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Citation	Yordanov, Y. S., Ma, C., Strauss, S. H., & Busov, V. B. (2014). EARLY BUD-BREAK 1 (EBB1) is a regulator of release from seasonal dormancy in poplar trees. Proceedings of the National Academy of Sciences, 111(27), 10001-10006. doi:10.1073/pnas.1405621111
DOI	10.1073/pnas.1405621111
Publisher	National Academy of Sciences of the United States of America
Version	Accepted Manuscript
Terms of Use	http://cdss.library.oregonstate.edu/sa-termsofuse

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EARLY BUD-BREAK 1 (_EBB1_) is a regulator of release from seasonal dormancy in poplar trees

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Running title: Regulation of bud-break

Keywords: Adaptation, meristem, _Populus_, phenology, AP2/ERF, dormancy, bud-break

Classification: _Biological Sciences_--Plant Biology

ABSTRACT

Trees from temperate latitudes transition between growth and dormancy to survive dehydration and freezing stress during winter months. We employed activation tagging to isolate a dominant mutation affecting release from dormancy, and identified the corresponding gene *EARLY BUD-BREAK 1* (*EBB1*). We demonstrate through positioning of the tag, expression analysis, and retransformation experiments that *EBB1* encodes a putative AP2/ERF transcription factor. Transgenic upregulation of the gene caused early bud-flush, while down-regulation delayed bud-break. Native *EBB1* expression was highest in actively-growing apices, undetectable during the dormancy period, but rapidly increased prior to bud-break. The *EBB1* transcript was localized in the L1/L2 layers of the shoot meristem and leaf primordia. *EBB1*-overexpressing transgenic plants displayed enlarged shoot meristems, open and poorly differentiated buds, and a higher rate of cell division in the apex. Transcriptome analyses of the *EBB1* transgenics identified 971 differentially-expressed genes whose expression correlated with the *EBB1* expression changes in the transgenic plants. Promoter analysis among the differentially expressed genes for presence of a canonical EBB1 binding site identified 65 putative target genes indicative of a broad regulatory context of EBB1 function. Our results suggest that EBB1 has a major and integrative role in reactivation of meristem activity after winter dormancy.

INTRODUCTION

Temporal modifications in plant growth and reproduction in conjunction with cyclical changes in climate are essential for adaptation to variable environments (35, 43). The annual alterations of growth and dormancy in forest trees from boreal and temperate regions in response to changing temperature and/or moisture regimes are very well-known examples of such cyclical changes. However, the molecular mechanisms governing these cycles remain poorly understood (49). Most cold injuries in trees occur due to frost damage as a result of either late spring frosts around the time of bud-break, or early fall frosts around the time of growth cessation (16). Spring and fall shoot phenology are polygenic traits and have very low genetic correlation (2), which suggests that a large number of independent genes control the onset and release from dormancy.

By definition dormancy is the absence of visible growth in any plant structure containing a meristem (34). The transition from active growth to dormancy is initiated in the fall by short day (SD) photoperiod causing initial cessation of shoot elongation (27, 31). This is followed by transformation of the apex into a bud (50). Bud formation is accompanied by acquisition of drought/freezing tolerance and poorly known physiological changes collectively known as endodormancy. Endodormancy results in the inability of the meristem and the youngest leaf primordia to respond to growth-promoting signals. Once endodormancy is established, growth is conditional upon meeting a chilling requirement (exposure for several months to low temperatures). Resumption of bud growth, known as bud-break, occurs after sufficient (variable species-specific duration) chilling and is controlled almost exclusively by high temperatures (28).

The timing of entry and release from dormancy are synchronized with local climates and are highly heritable (21, 28). Most research has focused on the early induction and establishment stages, mainly by seeking homologies to processes and types of dormancies characterized in annual plant species, or through correlative transcription profiling (49). For example, because light plays a major role in triggering growth cessation, photoreception via phytochromes (PHYs) has been viewed as an important control point in triggering the process (26, 30, 65). The integration of the light signal transduction into growth response is mediated via the FT/CO (FLOWERING TIME/CONSTANS) module (5, 29) and via regulatory proteins controlling circadian rhythms (1, 19, 45). One of the targets of FT/CO regulon is the *Populus*

