Original Paper

Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex

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Abstract Perennial plants such as the long-lived trees of boreal forest cycle between periods of active growth and dormancy. Transition from active growth to dormancy is induced by short day (SD) signal. Once dormancy is established, prolonged exposure to low temperature is required for breaking dormancy before warm temperatures can induce growth. We have studied global changes in gene expression in the apex of model plant hybrid aspen during the distinct stages of activity-dormancy cycle. Our data shows that all stages of activity-dormancy cycle in the apex are associated with substantial modulation of the transcriptome. Detailed analysis of core cell cycle genes indicates that with the exception of plant specific B-type CDKs, all of the other CDKs are regulated post-transcriptionally during growth cessation. SD signal appears to target the expression of cyclin genes that are down regulated during growth arrest. Several of the cold hardiness related genes e. g. dehydrins are induced during transition to dormancy although temperature is not reduced and the up-regulation of the expression of these genes does not appear to rely on SD mediated induction of classical CBF transcription factors. Our results suggest that transcriptional control plays a key role in modulation of hormones such as ABA and GA that are known to play a central role in various processes during activity-dormancy cycle. Analysis of histone and DNA modification genes indicates that chromatin remodeling could be involved in coordinating global changes in gene expression during activity-dormancy cycle.

Key words: Cell cycle, dormancy, hormone, microarray, poplar.

Perennial plants of boreal forests display cycles of active growth and dormancy. In order to survive the low winter temperatures, these plants need to arrest growth prior to the approach of winter and acquire tolerance to low temperatures (Rohde and Bhalerao 2007), using the associated reduction in day length as a signal to anticipate the approach of winter (Nitsch 1957). Once the day length falls below the permissive length for growth (referred to as the critical day length) cell division is arrested in apical and cambial meristems. Initially, this growth arrest is reversible upon transfer to long days, but continued exposure of plants that have undergone growth cessation to short days leads to the establishment of dormancy (Espinosa-Ruiz et al. 2004) and once dormancy is established, growth cannot be reinitiated simply by exposing plants to long days. Instead, exposure to chilling temperatures is essential for breaking dormancy, following which warm temperatures can reinitiate growth.

Exposure to short days not only leads to arrest of cell division in apical meristems but also initiates a range of developmental changes in the apex. These changes include termination of the initiation of leaf primordia and formation of bud scales enclosing the arrested leaf primordia in bud structures (Rohde et al. 2002). Simultaneously, plasmodesmatal connections are blocked, leading to symplastic isolation of the apical meristems (Rinne et al. 2001). Metabolism also shifts, to accumulation of storage compounds, and cold hardiness is increased (Weiser 1970; Ruttink et al. 2007). Subsequent reactivation of growth is accompanied by the swelling of buds, elongation of preformed internodes and outgrowth of leaf primordia. Many of the metabolic changes that occur during bud formation are reversed and storage compounds are utilized as sources of energy and substrates for growth and development (Druart et al. 2007).

In recent years considerable progress has been made in identifying components of the short day signaling pathway involved in growth arrest. It has been shown

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that photoreceptor phytochromes and the flowering time genes *FT* and *CO* are early acting components of the short day signaling pathway involved in growth cessation in hybrid aspen (Olsen et al. 1997; Bohlenius et al. 2006; Hsu et al. 2006). However, the downstream targets of this pathway remain largely unknown. In particular, our knowledge of the regulation of the transition to and release from dormancy at molecular levels remains rudimentary. Moreover the mechanisms underlying processes such as cold hardiness and the metabolic shifts that overlap growth cessation and dormancy, remain largely unexplored. Several lines of evidence suggest that short day signals could act via modulation of hormonal signaling. For example, it has been shown that exposure to short days leads to rapid downregulation of cellular GA levels (Olsen et al. 1997) and growth cessation in response to short days is delayed in hybrid aspen plants with enhanced levels of GAs (Eriksson et al. 2000). In addition, increases in ABA levels have been recorded following growth in short days, but these increases occur after growth cessation has been initiated (Rohde et al. 2002; Druart et al. 2007). While changes in hormonal levels may play a role in activity-dormancy transitions, it is also important to recognize the role of changes in hormone sensitivity in this process. For example, changes in sensitivity to indole-acetic acid (auxin) appear to play a key role in growth cessation and dormancy in the cambium (Little and Bonga 1974).

In recent years genomic approaches, *e.g.* microarray analyses and metabolic profiling, have been used to elucidate transcriptional and metabolic networks associated with various stages of activity-dormancy transitions in the model plant poplar (Schrader et al. 2004; Druart et al. 2007; Ruttink et al. 2007). However, these prior studies have not utilized the full genome microarrays, thus while they have been highly informative there are still major gaps in the knowledge they have provided. Moreover, there has been little molecular analysis of the release of bud dormancy. Consequently, the mechanism whereby exposure to chilling temperatures brings about release from dormancy is not well understood.

In contrast with previous studies (Schrader et al. 2004; Druart et al. 2007; Ruttink et al. 2007), here we report a full-genome microarray analysis of the activity-dormancy cycle in the apex of the model plant hybrid aspen. We have identified transcriptional changes that occur during the induction of growth cessation, transition to dormancy and subsequent release from dormancy and reactivation of growth in this plant. Our data identify several features of the transcriptional regulation of the activity-dormancy cycle that have not been previously reported, which should facilitate the selection of candidate genes for further analysis of this complex process at the molecular level.

Material and methods

Plant material and growth conditions

All experiments were performed using hybrid aspen (*Populus tremula* × *tremuloides*, clone T89). Tissue culture-grown plants were transferred to soil and grown for six weeks under long day conditions (18 h light/6 h dark), in the greenhouse at a temperature of circa 24°C. Plants were fertilized weekly during growth in the greenhouse. After six weeks, healthy plants were transferred to a climate chamber for short day (SD) treatment (eight hours light/16 h dark), in which the temperature was set to 20°C during the day and 15°C during the dark period. After five weeks of short day treatment, the temperature was reduced to 15°C during the day and 10°C during the night until the end of the experiment, at 11 weeks. To minimize the potential effects of variations in microclimate among different positions in the climate chamber, the positions of the plants were regularly changed. To induce release from dormancy, plants were subjected to low temperature (6°C) for four weeks in short days (C4). Following the low temperature treatment, plants were moved back to long day conditions in the greenhouse and signs of bud flush were checked every second day.

Samples from the plants' shoot apices (apices with leaves removed) and stems were collected after 0 (SD0), 3 (SD3), 5 (SD5) and 11 (SD11) weeks of short days and after 2 (C2) and 4 (C4) weeks of the low temperature treatment coupled with short days. Samples were also collected at the time (denoted "budbreak") of the first visible signs of budbreak (BB). All collected samples were immediately frozen in liquid nitrogen and stored at −80°C until further use. For microarrays, samples from timepoints SD0, SD5, SD11, C2, C4 and BB were used whereas for histone analysis stem samples were used.

Extraction of total histones and Western blotting

Total histones from stem pieces were extracted as described by Waterborg (1990). The protein concentration of the extracted histones was measured in a spectrophotometer using Bradford dye reagent from BIO-RAD. Approximately 10 µg of total histones were loaded on a 15% SDS-PAGE gel and blotted onto Immobilon-P PVDF membrane from Millipore (www.millipore.com). Multi-acetylated histone H3 was detected using rabbit anti-acetyl histone H3 antibody (www.millipore.com, catalogue no. 06-599) at 1 : 2500 dilution and horse-radish peroxidase-conjugated anti-rabbit IgG at 1 : 2500 dilution, then visualized using the ECL plus western blotting detection system and a Typhoon™ fluorescence scanner (www1.gelifesciences.com). Subsequently, antibodies were stripped from the membrane by incubation in stripping buffer (50 mM Tris pH 6.8, 50 mM DTT, 2% SDS) at 65°C for 30 min, then histone H3 was detected and visualized as described for acetylated histone H3, but using rabbit anti-histone H3 antibody (ab1791, Abcam, www.abcam.com) at 1 : 2500 dilution rather than the anti-acetyl histone H3 antibody. The amount of acetylated histone H3 present was calculated as the proportion of the total amount of histone H3 by dividing the value of the signal from the acetylated histone H3 by the signal from histone H3.

