanisms underlying the establishment of endodormancy and the inability of endodormant meristems to respond to growth-promotive signals have remained elusive.

We investigated whether the SD-regulated induction of cambial growth cessation and dormancy in hybrid aspen (*Populus tremula* × *tremuloides*) involves modulation of auxin (AUX)/indole-3-acetic acid (IAA) signaling, because several studies have shown that auxin is a key regulator of cell division in plants (8, 9) and reducing either auxin levels or auxin responsiveness leads to reductions in cambial cell division activity in trees (10, 11). Our results demonstrate distinct stage-specific changes in the auxin responsiveness of the transcriptome during the activity–dormancy transition and suggest a potential mechanism underlying SD-induced growth cessation and dormancy involving the modulation of auxin responsiveness.

Results

Temporal Separation of Ecodormancy and Endodormancy Under Short-Day Conditions. We exposed soil-grown hybrid aspen T89 (*Populus tremula* × *tremuloides*) plants to SDs and identified the timing of ecodormancy and endodormancy. Growth ceased after ca. 40 d under these conditions, and plants were in ecodormancy at this stage because growth could be reinitiated by transfer to long days (Fig. 1). In contrast, after exposure to 56 SDs, plants became endodormant. They were unable to resume growth after transfer to long days, and applied auxin could no longer stimulate cambial cell division as it normally does in actively growing trees (Fig. S1).

Transition to Endodormancy Coincides with Loss of Auxin Regulation of Polar Auxin Transport Machinery. Perturbing polar auxin transport (PAT) leads to a reduction in meristematic activity in *Arabidopsis* and other plants (12–14). We investigated regulation of PAT during different stages of the activity–dormancy transition. PAT is maintained during growth cessation and endodormancy because applied auxin could enter the PAT stream even in plants exposed to 56 short days (Fig. S2). We also investigated the ability of auxin to induce the expression of PAT components (11, 15, 16) during progressive stages of the activity–dormancy transition. During active growth, auxin could induce expression of cambially expressed genes encoding the PAT components *PttPAX3* and *PttPPL1* (Fig. 2); however, after exposure to 42 SDs, expression of the genes was no longer responsive to applied auxin, although PAT was still detectable at this stage (Fig. S2). The insensitivity of efflux

Author contributions: K.B., J. Schmidt, J. Schrader, T.R.H., L.B., and R.P.B. designed research; K.B., A.K., J. Schmidt, J. Schrader, and T.R.H. performed research; and A.K. and R.P.B. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1011506108/-/DCSupplemental.