Aintegumenta like 1 (AIL1), which controls meristem activity (32). In Arabidopsis, the ability of FT and other floral integrators to respond to inductive signals is controlled by a suite of MADS box genes like SHORT VEGETATIVE PHASE (SVP) and FLOWERING LOCUS C (FLC) (36). Similar MADS box genes, known as DORMANCY-ASSOCIATED MADS (DAM) genes (37) appear to be involved in regulation of bud dormancy of several woody perennial species (14). Involvement of ethylene and abscisic acid signaling in bud formation has been suggested based on overexpression of the dominant negative *ethylene triple response 1* (*etr1*) ethylene receptor and the ABSCISIC ACID INSENSITIVE 3 (ABI3) transcription factor (51, 53). Although both of these hormone modifications interfered with bud formation, neither was able to completely compromise endodormancy induction, suggesting that they do not exercise fundamental control over it. Modulation of auxin response was also found to be important for the transition to dormancy in poplar (3).

Even less is known about control of endodormancy and reinitiation of bud growth. Studies in *Picea*, *Vitis* and *Populus* have utilized transcription profiling to study gene expression during endodormancy and/or resumption of growth (38, 52, 63, 64). Homologies to vernalization have been invoked but critical differences exist as the vernalization-associated epigenetic mechanism requires sustained division while bud dormancy can be imposed and reset in the same meristem cells (49). Recently it has been shown that the plasmodesmata connections to the meristem are plugged during dormancy and need to be reopened before growth promoting signals like FT can reach to their targets tissues in the apex (48).

Expression of cell-cycle marker genes indicates that cambium meristem cells after endodormancy establishment are arrested in the G1-S transition and unable to respond to growth permissive conditions (20). Studies in *Arabidopsis* have identified many of the regulators of cell proliferation in SAM (9, 39) and expression of poplar homologs of these genes correlates with arrest of cell proliferation during dormancy (54). However functional characterization of these genes in relation to transitions between dormancy and active growth is absent and thus their role in regulation dormancy characteristics remains unclear.

Here we report the discovery and characterization of a gene that modifies the timing of bud-break phenology in a woody perennial plant. The gene encodes a putative AP2/ERF transcription factor that is involved in reactivation of cell division in the meristem and leaf primordia after winter dormancy.

RESULTS

Isolation of poplar early bud-break mutant

We isolated a mutant that showed accelerated bud-break under field conditions in a population of poplar activation tagging mutants (8). All four ramets (vegetative propagules) of the mutant, two pairs of which had been planted in two randomized locations in the ~one hectare field trial, flushed earlier than WT-717 (non-transgenic wild-type, *Populus tremula x Populus alba* INRA 717-IB4 genotype) and the large majority of other events that had been transformed with the same vector (Figure 1A). To validate these field observations we performed an experiment where we mimicked the induction and release from dormancy under growth chamber conditions (see Materials and methods). Similar to field observations the mutant event showed precocious bud-break approximately twice as fast as WT-717 plants (Figure 1B and C). Because of the effect on bud-break, we named the mutant *early bud-break 1 dominant* (*ebb1D*) and the corresponding gene *EARLY BUD-BREAK 1* (*EBB1*). Under field conditions leaves were similar in size to WT-717 and showed slight epinastic curvature (Figure S1B to D). Height growth was not affected ($P<0.05$) (Figure S1A), but diameter was slightly but significantly decreased ($P<0.05$) compared to WT-717 plants (Figure S1B).

Molecular characterization of *ebb1D*

To isolate the gene that conditions the mutant phenotype we first positioned the tag in the genome sequence. We used plasmid rescue to recover a 458 bp fragment of genomic DNA flanking the insertion site. BLASTn searches with the sequence of the fragment into the poplar genome sequence located the insertion on Chr08:12,804,945. Approximately 2.5 kb upstream of the insertion site we located a predicted gene model Potri.008G186300 (Figure 1D). No other gene was annotated within 10kb in either direction from the 4X 35S enhancers of the inserted T-DNA sequence. To verify that the proximal gene was activated, we designed primers based on the genome sequence and compared the expression of the candidate gene in WT-717 and mutant plants using RT-PCR. We used plant material from both greenhouse and field-grown plants with similar results for both sources. The gene corresponding to Potri.008G186300 was highly activated in *ebb1D* and undetectable in WT-717 plants (Figure 1E).