RNA extraction and double-stranded cDNA synthesis

RNA isolation was performed independently for each timepoint by pooling three apices. Tissue was ground in liquid nitrogen using a mortar and pestle. RNA was then extracted using an Aurum total RNA mini kit (Bio-Rad, www.bio-rad.com), concentrated by ethanol precipitation and 20 μg portions of total RNA were utilized for double-stranded cDNA synthesis, using a SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen, www.intvitrogen.com) according to the manufacturer's instructions. The resulting double-stranded cDNA, (400 ng at a concentration of 250 ng ml^{-1}) was checked for purity using a Nanodrop spectrophotometer and following this quality control step samples were sent to Nimblegen (www.nimblegen.com) for microarray hybridization.

Nimblegen oligoarrays

The *Populus trichocarpa* whole-genome expression oligoarray version 2.0 (www.nimblegen.com; Tuskan et al. 2006) consists of 65,965 probe sets corresponding to 55,970 gene models predicted in the *P. trichocarpa* genome sequence version 1.0 (Tuskan et al. 2006) and 9,995 aspen cDNA sequences (Sterky et al. 2004). Further details about the Populus version 2.0 oligoarray can be found as platform GPL2699 in the Gene Expression Omnibus (GEO) at NCBI (http://www.ncbi.nlm.nih.gov/geo) (Barrett et al. 2007). Hybridization procedures were applied and data were acquired at the NimbleGen facility (NimbleGen Systems, www.nimblegen.com). Robust Multichip Average (RMA) Normalization and data analysis were performed with in-house scripts using the R statistical package (http://www.r-project.org/). Custom packages for data analysis were built in R using the PlatformDesign and oligo tools. Sample comparisons were done with linear models implemented in the library limma (Smyth 2005). Gene annotations represent the top-scoring BLAST hits for each gene's predicted protein product as a query against the TAIR Arabidopsis protein database version 8 (http://www.arabidopsis.org/) (Swarbreck et al. 2008). A detected change in gene expression was considered significant if the adjusted p-value did not exceed 0.05 in a given comparison. For clustering and subsequent analyses only genes that showed at least a 2-fold change in a given comparison were considered (with the exception of ABA and GA metabolism in which 4-fold change was used as cut-off). Genes with at least a 2-fold change in transcription in selected time periods were clustered and analyzed separately unless stated otherwise Cluster analysis was performed using the K-means clustering (KMC) program with Euclidean distance of the MultiExperiment Viewer software package (http://www.tm4.org/mev.html).

Selection of genes

Poplar chromatin remodeling genes were identified using *Arabidopsis* chromatin remodeling genes described in chromDB (www.chromdb.org). A list of *Arabidopsis* genes involved in ABA and GA metabolism was obtained from the TAIR website (www.arabidopsis.org) and potential poplar orthologs of the *Arabidopsis* genes were obtained from the Nimblegen microarray data by searching the list for relevant ATg numbers. Gene models for the poplar *CBF* and *CBF-like* genes correspond to the classification by Zhuang (Zhuang et al. 2008). A list of poplar genes related to cold hardiness response was obtained as previously described (Druart et al. 2007).

Results and discussion

Analysis of the activity-dormancy cycle in hybrid aspen

To investigate the transcriptional networks underlying the physiological responses during distinct stages of the activity-dormancy cycle, we grew hybrid aspen clone T89 under short day conditions. In accordance with earlier observations (Esspinos-Ruis et al. 2004), growth ceased after five weeks and after six further weeks in short days dormancy was established as judged by the inability of the plants to grow after transfer to long days without exposure to chilling temperatures. Dormant plants were then exposed to four weeks of low temperature to induce release from dormancy. Once the low temperature-treated plants were transferred to the greenhouse and grown at room temperature (23°C ± 2°C) reactivation occurred within 2–3 weeks as judged by budbreak and the emergence and expansion of preformed leaves.

Distinct stages of the activity-dormancy cycle in the apex of hybrid aspen are associated with global changes in the transcriptome

We analyzed changes in gene expression during four key stages of the activity-dormancy cycle: induction of growth cessation (weeks 0–5 of SD treatment, designated SD 0–5), establishment of dormancy (weeks 5–11 of SD treatment, designated SD 5–11), release of dormancy (four weeks of cold treatment following 11 weeks of SD treatment, designated C4) and finally induction of budburst (two weeks of warm temperature treatment following the four weeks of cold treatment, designated BB). The induction of growth cessation after five weeks of short day treatment and the transition from growth cessation to dormancy between weeks 5 and 11 was associated with massive changes in gene expression (Table 1). Substantial changes in gene expression during the induction of growth cessation have been previously noted by (Ruttink et al. 2007), but the cited authors

Table 1. Numbers of transcripts found to be up-or down-regulated during growth cessation (0–5 weeks of short days), establishment of dormancy (5–11 weeks of short days), release from dormancy (11 weeks of short days and four weeks of cold treatment) and induction of budburst (two weeks of warm temperature followed by four weeks of cold treatment).

Period	Down	Up
SD 0–5	4181	4193
SD 5–11	3213	2126
SD11–C4	4792	3518
C4–BB	7515	7280

suggested that transition to dormancy in the bud may not depend significantly on transcriptional control since they detected changes in the expression of very few genes during this phase. However, our data clearly indicate that induction of dormancy involves considerable modulation of transcription. This raises questions regarding why this massive change in gene expression during dormancy establishment has not been noted in earlier studies. One reason could be that most of the cDNA microarray probes used in earlier investigations of activity-dormancy transitions in the poplar apex (Ruttink et al. 2007) originated from libraries constructed from tissues of actively growing plants (out of 19 cDNA libraries only two libraries were generated from dormant tissues). Moreover, cDNA libraries from dormant tissues typically have low complexity, being dominated by ESTs from a few genes whose transcripts are present at very high levels (Schrader et al. 2004). Consequently, ESTs for weakly expressed genes and corresponding probes are probably not well represented in the cDNA microarrays previously used. This may be why earlier experiments using cDNA arrays failed to detect the massive changes associated with dormancy establishment.

Low temperature signals inducing release from dormancy modulate global gene expression

How prolonged exposure to low temperatures leads to release from bud dormancy is not well understood and changes associated with release from dormancy have not been described (Rohde and Bhalerao 2007). We found much smaller changes in gene expression in dormant apices after exposure to low temperature for two weeks than after four weeks exposure (Table 1). In this respect it is worth noting that for the hybrid aspen clone used in this study four weeks of low temperature treatment is typically required for release from dormancy (Bhalerao RP, unpublished). This might be the underlying reason why four, but not two, weeks of low temperature treatment has a major impact on gene expression. The requirement for prolonged exposure to low temperature for dormancy release is similar to the requirements of some plants, e.g. winter annual ecotypes of *Arabidopsis* (Sung and Amasino 2005) for such exposure before flowering. This "vernalization" acts by downregulating the flowering repressor *FLC*, which requires the induction of *VIN3* (Bastow et al. 2004; Sung and Amasino 2004). Interestingly, Arabidopsis ecotypes vary in the timing of downregulation of *FLC* by low temperature (Shindo et al. 2006), suggesting that they may differ in "chilling requirements" as observed for dormancy release in buds. However, although release of bud dormancy by chilling shares certain similarities with vernalization, a limited set of orthologous genes appear to be involved in both the Arabidopsis and poplar responses to prolonged exposure to low temperature (Molnar, G et al. unpublished data), suggesting that targets of low temperature signals may differ at the global level between these two plant species.