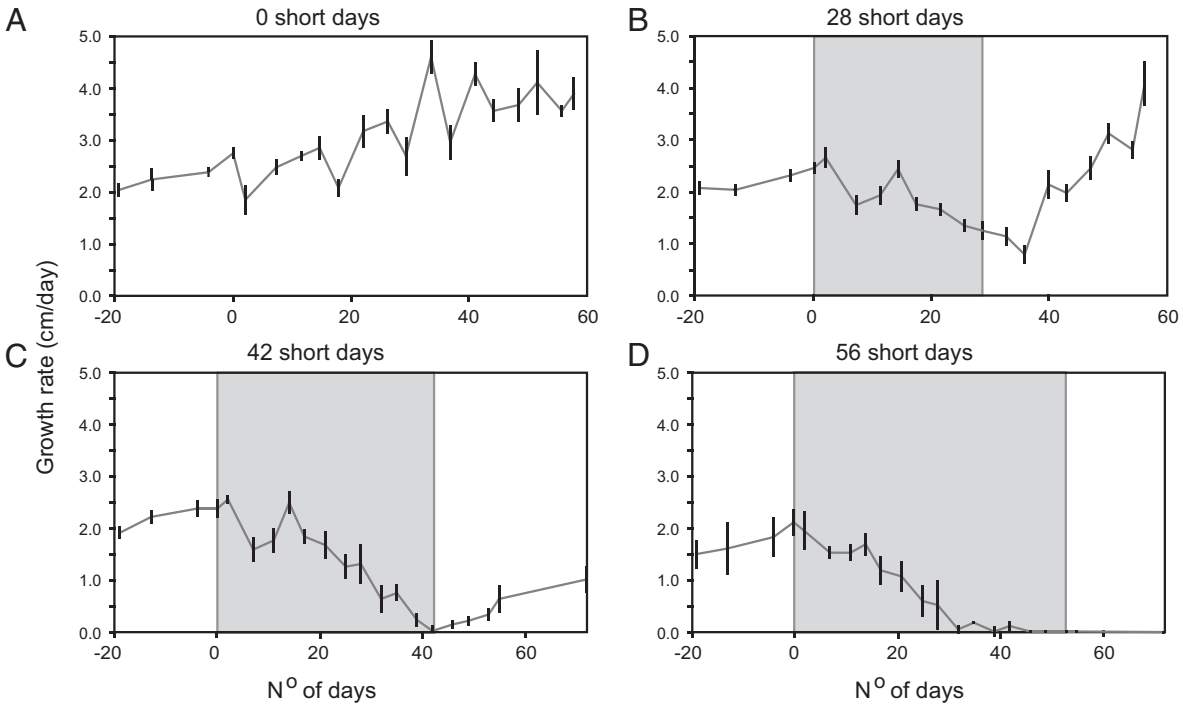

Fig. 1. Timing of ecodormancy and endodormancy in hybrid aspen (T89) plants. Soil-grown hybrid aspen plants were either grown continuously in long days (*A*) or initially in long days and then transferred to short days (*B–D*). The timing of endodormancy was determined by measuring growth rates of plants in long-day conditions, after exposure to 28 (*B*), 42 (*C*), and 56 (*D*) short days, as increases in height in centimeters per day. The periods of the short-day treatments are highlighted in gray (error bars = SD, $n = 3$).

and influx carrier expression to auxin was subsequently maintained during endodormancy.

Differential Changes in Auxin Responsiveness of *AUX/IAA* Genes During Progressive Stages of Activity–Dormancy Transition. Because AUX/IAA transcription factors play a key role in the transcriptional regulation of PAT-related genes by auxin (11, 16), we investigated the regulation of AUX/IAA genes by auxin after SD treatment (Fig. 3). The expression of the poplar *AUX/IAA* genes *PttIAA8*, *PttIAA1*, and *PttIAA5* was auxin-inducible in plants subjected to 28 SDs. *PttIAA8* and *PttIAA1* genes lost their auxin responsiveness after 42 SDs preceding the transition to endodormancy, simultaneously with the loss of auxin inducibility of PAT-related genes (Fig. 2). In contrast, *PttIAA5* expression remained auxin-responsive even in the endodormant plants. The transcript levels of all three of the *PttIAA* genes were down-regulated in plants after 56 SDs, suggesting an additional regulatory mechanism controlling steady-state transcript levels of AUX/IAA gene expression during endodormancy.

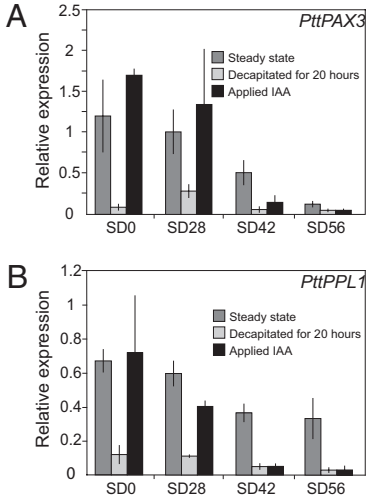

Fig. 2. Modulation of auxin regulation of the expression of PAT component genes by short-day treatment. Transcript levels of *PttPAX3* (*A*) and *PttPPL1* (*B*) are shown relative to that of 18S rRNA. Each value is the average of three replicates, and the SD is indicated. The differences in transcript levels between auxin-depleted and auxin-applied samples were significant according to a *t* test ($P < 0.001$) at time points short day 0 (SD0) and SD28.