EBB1 corresponds to an AP2/ERF-domain protein

We amplified, cloned, and sequenced full length cDNA of the putative *EBB1* gene. Sequence analysis indicated that the gene encodes an AP2/ERF domain putative transcription factor corresponding to a gene in the *P. trichocarpa* genome annotated as *PoptERF61*. AP2/ERF is a superfamily (e.g., at least 147 members in *Arabidopsis*) of plant-specific transcription factors (41, 47). EBB1 is most similar to a small subfamily of seven members in Arabidopsis, including four that are functionally characterized in *Arabidopsis* (e.g., ENHANCED SHOOT REGENERATION (ESR1)/DORNROSCHËN (DRN), ESR2/DRN-like (DRNL), LEAFY PETIOLE (LEP), and PUCHI) (4, 24, 33, 61). The subfamily is comprised of nine members in poplar, none of which have been characterized to date (Figure 2C). We performed phylogenetic analysis using all 9 *P. trichocarpa* proteins identified in the genome sequence (Table S1), EBB1, all seven *Arabidopsis* proteins, and two monocot proteins FIZZLE PANNICLE (FZP) (rice)/BRACHLESS SILKLESS (BD1) (maize) that have been functionally studied (Figure 1F). EBB1, its *P. trichocarpa* close homolog PoptERF61, and its putative paralog PoptERF60, were clustered with very high bootstrap confidence in a common lineage along with ESR2/DRNL (Figure 1F).

Recapitulation of *ebb1D* phenotype

Because of the localization of the tag and the activation of the gene proximal to the insertion site, we hypothesized that PoptERF61 corresponded to the *EBB1* gene. Therefore we proceeded to recapitulate the early bud-break mutant phenotype. We fused the *EBB1* cDNA to the strong *CaM35S* promoter that is commonly used for overexpression in dicotyledonous plants, together with the OCS (octopine synthase) terminator, and transformed this gene construct into the same WT-717 poplar genetic background. We recovered 21 independent events, PCR-verified them for the presence of the transgene, and performed RT-PCR on a subset to verify overexpression (hereafter referred to as *EBB1-oe* transgenics). During *in vitro* regeneration, we observed an increase in rate of shoot regeneration (Figure S3). In addition, the *EBB1-oe* plants produced prolific shoot regeneration from callus tissues produced on the surface of cut stems (Figure S4). To recapitulate *EBB1'*s effect on bud-break we performed a controlled growth chamber experiment with 15 independent events. No effect of *EBB1* overexpression was observed on growth cessation and timing of bud-set, however, all *EBB1-oe* plants showed precocious early

bud-break (Figure 2 A-C). On average *EBB1-oe* plants flushed approximately twice as fast as the WT-717 controls (Figure 2B). More than half (75%) of all *EBB1-oe* plants flushed as early as the first week, and some even as early as 1 day, after transfer to LD and high temperature. Bud-break under field conditions was similarly precocious in all *EBB1-oe* transgenic events.

EBB1-oe plants were generally showed phenotypic abnormalities more severe than in the original mutant, likely a result of the stronger and ubiquitous expression expected from the 35S promoter (Figure S2). These included smaller epinastic leaves (Figure S2A) and an increased proliferation of sylleptic branches (Figure S2C).

Suppression of *EBB1* delays bud-break

Using artificial microRNA we successfully downregulated expression of *EBB1* in four independent lines (called *amiEBB1*) (Figure S6). None of the lines with suppressed expression of *EBB1* showed unusual phenotypes during *in vitro* development and the first year of greenhouse growth. We next studied the effect of *EBB1* suppression on bud phenology (see also Materials and Methods). No effect of *EBB1* down-regulation was observed on growth cessation, timing of bud-set, and bud formation; however, all *EBB1*-suppressed plants showed delayed bud-break (Figure 2D-F). In contrast to the overexpression events, all *amiEBB1* plants flushed significantly later than the WT-717 controls (Figure 2E and F).

Anatomical changes in EBB1 transgenics buds and apices

We inspected the cellular and tissue organization of the bud and actively growing apex in WT-717 and *EBB1-oe* transgenics (Figure 3). The poplar buds consisted of tightly folded and packed bud scales, stipules, and embryonic leaves (51). In all examined *EBB1-oe* transgenics (10 ramets from five different lines), bud scales and embryonic leaves appeared to be thicker at the base and less folded compared to WT-717 plants (Figure 3A, B, D, E). Thus the overall bud shape in *EBB1-oe* plants appeared more oval (Figure 3D) and the meristem was more open and exposed (Figure 3E). No changes in bud morphology were observed in the *EBB1*-suppressed plants.