Expression patterns of several cell cycle-related genes mirror activity and dormancy cycles in the apex

Short day signals are thought to induce cell cycle arrest in the cambial meristem by causing a reduction in the activity of cell cycle-dependent kinases (CDKs) (Espinosa-Ruiz et al. 2004), which could be due to a reduction in the level of cyclins and/or induction of CDK inhibitors. In addition, reductions in levels of transcripts encoding several cell cycle regulators have been observed in the apex during the transition to dormancy (Ruttink et al. 2007). However, previous microarray studies (Schrader et al. 2004; Druart et al. 2007; Ruttink et al. 2007) did not investigate the expression of the orthologs of several CDKs, cyclins and KRPs that have been described in *Arabidopsis* recently (Menges et al. 2005). Therefore, we identified poplar orthologs of *Arabidopsis* core cell cycle genes described by Menges et al. (2005) that were represented on the full genome array to obtain a more complete picture of the transcriptional regulation of cell cycle genes during the transition to dormancy and its subsequent release in the apex (supplementary Table 1 and Figure 1). During the induction of growth cessation (S0–S5) and dormancy (S5–S11) the expression of 36 genes was downregulated, but no genes whose transcript levels were upregulated during this phase were detected. Subsequent cold treatment (S11–C4) led to a further downregulation of the expression of 22 of these 36 genes and, again, there were no genes whose expression was upregulated. Induction of budburst was associated with a dramatic transcriptional switch, in which the expression of 42 genes was upregulated. However, it should be noted that only four genes were solely expressed in the reactivation stage. Thus, the expression patterns of all core cell cycle genes that displayed significant changes are consistent with expected changes during growth cessation and reactivation cycles.

Most CDKs are regulated at the post-transcriptional level during the activity-dormancy cycle

Espinosa-Ruiz et al (2004) have previously shown that *CDKA* is post-transcriptionally regulated during the transition to dormancy in the cambium, but our results indicate that nearly the entire *CDK* gene family, with the exception of the plant specific *CDKB*, is post-transcriptionally regulated during growth cessation and the transition to dormancy in the buds (Figure 1A). Although *CDKA* expression reportedly rarely varies in other experimental systems, e.g. *Arabidopsis* cell

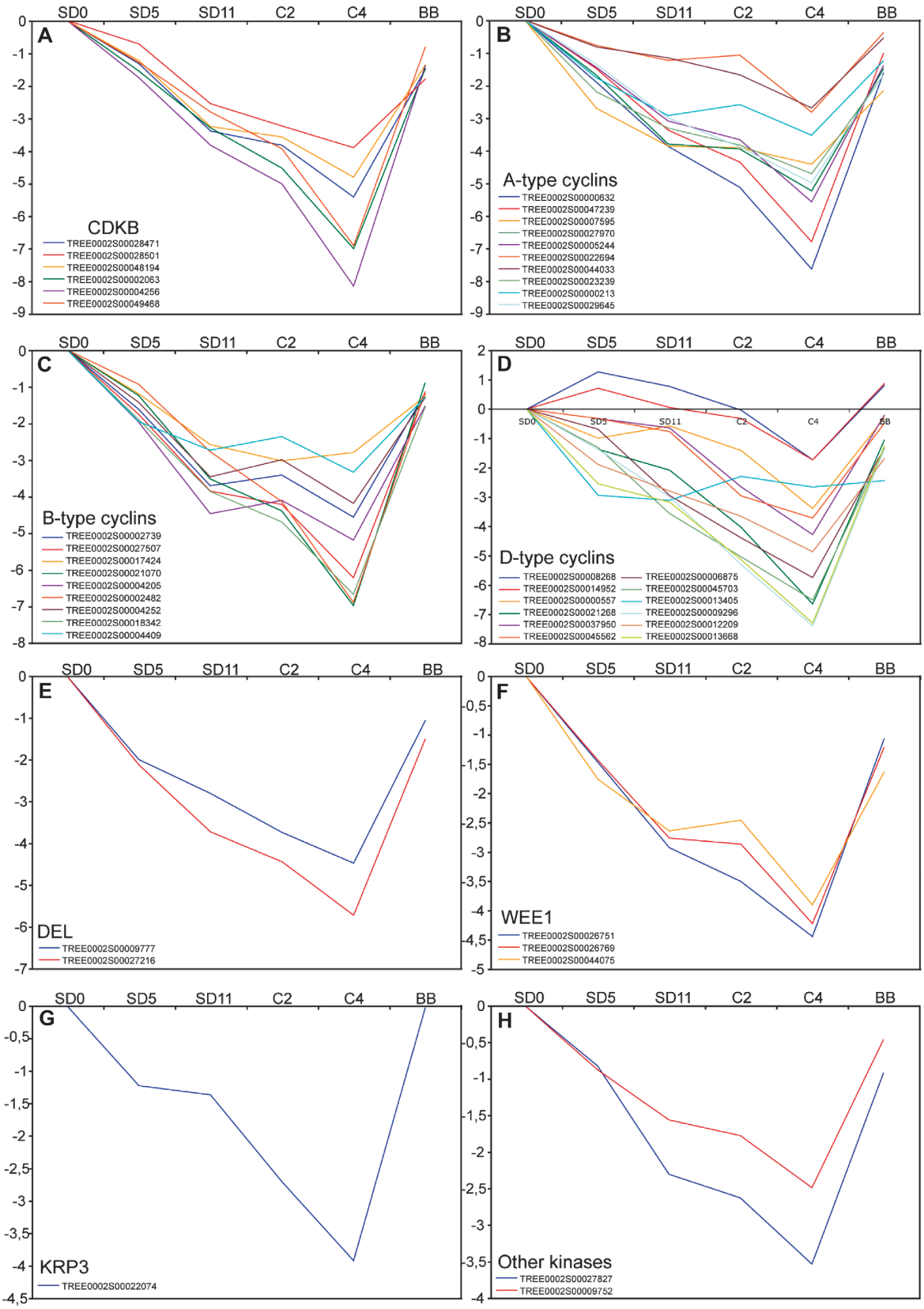

Figure 1. Expression pattern of cell cycle genes during activity-dormancy cycle. X-axis indicates exposure to short days in weeks (SDO, SD5 and SD11) followed by low temperature treatment for 2 (C2) and 4 (C4) weeks and BB-bud burst. Y-axis indicates change in transcript levels in $\log_2$ scale (see supplementary table 1 for details).

cultures (Hemerly et al. 1993), the lack of significant transcriptional control of other *CDK*s during the induction of dormancy conflicts with earlier observations in *Arabidopsis* (Menges et al. 2005). Currently we have no explanation for this difference in the regulation of *CDK*s during dormancy compared with other experimental systems. It is possible that dormancy is exceptional in this respect, and it remains to be seen whether these *CDK*s are also post-transcriptionally regulated during differentiation processes in organs such as roots and leaves when cell division ceases. Intriguingly, *WEE1* kinase expression was also downregulated during dormancy, despite being a negative regulator of CDKA activity (Parker et al. 1991) (Figure 1F). While our expectation was that the expression of this negative regulator of CDKs could be upregulated as growth cessation occurs, the contrary result might reflect the additional role of this kinase in the DNA integrity checkpoint (Michael and Newport, 1998). Since no replication occurs after cessation of cell division it may not be necessary to maintain high expression of this gene since there may be no need for quality control of newly synthesized DNA.

D-type cyclins display complex expression pattern during the activity-dormancy cycle

As expected, the expression of most of the A- and B-type cyclins was downregulated during growth cessation, in agreement with their roles as positive regulators of cell proliferation (Figure 1B, C). In contrast, considerable variation was observed in the expression of D-type cyclins. Cyclin D3; 1, D3; 2 and D6; 1 appeared to be under strong transcriptional control, being downregulated like the A- and B-type cyclins (Figure 1D). Surprisingly, transcript levels of Cyclin D1; 1 genes slightly, but significantly, increased during the first phase of short day treatment (SD0–SD5) and were not downregulated during growth cessation or induction of dormancy. Interestingly, of the three Cyclin D3; 1 genes, only one was downregulated during growth cessation and induction of dormancy, while two were downregulated only after cold treatment (Figure 1D). The expression of the downregulated Cyclin D3 genes was subsequently reversed during the budburst. Of the D-type cyclins, Cyclin D5; 1 showed a unique pattern, being downregulated during growth cessation and dormancy, but not responding to cold treatment or being up-regulated during budburst (Figure 1D). The divergent expression patterns of D-type cyclins could indicate that this class of cyclins plays a more complex role in the regulation of growth cessation-related processes than simply acting as on-off switches that regulate the activity of CDKs.