Global Stage-Specific Changes in Expression of Auxin-Responsive Genes During the Transition to Dormancy. We next analyzed whether SD treatment leads to global changes in auxin responsiveness of the transcriptome during the distinct stages of the activity–dormancy transition. The transcript profiling analysis using poplar cDNA microarrrays indicates that a major change in auxin responsiveness occurs after 28 SDs, with 607 transcripts losing their ability to respond to auxin (Dataset S1). Following this, there is a progressive loss of auxin responsiveness, with 83 and 84 transcripts losing their auxin responsiveness in plants subjected to 42 and 56 SDs, respectively. A subset of 238 transcripts still retains auxin responsiveness in endodormant plants, however, as observed in our initial analysis for *PttIAA5* (Dataset S1).

Promoter Analysis of Auxin-Responsive Genes. To date, several auxin response elements involved in regulating gene expression in response to auxin have been reported (17). Of these, TGTC_C is the best-characterized auxin response element (18). Therefore, we scanned the promoters of all genes (1 kb upstream of the transcription start site) for exact occurrences of this well-characterized auxin response element and investigated its correlation with the pattern of loss of auxin responsiveness after

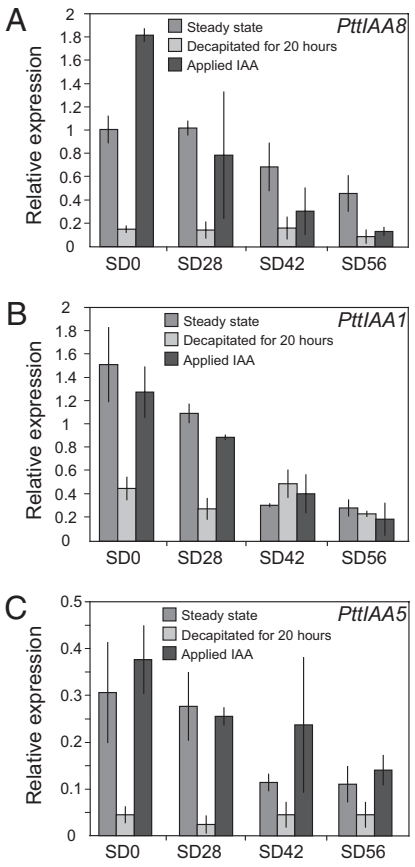

Fig. 3. Alteration of auxin responsiveness of poplar *AUX/IAA* genes following short-day treatment. Transcript levels of *PttIAA8* (*A*), *PttIAA1* (*B*), and *PttIAA5* (*C*) are shown relative to that of 18S rRNA. Each value is the average of three replicates, and the SD is indicated. The differences in transcript levels between auxin-depleted and auxin-applied samples were significant according to a *t* test ($P < 0.001$) for *PttIAA8* and *PttIAA1* at short day 0 (SD0) and SD28, whereas the differences in transcript levels between auxin-depleted and auxin-applied samples were significant for *PttIAA5* according to a *t* test ($P < 0.001$) at time points SD0 and SD28 and ($P < 0.01$) at time points SD42 and SD56.

SD treatment. Although the element is present at least once in about every second gene (8,206 of 15,883 genes on the array), it was significantly overrepresented in auxin-responsive genes compared with the other genes on the array ($P < 4.14E-04$). Genes that lose their auxin responsiveness late (i.e., after 56 SDs) when endodormancy is established also have a significantly higher number of the element compared both with all auxin-responsive genes ($P < 0.021$) and with the genes losing responsiveness after 28 SDs ($P < 0.0015$; details provided in Dataset S2). These data suggest a trend in which genes that maintain the auxin response longer after SD treatment have more occurrences of the auxin response element.