We also studied the organization of non-dormant actively growing apices. The meristem dome was visibly enlarged (Figure 5C and F). We studied if cell division activity was increased

in the apex of transgenic plants. Indeed, *EBB1-oe* plants displayed approximately 80% higher cell division rate than WT-717 plants (Figure S5).

EBB1 native expression and localization

We studied *EBB1* expression in WT-717 plants. *EBB1* transcript was detected in the apex and stems with highest expression in the apices (Figure 4A).

Because *EBB1* expression was highest in growing apices we performed *in situ* RT-PCR to understand its tissue localization in WT-717 plants (Figure 4B). We used *EBB1-oe* transgenic plant as a positive control to verify that the transgene was expressed in all apical tissues. As expected, we detected ubiquitous and high expression throughout the *EBB1-oe* shoot apex. No signal was detected in –RT negative control. In WT-717 apices *EBB1* transcript was detected in the L1/L2 layer of the meristem dome, extending into the emerging leaf primordia.

We also found that *EBB1* was cytokinin-induced and also required treatment with an auxin such as 2,4-D; cytokinin or 2,4-D alone were unable to induce *EBB1* expression (Figure 4C). During natural bud-set to bud-break in wild aspen (*Populus tremuloides*) trees, *EBB1* expression was undetectable for most of the dormancy period, but increased prior to bud-break (Figure 4D).

EBB1 transgenic modifications led to major genome-wide transcriptome changes

We used microarrays to study the transcriptomic changes in the EBB1-modified transgenics. Our analysis focused on apices because of the native predominant expression of *EBB1* in this tissue. We first identified differentially expressed genes in the transgenic plants. The significantly regulated genes were then subjected to co-expression analysis with the expression of the EBB1 gene in the three genotypes (e.g., WT-717, *EBB1-oe* and *amiEBB1*). A total of 971 differentially expressed genes that correlated with EBB1 expression in the three genotypes were identified (Datasets S3). Out of the 971 genes, 416 were positively- and 555 negatively-correlated with *EBB1-* expression (Datasets S3). The expression changes identified through the microarray studies were successfully validated by reverse transcription RT-PCR for a subset of 12 genes (6 positively and 6 negatively-regulated) (Figure S6).

We performed functional classification of the differentially expressed genes using gene ontology (GO) analysis (Datasets S4). Several groups of biological processes categories were significantly affected in the *EBB1* transgenics. For example, genes involved in brassinosteroids

biosynthesis, jasmonic acid biosynthesis, receptor linked signaling pathways, and growth were significantly enriched (Datasets S4).

Identification of EBB1 putative direct targets

In Arabidopsis, the EBB1 close orthologs (DRN/ESR1 and DRNL/ESR2) have been found to be positive regulators that bind to a GCC-box sequence (Nomura et al 2009; Eklund et al., 2011). Therefore, to identify putative direct targets of EBB1, we searched the promoter regions (-3000bp) of the 416 positively regulated genes for presence of a GCC box. A total of 65 genes were identified (Dataset S7). Interestingly, GO analysis of the EBB1 putative target genes identified enrichment of a large number (43) of biological processes (Dataset S8). Among the most represented/enriched were nitrogen metabolic processes (13), developmental process (11), response to stimulus (12) and regulation of transcription (6).

Dormancy-induction and EBB1 share common regulons

We compared the differentially expressed genes in the *EBB1* transgenics with recently published genes regulated during induction of bud dormancy in the same poplar genotype that we used in this study （54）. This analysis discovered 265 (132 positively- and 133 negatively-regulated) common genes (Figure 7A), representing a significant enrichment of bud-dormancy-related genes in *EBB1* transgenics (27.3% of all differentially expressed genes; $P<0.001$, Fisher exact tests; Tables S5). Classification of the common gene set by gene ontology (GO) (Datasets S6) identified many genes that have been associated with entry into dormancy, including response to water deprivation, response to temperature, light exposure, red/far led light quality, and abscisic acid signaling.