Low temperature effects on the expression of cell cycle genes during activity-dormancy cycle

It has been unclear how a low temperature signal that induces release from dormancy simultaneously prevents reactivation. In this regard, it is worth noting that for several of the cell cycle genes described above, cold treatment led to further downregulation of expression (Supplementary Table 1, Figure 1). As outlined above, our data indicate that cold treatment further downregulates the expression of several cell cycle regulators. Based on this observation we suggest that although prolonged exposure to low temperature induces release from dormancy, it simultaneously prevents precocious reactivation of cell division by further downregulating the expression of cell cycle genes after dormancy has been established, thereby fulfilling two superficially paradoxical functions, namely release from dormancy and inhibition of reactivation. Interestingly, no cell cycle-related genes showed increased expression following cold treatment (Supplementary Table 1). Therefore, the release from dormancy may not involve simple transcriptional induction of core cell cycle regulators. Previous findings of increases in the transcript levels of cell cycle genes after the initial phase of cambial reactivation support this conclusion (Druart et al. 2007). Thus, the mechanism controlling the ability to restart growth after prolonged exposure to low temperature must act at the post-transcriptional level, at least with respect to cell cycle genes.

Transcriptional regulation of cold hardiness induction

Perennial plants, *e.g.* trees in boreal forests, initiate the acquisition of cold hardiness prior to the onset of winter (Weiser 1970). Earlier have suggested that the first stage of cold hardiness induction is controlled by short day signals, while the second stage is induced by day length signals acting in concert with low temperature and the final stage is dependent on low temperature signals alone observations (Weiser 1970; Welling et al. 2002). We have previously shown that these three stages of cold hardiness are reflected in three distinct expression patterns of cold hardiness-related genes in the cambium during the transition to dormancy (Druart et al. 2007). In agreement with those findings, we found here that the expression of several cold hardiness-related genes e.g. *CORs*, *DEHYDRINS* and *LTPs* was upregulated (supplementary Table 2) after five weeks of SD treatment. However, there are other genes whose expression was induced between five and 11 weeks of short days. Since the temperature was not lowered to 4 degrees throughout the 11 weeks of SD treatment, the upregulation of cold hardiness-related genes during this phase most likely represents a response to short day signals. These findings suggest that cold-hardiness-

related genes could be further divided into a set that are induced relatively early, e.g. after five weeks of SD treatment, and a set that are induced later.

We further investigated whether CBF transcription factors that are responsive to cold in birch and hybrid aspen (Benedict et al. 2006; Welling and Palva 2008) and have been shown to regulate the expression of several cold hardiness-related genes in a variety of plants, including poplar (Fowler et al. 2001; Benedict et al. 2006) are involved in SD regulated induction of cold hardiness. In poplar, the *CBF* family is represented by six genes, four of which are highly similar to Arabidopsis *CBF*s (Benedict et al. 2006). None of the closest orthologs of *CBFs* (*CBF1–4*) were significantly upregulated during the first five weeks of short days, but the expression of one of the *CBF*-related genes analyzed, *CBFL2,* was transiently upregulated and subsequently downregulated to almost the same level as before the SD treatment (Supplemental Table 3; Figure 2). In contrast with other *CBF* genes, *CBFL1* was downregulated after short day treatment, and whether this gene is a negative regulator of cold hardiness induction and is therefore downregulated remains to be seen. In this respect it is worth noting that *Arabidopsis CBF2* has been shown to be a negative regulator of low temperature responsive genes (Novillo et al. 2004) and it is possible that *CBFL1* performs a similar function in hybrid aspen. Our results regarding the lack of significant up-regulation of *CBFs* during SD treatment are perhaps not surprising given that these genes have been primarily shown to respond to low temperature, and during our SD treatment the temperature was not reduced. Thus, it is likely that *CBFs1–4* are involved in low temperature-mediated induction of later stages of cold hardiness. Although downregulation of *CBFL1* and induction of *CBFL2* could mediate the SD induction of the first stage of cold hardiness, this does not exclude the possibility that several other transcription factors could also be involved in this process; possible candidates include the newly discovered *CAMTA* transcription factors (Doherty et al. 2009) and other transcription factors, e.g. *ZAT12* (Vogel et al. 2005) and *PttDRTY* (Druart et al. 2007), described in *Arabidopsis* and hybrid aspen. However, analysis of these transcription factors did not reveal significant induction during SD treatment (data not shown), suggesting that either post-transcriptional regulation of key transcription factors is involved in SD-regulated cold hardiness induction or that this process involves largely different transcription factors from those described so far in *Arabidopsis*.

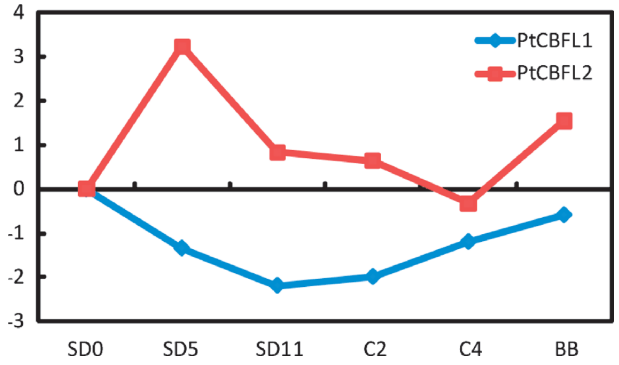

Figure 2. Expression profiles of *CBF* and *CBF*-like genes during growth cessation and dormancy induction (0–11 weeks SD) and dormancy release (11 weeks SD-BB). Y-axis indicates change in transcript levels in $\log_2$ scale (see supplementary Table 3 for details).

Another aspect of the SD regulation of cold hardiness induction in trees that has not been previously investigated is the role of chromatin remodeling, which appears to be involved in the regulation of cold responsive genes in *Arabidopsis* since (*inter alia*) the induction of cold responsive genes is reduced in *Arabidopsis* GCN5-like histone acetyl transferase mutants (Grant et al. 1997; Vlachonasios et al. 2003). In this respect it should be noted that a gene encoding an ortholog of *GCN5*-like histone acetyltransferase was upregulated in the apex following short day treatment in our experiments (supplementary Table 7). Moreover, mutations in *FVE* (a gene which is thought to be part of the chromatin remodeling machinery) lead to increased expression of *COR* genes in Arabidopsis (Kim et al. 2004). Interestingly, we found that one of the poplar orthologs of Arabidopsis *FVE* (supplementary Table 3) was downregulated during our SD treatment, thus SD-induced reduction in *FVE* expression could also contribute to the induction of cold hardiness in trees.

Transcriptional regulation of ABA metabolism related genes during activity-dormancy cycle

ABA measurements suggest a role for this hormone in activity-dormancy transition in trees since it is known that ABA levels increase during SD treatment in the apex (Rohde et al. 2002) and in the cambium in poplar plants (Druart et al. 2007). Moreover, ABA is also thought to be involved in the process of bud development (Rohde et al. 2002) since modulating the expression of the poplar ortholog of *ABI3*, a transcription factor acting in ABA signaling in *Arabidopsis* (Rohde et al. 2002), leads to an abnormal bud structure. However, the source of ABA in the apex during dormancy induction and bud formation is elusive. Therefore we analyzed the regulation of genes encoding enzymes involved in ABA metabolism (supplementary Table 4, Figure 3) during the distinct stages of the activity-dormancy cycle. Our data (Supplementary Table 4, Figure 3A) indicate that several of the ABA biosynthesis-related genes such as *NCED6a*, *NCED6b* and *NCED3*, catalyzing the first step of ABA production (Schwartz et al. 1997; Iuchi et al. 2001), and the alcohol dehydrogenase *ABA2a*, catalyzing the conversion of xanthoxin to abscisic aldehyde (Gonzalez-