Establishment of Endodormancy Is Correlated with the Loss of Ubiquitination of PttIAA3. The degradation of the AUX/IAA proteins is mediated by the proteasomal pathway, which typically involves ubiquitination by E3-ubiquitin ligases (19). We investigated whether the changes in auxin responsiveness during the activity–dormancy transition involve alterations in the proteasomal degradation of AUX/IAA proteins as shown for *Arabidopsis* AUX/IAA proteins (20–24). For this, we analyzed the ubiquitination of a GST fusion of *PttIAA3*, a poplar *AUX/IAA* gene expressed in the cambial meristem in hybrid aspen (25). This PttIAA3 protein contains the conserved domain II found in all the poplar AUX/IAA genes that has been shown to be necessary and sufficient to mediate degradation by auxin. Our results show that GST-PttIAA3 is efficiently ubiquinated by extracts from actively growing and ecodormant plant tissues, and several bands of ubiquitinated GST-PttIAA3 can be detected following incubation with 6-histidine–tagged ubiquitin and electrophoretic separation (Fig. 4). These multiple bands of ubiquitinated GST-PttIAA3 indicate that GST-PttIAA3 is polyubiquitinated by the extracts. In contrast, with extracts from actively growing and ecodormant plants, extracts from endodormant plants are unable to ubiquitinate GST-PttIAA3 (Fig. 4).

***PttTIR1* but Not *PttAXR1* Expression Is Down-Regulated Preceding Endodormancy.** Because GST-PttIAA3 could no longer be ubiquitinated by extracts from endodormant plants, we investigated whether this could be attributable to a lack of expression of *PttTIR1* or *PttAXR1*, both of which are key components of the SCF^{TIR} complex (26, 27) involved in AUX/IAA degradation (Fig. 5). Although both of these genes are expressed through the entire activity–endodormancy transition, *PttTIR1* expression was reduced by 50% after 42 SDs preceding endodormancy, whereas SD treatment had little effect on the expression of *PtAXR1*.

Discussion

Perception of SD induces the cessation of cambial cell division, followed by a gradual transition to dormancy in hybrid aspen and other tree species (2, 3). Early analyses suggested that SDs could induce the cessation of cambial cell division by reducing cambial auxin levels (28, 29). This hypothesis was subsequently rejected, however, when high-resolution MS analysis indicated that no reduction in cambial auxin levels occurred during the activity–dormancy cycle (30). These results were surprising, because if cambial auxin levels remain unchanged during growth cessation, what would be the mechanism that inhibits cell division, a process normally promoted by auxin? Therefore, we explored an alternative hypothesis that SDs could act by modulating auxin responsiveness rather than cambial auxin content during the activity–dormancy transition.

Our RT-PCR and microarray data show that specific stages of the activity–dormancy cycle are associated with changes in the auxin responsiveness of distinct sets of genes. The first major change in auxin responsiveness coincides with the induction of growth cessation, with several genes losing their ability to respond to auxin. Gene expression data, coupled with prior results

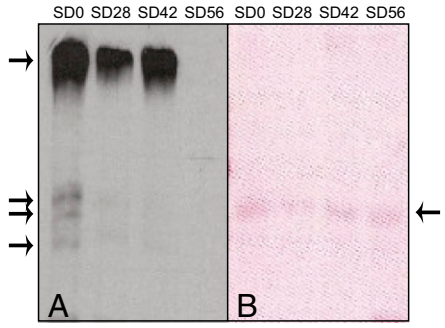

Fig. 4. (*A*) Ubiquitination analysis of purified GST-PttIAA3 protein. Plant extracts supplemented with purified 6-histidine–tagged ubiquitin from plants exposed to short day 0 (SD0), SD28, SD42, and SD56 were used for ubiquitination analysis of purified GST-PttIAA3 protein, as described in *Materials and Methods*. Ubiquitination was detected using anti–6-histidine antibody. Arrows indicate ubiquitinated protein bands. (*B*) Ponceau-stained membrane after transfer of proteins before Western blot analysis. The arrow indicates GST-PttIAA3.