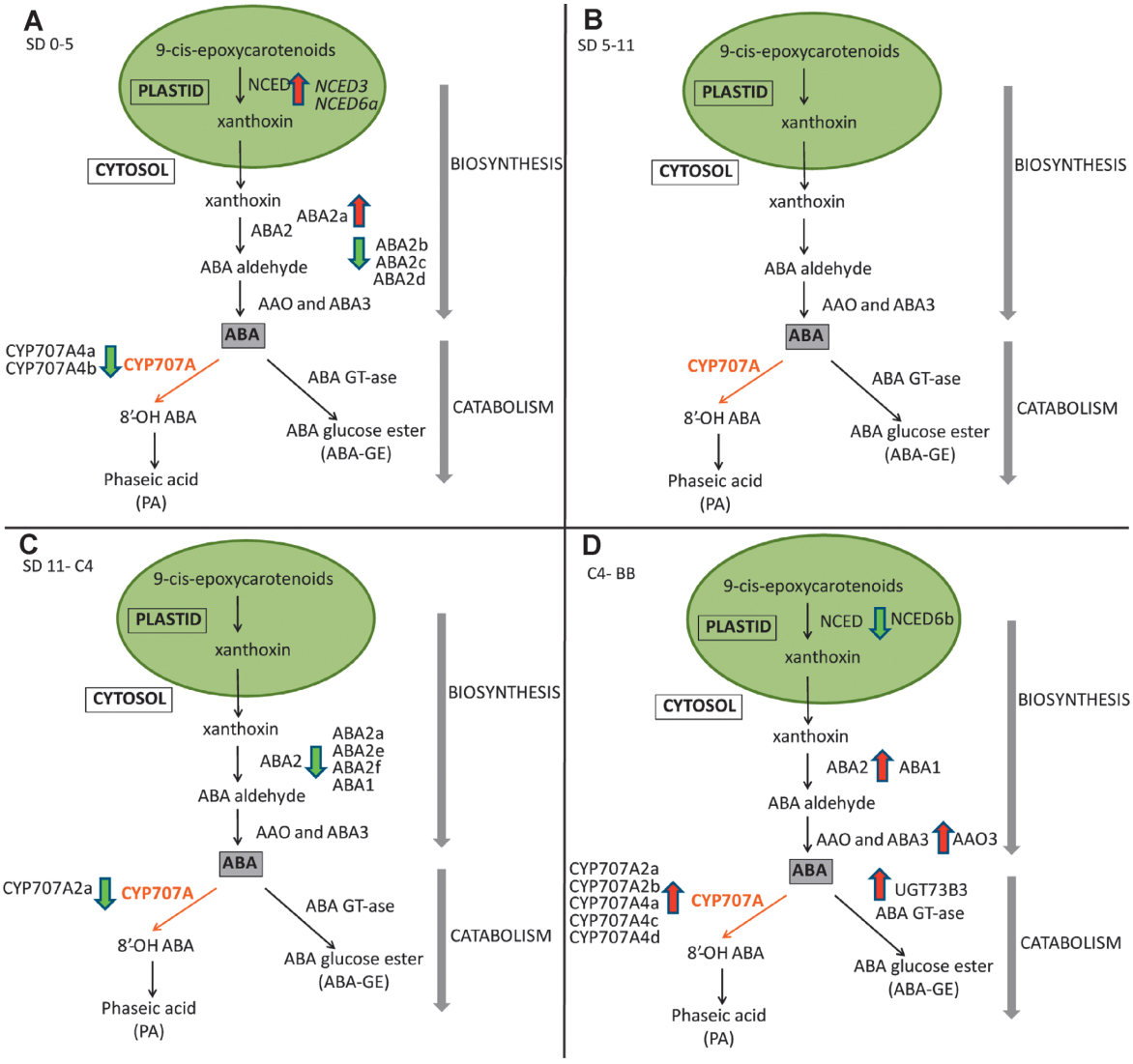

Figure 3. Transcriptional regulation of ABA metabolism-related genes during the active-dormancy cycle. (A) 0–5 weeks SD. (B) 5–11 weeks SD. (C) 11 weeks SD-4 weeks cold treatment. (D) 4 weeks cold treatment-BB. Downward arrows indicate downregulation and upward arrows indicate upregulation of transcripts. Gray arrows mark synthesis and catabolism steps.

Guzman et al. 2002), are upregulated after five weeks of short day treatment. In contrast, genes responsible for ABA inactivation, such as *CYP707A* (Kushiro et al. 2004; Saito et al. 2004), which catalyses the inactivation of ABA by hydroxylation, are downregulated at the same time. Thus, SD-induced increases in ABA levels could involve induction of genes involved in the biosynthesis of the hormone and simultaneous down-regulation of those involved in its breakdown in the apex. While we cannot definitively exclude the possibility that transport of ABA from leaves to the apex is involved in the regulation of growth cessation and dormancy, our gene expression data suggest that the increase in ABA levels in the apex after SD treatment may occur independently of ABA biosynthesis in the leaves.

We also investigated the transcriptional regulation of ABA metabolism-related genes during release from dormancy and budburst (supplementary Table 4, Figure 3C, D). Interestingly, release from dormancy promoted by prolonged exposure to low temperature was associated with the downregulation of the expression of *ABA2a*, *ABA2e*, *ABA2f* and *ABA1*, all of which encode enzymes that catalyze intermediate steps of ABA biosynthesis. Further, as budburst is initiated *CYP707A* appears to be upregulated whereas the expression of *NCED6a* and *NCED6b* (which encode enzymes catalyzing the first step of ABA biosynthesis) is downregulated. Before budburst the genes encoding enzymes catalyzing intermediate steps of ABA production such as *ABA1*, *ABA2a*, *ABA2e* and *AOO3* are upregulated. However, it is unlikely that ABA levels increase at this stage since the expression of key biosynthetic genes, e.g. *NCED6*, is downregulated whereas the expression of genes involved in its

inactivation, e.g. *CYP707A* and *UGT73B3* (which compartmentalizes ABA in the vacuole) (Xu et al. 2002), is upregulated simultaneously. Thus, cold treatment could potentially promote release from dormancy and budburst by reducing the levels of ABA in a similar manner to that shown for seed dormancy and germination potential (Okamoto et al. 2006; Seo et al. 2006).

ABA receptor components are induced in the apex after short day treatment

The ABA receptor encoded by the RCAR gene family has been recently described in the model plant *Arabidopsis* (Ma et al. 2009) where it has been shown that the binding of the RCAR-ABA complex to ABI protein phosphatase PP2C leads to the activation of ABA signaling in Arabidopsis. It is possible that ABA regulation of growth cessation and dormancy could involve not just increases in levels but also a change in sensitivity to the hormone. Therefore, we investigated the expression of the poplar orthologs of *ABI1*/*ABI2* protein phosphatase and *RCAR1*/*RCAR2* genes. The expression of *ABI* protein phosphatase as well as a subset of *RCARs* was upregulated after SD treatment (supplementary Table 5, Figure 4) and one of the upregulated genes was downregulated as budburst was initiated. The induction of *RCARs* coincided with the transition to dormancy between 5–11 weeks of SD treatment. This, together with earlier results showing upregulation of ABA post-growth cessation (Druart et al. 2007; Ruttink et al. 2007), strongly indicates that SD signals could potentially enhance the sensitivity of cells to ABA after SD treatment. Thus, our data suggest that ABA signaling could play a key role in the induction of growth cessation and dormancy as well as related processes, e.g. the induction of cold hardiness, via increases in ABA levels and/or enhancement of sensitivity to ABA.

Simultaneous downregulation of GA20 oxidase and induction of GA catabolism-related genes underlies the reduction of GA levels after SD treatment

Reduction of GA levels induced by exposure to short days has been postulated to play an important role in triggering growth cessation in the apex (Junttila and Jensen 1988; Olsen et al. 1997), and it has been shown that an increase in GA levels in hybrid aspen leads to a corresponding delay in growth cessation (Eriksson and Moritz 2002). Surprisingly, our data (supplementary Table 6, Figure 5) indicate that transcripts of *GA1a*, *KAO1* as well as *GA5a* and *GA5b,* which are involved in the early steps of GA biosynthesis (Xu et al. 1995), are strongly upregulated after five weeks of SD treatment, whereas the expression of *GA20OX2a*, which plays a key role in the production of active GAs in poplar (Eriksson and Moritz 2002), is downregulated during this phase. Simultaneously, expression of the genes involved in inactivation of active GAs through 8-hydroxylation, such as *GA2OX8a*, *GA2OX6*, *GA2OX2* and *GA2OX4* (Hedden and Proebsting 1999), also appears to be significantly upregulated. Subsequent short day treatment between 5 and 11 weeks led to a further downregulation of the expression of many of the genes involved in biosynthesis of GAs as dormancy was established. These findings, together with measurements of GA levels in hybrid aspen after short day treatment (Olsen et al. 1997), show *GA20OX2a* and the hydroxylation-related genes *GA2OX8a*, *GA2OX6*, *GA2OX2* and *GA2OX4* to be the key targets of SD signals in downregulating the levels of active GAs (Figure 5A).

Reactivation and budburst are accompanied by induction of GA biosynthetic genes

We also investigated the regulation of GA metabolism related genes during the release from dormancy and budburst. Our data indicate that exposure to low temperature leads to upregulation of *GA20OX2c* and

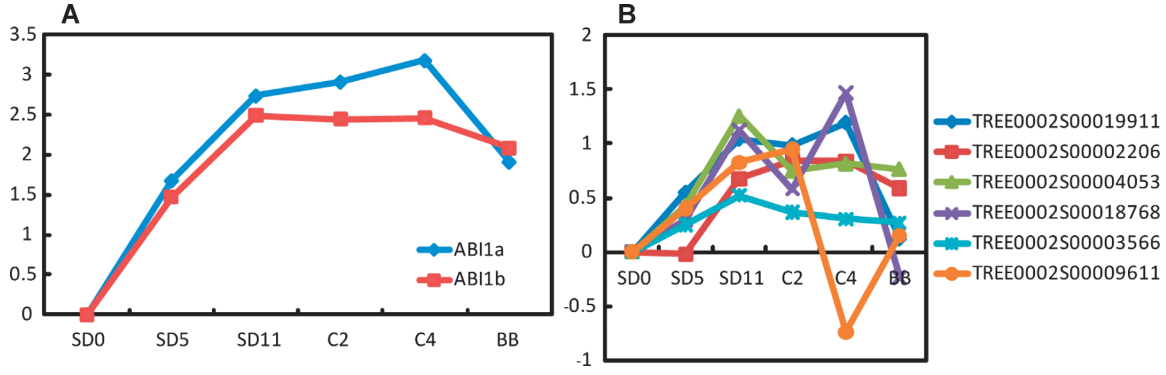

Figure 4. Expression of poplar *ABI* (A) and *RCAR* (B) genes during the activity-dormancy cycle. X-axis indicates exposure to short days in weeks (SDO, SD5 and SD11) followed by low temperature treatment for 2 (C2) and 4 (C4) weeks and BB-bud burst. Y-axis indicates change in transcript levels in $\log_2$ scale. For *RCAR* genes, Nimblegen identifiers are shown (see supplementary Table 5 for details).

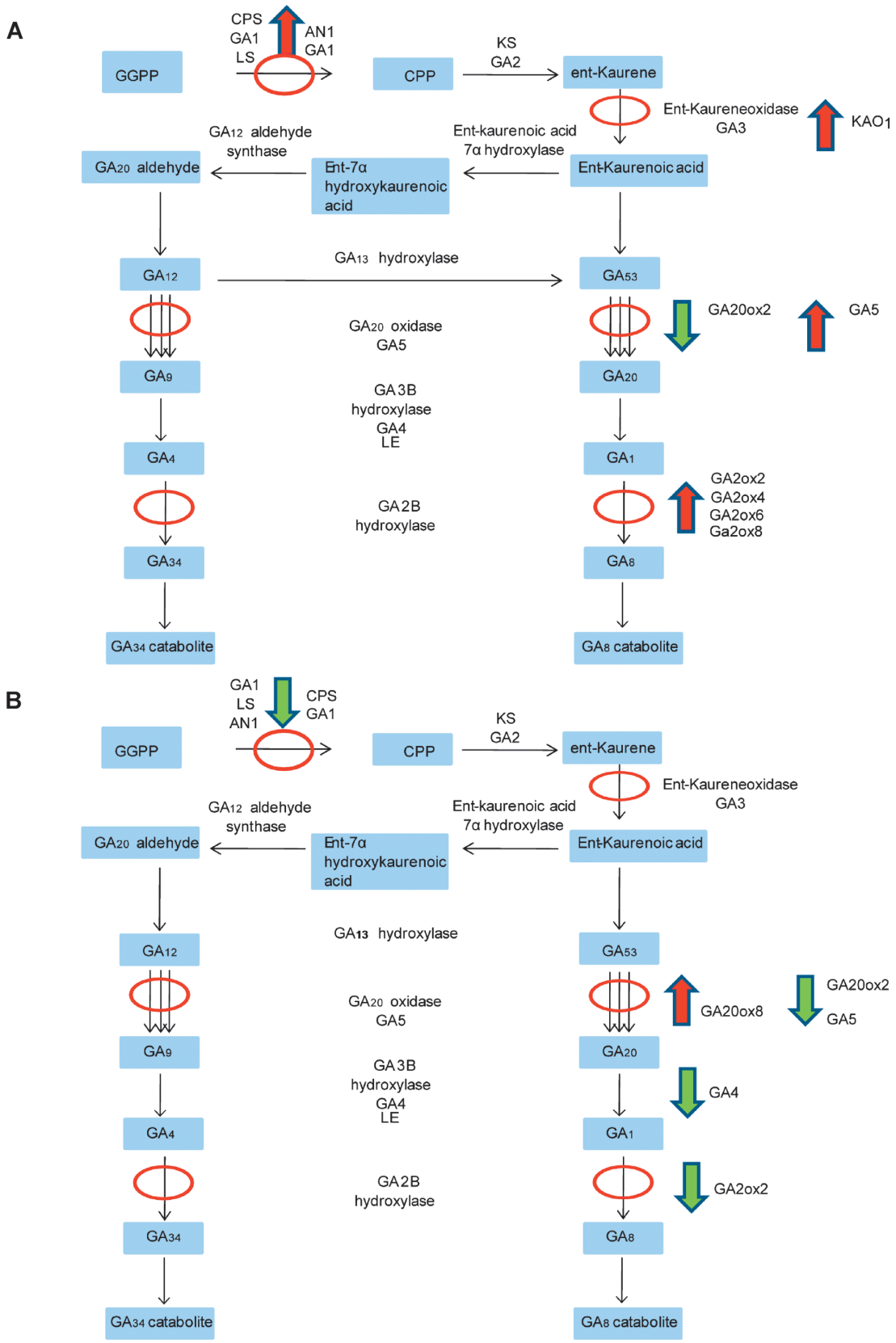

Figure 5. Transcriptional regulation of GA metabolism during the activity-dormancy cycle. (A) 0–5 weeks SD. (B) 5–11 weeks SD. Upward and downward arrows indicate up- and down-regulation of transcripts.