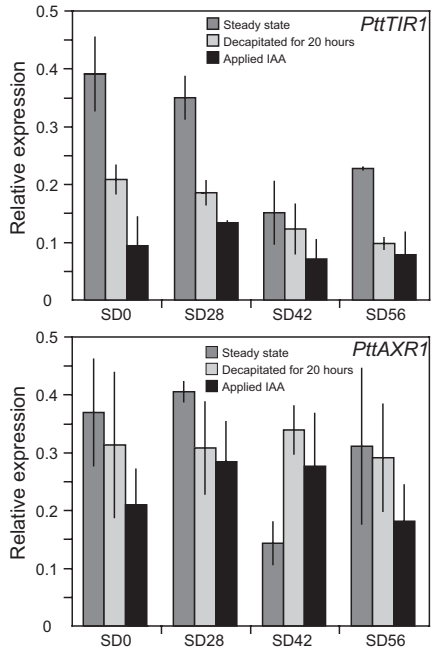

Fig. 5. Expression analysis of *PttTIR1* and *PttAXR1* following short-day treatment. Expression levels are plotted relative to that of 18S rRNA. For each time point, the average of three measurements is shown with the SD. The *x* axis indicates the time in short day 0 (SD0), SD28, SD42, and SD56. The differences in steady-state transcript levels for *PttTIR1* were significant according to a *t* test ($P < 0.01$) between time points SD28 and SD42 and between time points SD28 and SD56.

(11) showing that perturbing auxin responsiveness reduces cambial cell division, strongly suggest that SDs could potentially induce the cessation of cambial cell division by modulating auxin responses. Further support for this hypothesis comes from analysis of two hybrid poplar clones that vary in their timing of cambial cell division arrest after SD treatment (31). In these hybrid poplar clones, the change in the expression of auxin-responsive genes is earlier and stronger in the clone that undergoes cambial cell division arrest earlier than in the late clone.

The indications that changes in auxin responsiveness may be involved in cambial growth cessation raise questions regarding how this could lead to the termination of cell division. One possibility is that SD could interfere with auxin-promoted expression of cell cycle genes, causing their down-regulation and leading to growth cessation. Only 2 of the 25 core cell cycle-related genes down-regulated during cambial growth cessation (32) were positively regulated by auxin (11) (Dataset S1). Thus, the most likely explanation is that the change in auxin responsiveness can only indirectly lead to the down-regulation of the cell cycle genes, thereby inducing growth cessation. The change in auxin responsiveness could also negatively affect other hormonal pathways (e.g., ethylene and gibberellin synthesis and/or signaling) that promote cambial cell division and are also involved in the activity–dormancy transition (33, 34).

Following growth cessation, continued exposure to short days leads to the establishment of endodormancy. An important change preceding this development is the uncoupling of the PAT machinery from its regulation by auxin at the transcriptional level, as suggested by the loss of auxin responsiveness in the expression of the cambially expressed *PttPIN1* and *PttPAX3* genes. The ability of auxin to regulate its own transport provides a dynamic regulatory mechanism that can balance increases in auxin by increases in transport capacity. Such a mechanism is highly useful when, for instance, environmental signals promote growth by enhancing auxin synthesis (35, 36). Under natural conditions, the transition to endodormancy is initiated after growth cessation occurs, as winter approaches. At this stage, there is little if any auxin synthesis in source tissue (e.g., leaves) and little likelihood of a sudden change in auxin levels; in addition, the growth rate is low. Therefore, it may not be necessary to maintain dynamic regulation of PAT once the transition to endodormancy is initiated, which could explain the uncoupling of PAT from its regulation by auxin at this stage.

Interestingly, the establishment of endodormancy does not lead to a complete loss of auxin responsiveness per se, because a subset of genes still retains auxin responsiveness at this stage. This finding supports the idea that auxin regulates several aspects of cambial activity in addition to cambial cell division (e.g., maintenance of the meristem identity of the cambium) (11, 37). Thus, although cambial cell division ceases, the meristem identity of the cambium still needs to be maintained, necessitating continued auxin responsiveness even in the endodormant state. Taken together, these results indicate the presence of multiple auxin signaling pathways that are differentially modulated during growth in short days.