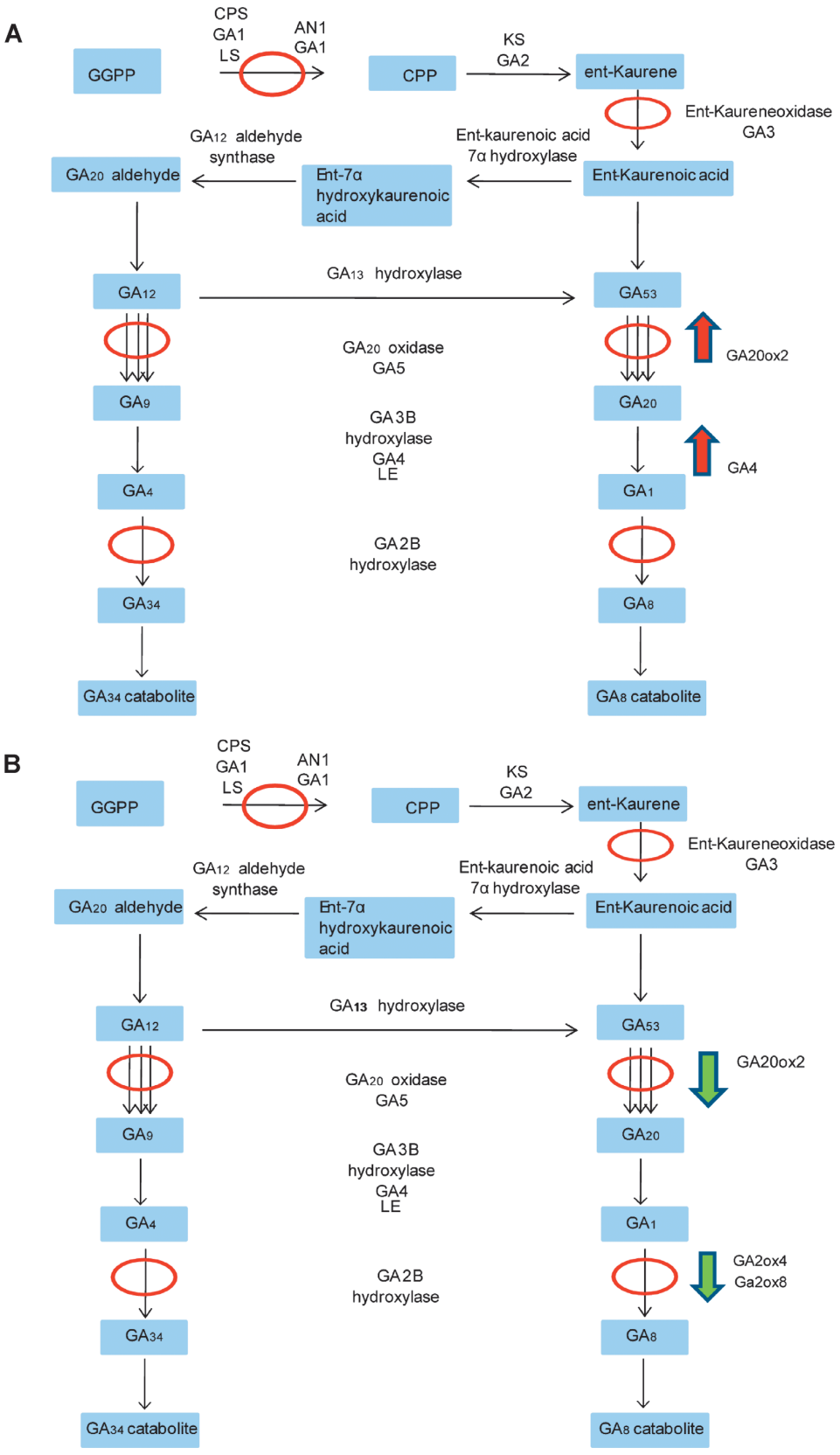

Figure 6. Transcriptional regulation of GA metabolism during dormancy release. (A) 11 weeks SD-4 weeks cold treatment. (B) 4 weeks cold treatment-BB (bud burst). Upward and downward arrows indicate up- and down-regulation of transcripts.

GA20OX2d, and *GA4b*, while there is a simultaneous downregulation of several catabolic genes such as *GA2OX8a*, *GA2OX8b*, *GA2OX2, GA2OX4* and *GA2OX6* either after cold treatment or as budburst is initiated (supplementary Table 6, Figure 6). Thus, cold treatment could potentially promote the production of active GAs, thereby contributing to budburst. Interestingly, these observations are similar to previous observations of transient cold-induced increases in GA levels during the reactivation of the poplar cambium and seed germination in *Arabidopsis* (Yamauchi et al. 2004; Druart et al. 2007). However, in contrast to seed germination, where a key role has been ascribed to gibberellin-3-oxidase genes (Yamauchi et al. 2004; Mitchum et al. 2006), we did not detect any change in the expression of the corresponding genes in hybrid aspen during dormancy release.

Taken together, our gene expression analyses indicate that transcriptional regulation could play a key role in the regulation of GA and ABA levels during distinct stages of the activity-dormancy cycle. Moreover, the gene expression data presented here, as well as hormonal measurements from earlier studies, indicate that GA and ABA metabolism display opposite responses to short days, similar to the opposite regulatory patterns of these hormonal pathways in seeds, in which GA and ABA play mutually antagonistic roles (Penfield and King 2009).

Global regulation of gene expression by chromatin remodeling during activity-dormancy cycle

Distinct stages of the activity-dormancy cycle involve highly coordinated regulation of transcriptional programs underlying the different physiological responses (Druart et al. 2007; Ruttink et al. 2007). Our knowledge of the mechanisms whereby these massive changes in gene expression are coordinated at the genomic level remains rudimentary. However, global regulation of gene expression often involves modulation of chromatin structure via modifications of histones and/or modification of DNA through methylation (Kouzarides 2007). Therefore, we investigated the regulation of genes encoding components of the chromatin remodeling machinery and DNA methylation during the distinct stages of the activity-dormancy cycle (Supplementary Table 7)

Interestingly, the expression of two of the putative histone deacetylases (*HDA14* and *HDA08*), a gene encoding a histone lysine methyltransferase (*SUVR3*) and a gene involved in histone ubiquitination (*HUB2*) was upregulated after five weeks of SD treatment as transition to dormancy was induced (Figure 7A). Simultaneously, the expression of several genes of the trithorax family that counteract the repression induced by the polycomb repression complex (Ringrose and Paro 2004) was downregulated during growth cessation and induction of dormancy (Figure 7B). Another contributor to gene repression during the activity-dormancy transition could be the downregulation of poplar orthologs of the Arabidopsis *DEMETER* gene (Figure 7B). *DEMETER* encodes DNA glycosylase (Gehring et al. 2006) and its downregulation could potentially lead to a further increase in DNA methylation and hence to chromatin compaction and gene repression following SD treatment. Although changes in DNA methylation during dormancy have not been reported in perennial trees to date, these results indicate that further investigation of this regulatory mechanism in growth cessation and dormancy regulation is warranted. Based on these findings we propose the hypothesis outlined in Figure 8 for repression of gene expression during growth cessation and induction of dormancy. According to this model, compaction of chromatin due to a combination of histone deacetylation, DNA methylation and histone ubiquitination, with simultaneous downregulation of trithorax genes, leads to repression of target genes, as has been shown to occur in well-studied systems such as *Drosophila* (Kouzarides 2007). While the role of *HDA*-related genes in regulation of dormancy is not well established, *hub2* mutants in Arabidopsis display reduced seed dormancy (Liu et al. 2007), suggesting a conserved mechanism in the regulation of embryonic dormancy in annual plants and post-embryonic bud dormancy in

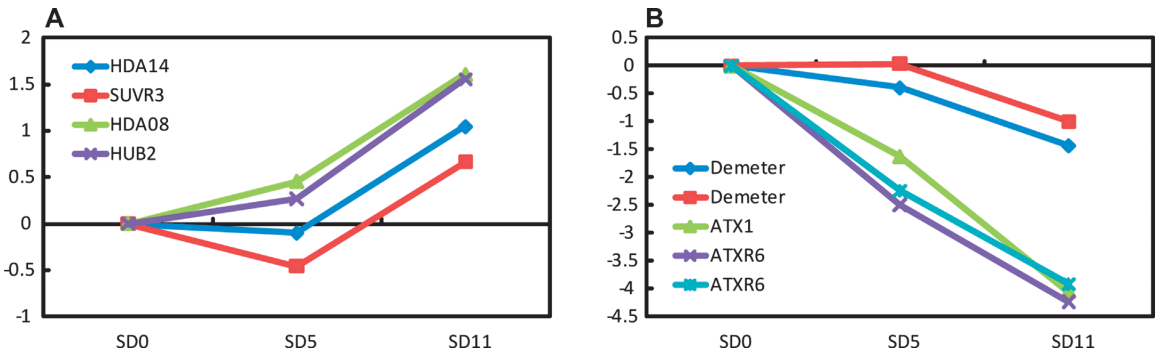

Figure 7. Expression patterns of selected chromatin remodeling and DNA modifying factors during growth cessation and dormancy induction. X-axis indicates time in short days in weeks and Y-axis indicates change in transcript levels in $\log_2$ scale.

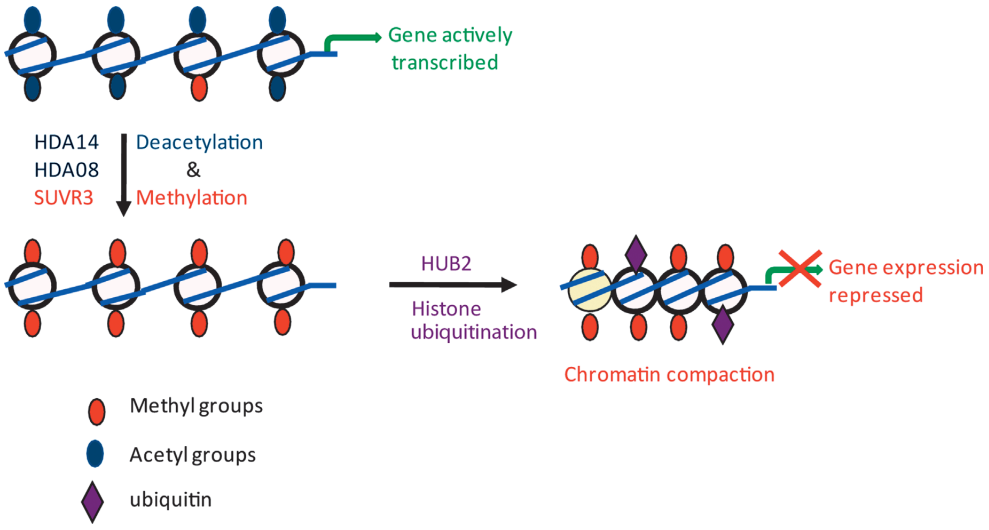

Figure 8. Proposed model for repression of gene expression via chromatin remodeling and DNA methylation during the activity-dormancy transition.

perennial plants.

The activity-dormancy cycle does not involve global changes in histone H3 acetylation

Although modification of histones has been linked to global changes in gene expression, histone modifications have not been analyzed in perennial trees, especially in the context of dormancy. To date, the only data on histone modification during post-embryonic dormancy have come from the analysis of potato tubers (Law and Suttle 2004), in which alterations in histone acetylation patterns (including increases in levels of acetylated histone H3 and H4) during release from endodormancy have been observed (Law and Suttle 2004). However, it is not clear from these results whether this change in histone acetylation is essential for release from dormancy or reflects a general increase in transcription as activation occurs. Nevertheless, such changes in histone acetylation could potentially underlie changes in gene expression that accompany distinct stages of the activity-dormancy cycle since they could allow global coordination of gene expression changes. Moreover, our microarray data indicate that changes in the expression of genes encoding histone modification enzymes occur. Therefore, we firstly analyzed whether histone acetylation was altered globally during the activity-dormancy cycle, focusing on acetylation of histone H3, since increases in the acetylation of this histone are typically associated with the activation of gene expression, while deacetylation is associated with repression (Kouzarides 2007). In contrast with the data presented for potato tubers, our results do not suggest that the levels of acetylated histone H3 changed during the activity-dormancy cycle in hybrid aspen (Figure 9). These findings suggest either that acetylation of other

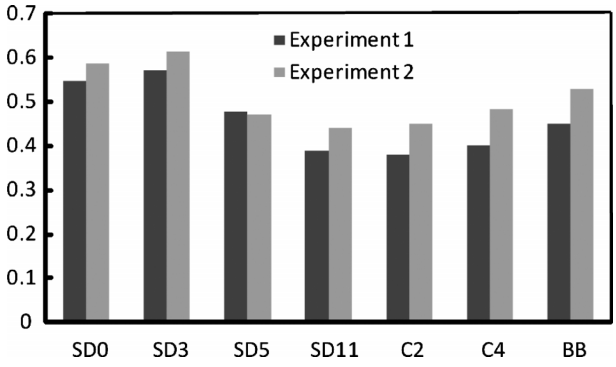

Figure 9. Relative acetylation levels of histone H3 during activity-dormancy cycle. Y-axis indicates ratio of acetylated histone H3 to total histone H3. Results from 2 independent experiments are shown.

histones could be more important during the activity-dormancy cycle in perennial trees or that histone acetylation in DNA regions containing genes that are repressed could be reduced but, simultaneously, histone acetylation in regions containing genes that are activated during the same period might increase and thus there may be little global change in histone acetylation. Such balancing of histone H3 acetylation at some loci with deacetylation at others is supported by the observation that a simultaneous induction of genes encoding histone acetylases (e.g. *GCN5*) and histone deacetylases (e.g. *HDA14*) expression occurs during growth cessation and induction of dormancy. Indeed, this result is not surprising given our data showing that although growth ceases and dormancy is induced, general transcription levels probably do not change under the experimental conditions used here. For example, repression of cell cycle-related genes overlaps with the induction of cold hardiness-related genes during the transition to dormancy. Importantly, in view of these findings, the

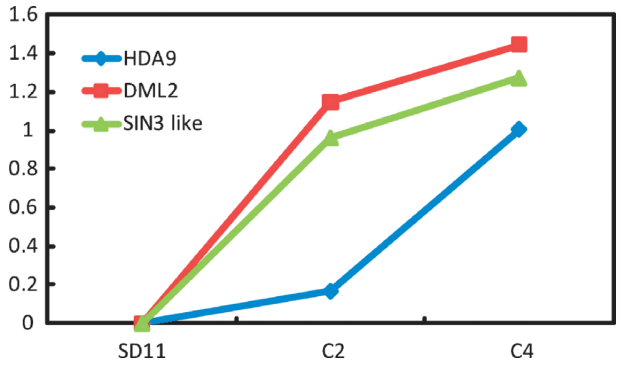

Figure 10. Expression of the chromatin remodeling genes HDA9, DML2 and SIN3 during dormancy release, after SD treatment for 11 weeks (SD11), and after 2 and 4 weeks of cold treatment (C2 and C4, respectively). Y-axis indicates expression change in $\log_2$ scale.

mechanism that targets chromatin remodeling proteins to the chromatin of specific loci that are either repressed or activated during the distinct stages of the activity-dormancy cycle needs to addressed in the future.

Role of chromatin remodeling in release from dormancy

We also investigated if the expression of chromatin remodeling components is altered during release from dormancy. As a first step, we investigated whether dormancy release simply involves reversal of the expression of chromatin remodeling genes induced during dormancy transition. However, with the exception of *EZA*/*SWINGER* (which is related to *Enhancer of Zeste* of *Drosophila*), most of the chromatin remodelling genes hypothesized to be involved in dormancy establishment and global modulation of gene expression during this process were not downregulated following exposure to low temperature. Thus, a simple low temperature-mediated downregulation of chromatin remodeling genes promoting dormancy may not be the primary mechanism of dormancy release. Another possibility is that low temperature signals could induce the expression of a completely novel set of genes that counter the action of dormancy-promoting genes, thereby inducing release from dormancy. We found that low temperature induces the expression of two different histone deacetylases, *HDA9* and *SIN3* (supplementary Table 7, Figure 10). While the targets of these histone deactylases are not known, they could serve to induce the expression of genes that counteract dormancy-promoting genes. In accordance with this hypothesis, cold temperature exposure led to the repression of a large number of genes, which could be mediated by these two histone deacetylases. It has been shown that *SIN3* interacts with *AGL15*, a transcription factor of the MADS box family in *Arabidopsis* (Hill et al. 2008), and this interaction could be involved in repression of *SIN3* targets. Although poplar *AGL15* is not induced by low temperature signals, this does not exclude the possibility of *SIN3* involvement in dormancy release via interaction with *AGL15* since it is plausible that *AGL15* or related MADS box genes simply serve to target *SIN3* to specific loci. Another gene whose expression is induced by low temperature and could be involved in dormancy release is the *DML* (Demeter related) (supplementary Table 7). This DNA glycosylase could serve to demethylate and activate genes contributing to the release from dormancy. For example, it has been shown in *Arabidopsis* that expression *DEMETER* in the central cell of the female gametophyte is essential for expression of imprinted genes such as *FWA* and *FIS2* (Kinoshita et al. 2004). Whether *DML* performs a similar function and counteracts the effect of polycomb repression complex 2-regulated genes and thus contributes to dormancy release in trees remains to be seen.

In conclusion our study reveals that transcriptional control could play a key role in regulation of the physiological responses that characterize the distinct phases of activity-dormancy cycle. We conclude that downregulation of the expression of cyclins by SD signal could a key mechanism in the induction of growth cessation. We show that environmental signals can modulate various aspects of activity-dormancy cycle by modulating the level of key hormones such ABA and GA by acting at the transcriptional level. Since hormones such ABA can also regulate several physiological responses that overlap dormancy transition temporally, it could allow for SD signal to coordinately regulate several physiological responses during activity-dormancy cycle via change in the level of hormones such ABA. Importantly, chromatin remodeling could be a key mechanism underlying the coordinated changes in global gene expression during activity-dormancy cycle. However the analysis of histone acetylation highlights the complexity of chromatin remodeling and suggests that there is probably a mechanism that would target the histone and DNA modification enzymes to distinct loci during the distinct stages of activity-dormancy cycle. Taken together our data reports several novel findings on how activity-dormancy cycle could be regulated at the molecular level.

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