Our analysis of auxin responsiveness during the progressive stages of the activity–dormancy transition raises several questions. First, what is the mechanism underlying temporal differences in the loss of auxin responsiveness after SD treatment? Promoter analysis of auxin-responsive genes suggests that these temporal differences in loss of auxin responsiveness between early (auxin response lost after 28 SDs) and late (auxin response lost after 56 SDs) genes could at least partially be explained by differences in the number of auxin response elements in the promoters of the early and late genes. Although TGTC_C is the best-characterized auxin response element, several other less well-characterized auxin response elements have also been reported (17). We found very few of these less well-characterized elements to be present in the promoters of the auxin response genes investigated here, however. Nevertheless, the possibility cannot be excluded that differences in the occurrence of these less well-characterized elements in the promoters of auxin response genes may also be a factor in differential regulation of auxin-responsive gene expression during activity–dormancy transitions.

Second, what is the mechanism by which SD can modulate auxin responsiveness? One possibility is suggested by the recent observation that the circadian clock influences auxin signaling in *Arabidopsis* (38). Thus, alteration of auxin response during growth cessation could potentially involve modulation of the output of the circadian clock by the SD in perennials. Although attractive as a hypothesis, the link between the circadian clock and auxin response modulation by SD requires more knowledge about the clock components in tree species and their regulation of auxin response.

Another key question that arises from our observations is that of which genes mediate the input from SD in modulation of the auxin responsiveness of gene expression. Our microarray data (32) show that *PU11616*, which encodes a poplar auxin response factor (ARF) orthologous to *Arabidopsis ARF2*, is up-regulated during the induction of growth cessation and transition to dormancy. *ARF2* acts as a transcriptional repressor (39, 40) and is a negative regulator of cell division in *Arabidopsis* (41). In contrast, *PU01758*, which encodes a poplar homolog of the *Arabidopsis* factor *ARF6* that has an activator domain and appears to be a transcriptional activator (18, 42), is down-regulated during the transition to dormancy (32). These results suggest that up-regulation of negatively acting *ARFs*, with simultaneous down-regulation of activator *ARFs*, could lead to a general reduction in auxin responsiveness during the transition to dormancy. Both *ARF2* and *ARF6* are part of a small gene family (43), and the possible roles of these genes and their close homologs in the regulation of auxin responses during growth cessation and dor-

mancy clearly warrant further attention. In addition to ARFs, however, several other factors (e.g., *PICKLE*, *TOPLESS*) and other transcriptional regulators have been implicated in the regulation of auxin-responsive gene expression (44); therefore, it is likely that these may also be involved in the modulation of auxin response during the activity–dormancy cycle.

Although loss of auxin responsiveness of the cambial cell cycle machinery during endodormancy has been reported (3), the molecular basis of this has not yet been elucidated. We propose that the altered auxin responsiveness during endodormancy could be at least partially attributable to the stabilization of PttIAA3, and potentially to that of other AUX/IAA repressors resulting from perturbed SKP-Cullin-F-box^{TIR} (SCF^{TIR}) function. This hypothesis is supported by data showing that the expression of mutated versions of AUX/IAA proteins (e.g., PttIAA3) that can lead to their stabilization reduces cell division in hybrid aspen plants (11). The lack of ubiquitination, suggesting altered activity of the SCF^{TIR} complex during endodormancy, led us to investigate the possibility whether this could be attributable to the lack of expression of *PttTIR1* and *PttAXR1*, which are key components of this complex. We focused on analysis of these two components because TIR1 is the auxin receptor and is specifically required for the degradation of AUX/IAA proteins by the SCF^{TIR} complex. Although AXR1 is also involved in other processes, it is, nevertheless, required for AUX/IAA degradation by the SCF^{TIR} complex (44). Our data suggest that the alteration in the SCF^{TIR} complex is not attributable to the lack of *PttTIR1* and *PttAXR1* expression, because both of these are expressed during endodormancy. Although *PttTIR1* expression is reduced in the endodormant state by 50%, it seems less likely that this reduction alone could significantly affect SCF^{TIR} activity, because the SCF^{TIR} complex involves several additional proteins (44). Moreover, in *Arabidopsis*, the expression of the entire TIR1/AFB gene family needs to be reduced by 50% to stabilize AXR3 (45). Thus, it is likely that the reduction in the SCF^{TIR} complex during endodormancy might involve a cumulative reduction in the level of several components of the SCF^{TIR} complex.

Based on our observations, we present a model for the modulation of auxin responsiveness during the two key stages of the activity–dormancy transition, namely, growth cessation/ecodormancy and endodormancy. During the induction of growth cessation, auxin responsiveness could be altered via changes in the expression of ARFs, and potentially other transcriptional regulators as well. This primarily transcriptional control could allow for rapid reversal of changes in auxin responsiveness on transfer to permissive growth conditions (e.g., long days). Continued growth under short-day conditions could affect the SCF^{TIR} complex, however, preventing the ubiquitination and subsequent degradation of the AUX/IAA repressors (e.g., PttIAA3) that are expressed in the cambial meristem throughout the activity–dormancy cycle (25), leading to a mechanistically distinct loss of auxin responsiveness in the endodormant state. Furthermore, stabilization of the repressor AUX/IAA proteins would interfere with the action of activator ARFs and reinforce the action of repressor ARFs (e.g., ARF2), thereby leading to the down-regulation of steady-state transcript levels of a set of genes, including *PttPAX3*, as endodormancy is induced. Thus, a reduction in auxin responsiveness during endodormancy may involve a combination of reduced SCF^{TIR} activity and ARF regulation.

In summary, we have identified several aspects of the regulation and role of auxin signaling in the SD controlled activity–dormancy transition in perennial plants. Importantly, SD-mediated regulation of growth cessation and dormancy in perennial plants appears to involve modulation of auxin responsiveness rather than auxin levels. This mechanism is in contrast to the majority of cellular responses regulated by auxin, which are mediated primarily via changes in cellular auxin contents (46). Furthermore, auxin appears to play a role not only in endodormancy, as previously postulated (3), but potentially in the induction of growth cessation. The future challenges will lie in identifying the molecular regulators mediating short-day modulation of auxin responsiveness in perennial plants.

Materials and Methods

Plant Material and Growth Conditions. Hybrid aspen (*Populus tremula* × *tremuloides*) clone T89 plantlets were grown under long-day conditions in a greenhouse; they were then shifted to growth chambers and grown further for a week under long-day conditions to acclimatize before initiation of short-day treatment in controlled environment growth chambers. Detailed growth conditions are provided in *SI Materials and Methods*.

Auxin Treatment. To identify auxin-responsive genes, auxin treatments were performed as follows. At each time point described above, three plants were decapitated at internode 11, a 1-cm-long piece of each of their stems was collected (as an untreated control), and lanolin was applied to the decapitated stems. Twenty hours later, another (auxin-depleted) 1-cm sample was collected from each stem; auxin was then applied to the cut stems in lanolin, and final (auxin-treated) samples were collected after a further 4 h. At each sampling point, sampled tissues were immediately frozen in liquid nitrogen and kept at −70 °C until analysis.

RNA Isolation, First-Strand cDNA Preparation, and RT-PCR Analysis. RNA was isolated and cDNA was generated using standard methods as described in *SI Materials and Methods*. RT-PCR was performed as described by Schrader et al. (15). The gene-specific primers used for RT-PCR analysis are described in Table S1.

Microarray Probe Preparation. Preparation of labeled probes for microarray hybridization was performed as described previously (47). Briefly, mRNA prepared from 1 μg of total RNA isolated from stem tissues (the same as those used for RT-PCR analysis of auxin response after short days as described above) was used to generate cDNA, and amplified cDNA was labeled, followed by purification of labeled probes using a QIAquick PCR purification kit (Qiagen) as described previously (32) (detailed description is provided in *SI Materials and Methods*).

Microarray Array Hybridizations and Data Analysis. Hybridizations were performed on the POP2 cDNA array, as described by Moreau et al. (47), according to the experimental design illustrated in Fig. S3. Slides were scanned, and spot data were extracted and analyzed. The resulting raw data were imported into the poplar microarray database UPSC-BASE (48) for subsequent analysis. After applying normalization and array quality control measures, flagged spots were filtered out of the dataset. *B*-statistics included in the LIMMA R-package (49) (http://bioinf.wehi.edu.au/limma/) were used to obtain relative expression values and statistical values compared with an *in silico* reference or a reference sample of choice and to identify differentially expressed genes. To generate lists of differentially expressed genes, an arbitrary log-odds cutoff of $B \geq 5$ was set. To identify auxin-regulated genes at each time point, the auxin-depleted sample was set as the reference for the full dataset and compared with the auxin-induced sample from the corresponding time point. To analyze the expression profiles of the identified auxin-responsive genes further, the dataset was divided into three time series subsets (full method is provided in *SI Materials and Methods*).

Promoter Analysis. Probes on the microarray were mapped to gene models, and promoters were taken from the Populus Genome Integrative Explorer (PopGenIE) online resource (http://popgenie.org/) (50). We scanned both strands of the promoters for exact matches to the auxin response element TGTC_C (or G_GACA) in 1-kb promoters (the position _ is allowed to take on any nucleotide). We then analyzed five gene sets of interest: (*i–iii*) genes losing auxin response after 28, 42, and 56 short days, respectively; (*iv*) genes never losing auxin response; and (*v*) all genes with auxin response (i.e., all the genes in sets *i–iv*). Three statistical tests were conducted. First, we tested for overrepresentation of the response element in each of the five gene sets compared with the remaining genes on the array using the hypergeometric distribution. Second, we tested for differences in the average number of elements between each of the five gene sets and the remaining genes on the array using Fisher's exact test. Finally, we tested for differences in the average number of elements between the five gene sets themselves, again using Fisher's exact test.

Auxin Measurements and PAT Analysis. Measurements of PAT were performed using radiolabeled IAA on three independent stems for each time point according to Schrader et al. (15).

Production of GST-PttIAA3 Fusion Protein. PttIAA3 was cloned into plasmid pGEX-5 × 1 (Amersham, Inc.), and the resulting plasmid was introduced into *Escherichia coli* strain BL21DE3 for protein production. GST-PttIAA3 fusion protein was purified according to the manufacturer's instructions (details are provided in *SI Materials and Methods*).

Ubiquitination Analysis. Total proteins were extracted from stem tissues of plants at each time point before and after SD treatment (0, 28, 42, and 56 short days). One hundred twenty micrograms of total protein from each extract was used to assay ubiquitination activity. After the ubiquitination reaction, GT-Sepharose beads were added to bind ubiquitinated protein. Bound proteins were electrophoretically separated and transferred to a PVDF membrane overnight as previously described (2). Ubiquitination of GST-PttIAA3 was detected using anti-6XHis antibody (Qiagen) (a detailed description is provided in *SI Materials and Methods*).

Generation of 6XHIS-Ubiquitin Fusion Protein. *Arabidopsis* cDNA coding for the UBQ11 gene was amplified by PCR and cloned into the pET28a vector. For protein production, pET28a-UBQ11 was transformed into bacterial strain BL21DE3 and 6XHIS-Ubiquitin was purified using a Ni^{2+}-NTA matrix (Qiagen). The His6-UBQ protein eluted from the Ni^{2+}-NTA beads was dialyzed, and portions were stored at −80 °C (details are provided in *SI Materials and Methods*).

ACKNOWLEDGMENTS. We thank Ingela Sandström, David Jonsén, Nathalie Druart, and Manuel Lopez-Vernaza for technical support. This work was funded by grants from Vetenskapsrådet and an Excellence grant from the Swedish University of Agricultural Sciences (to R.P.B.).

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