We calculated the mean of expression for all 265 commonly regulated genes separately for negatively- and positively-regulated genes in *EBB1* transgenics, and studied their expression dynamics during the six weeks of dormancy induction using data from a previously published study （54）(Figure 7B). Surprisingly, large numbers of genes (over 130 genes in each category) showed distinct and opposing patterns in their expression during poplar bud dormancy induction. Genes that were up-regulated in *EBB1-oe* and downregulated in the EBB1-suppressed plants showed reduced expression during dormancy induction. Conversely, genes that were down-regulated in *EBB1-oe* but upregulated in the *amiEBB1* plants showed elevated expression during

dormancy induction. Therefore *EBB1* appear to negatively affect expression of genes that are typically upregulated during dormancy induction, and to positively impact transcript abundance of genes that are normally repressed during dormancy induction.

DISCUSSION

We report the isolation and characterization of the EARLY BUD-BREAK 1 (EBB1) gene that regulates the re-initiation of shoot growth after winter dormancy in *Populus*. Two main lines of experimental evidence strongly suggest that *EBB1* plays an important role in the resumption of growth after winter dormancy. First, overexpression of the gene is sufficient to accelerate bud-burst while downregulation delays bud-break. Second, *EBB1* transcript levels in buds are undetectable during the majority of the dormancy period but sharply increase prior and during bud-break. There is very little information on the regulation of bud-break. To date the only other gene than EBB1 that has been implicated in control of bud-break is the poplar ortholog of CENTRORADIALIS (CEN) (PopCEN1). CEN is known as TERMINAL FLOWER1 (TFL1) in Arabidopsis and is a repressor of flowering (7). Similarly to Arabidopsis, PopCEN1 overexpression delayed flowering, while RNAi suppression of both paralogs (PopCEN1 and 2) significantly accelerated flowering in poplar (40). In support to the growing body of evidence for a link between regulation of flowering and bud phenology in perennials (25), PopCEN1 overexpressors displayed delayed bud- break while RNAi transgenics showed precocious bud flush (40). It is still unclear if EBB1 play part in this regulatory module. EBB1 shows highest sequence homology to DRN/ESR1 and DRN-like (DRNL)/ESR2 from Arabidopsis (33). To our knowledge, there has been no information to date of a mechanistic connection between the flowering time integrators and DRN/ DRNL.

And third, we found a very significant enrichment of dormancy-associated transcripts (54) in apices of *EBB1*-oe plants. In Arabidopsis, DRN and DRNL are considered paralogs with largely overlapping but also some distinct functions (12). Comparative sequence analysis indicates that despite the recent whole-genome duplication (59), poplar genome has the same number of orthologous genes - EBB1/PoptERF61 and PoptERF60 (Figure 2C). The phylogenetic sequence analysis indicates that both EBB1/PoptERF61 and PoptERF60 are more similar to DRNL. However, EBB1 tissue localization and expression in response to hormones is reminiscent to these of DRN gene (see more discussion below) (4). Thus it is difficult to draw

functional parallels between the paralogous pairs in poplar and Arabidopsis based solely on their sequence orthology. DRN and DRNL genes are considered to be part of one of the three independent pathways that maintain growth and organization of the shoot apical meristem (SAM) (9, 12). *In vitro*, DRN ectopic expression causes enhanced shoot regeneration from root cultures, suggesting that DRN promotes *de novo* meristem formation and activity. *EBB1* shows expression and localization that resemble these of DRN. *EBB1,* like *DRN,* was induced by a combination of cytokinin and auxin treatment (4), and it was expressed predominantly in the L1/L2 layers of SAM and leaf primordia (33). Similar to DRN positive effect on shoot regeneration from root cultures (4), *EBB1* upregulation caused spontaneous regeneration from tissues that typically do not produce shoots (Figure S4), and enhanced shoot regeneration from leaf disks (Figure S5). Increased cell division activity in the SAM of *EBB1-oe* transgenics further supports the role of EBB1 in activation of SAM via stimulation of cell proliferation. Therefore we believe that EBB1 is involved in bud-break after winter dormancy through restarting of cell proliferation in SAM and leaf primordia.

The localization of *EBB1* in the L1/L2 layers of SAM and leaf primordia implies that growth after winter dormancy reinitiates in L1/L2. L1/L2, and particularly L1 from which epidermis is derived, may promote and restrict growth of the entire leaf/shoot by spreading growth promoting signals to the inner layers (22, 56). The enrichment of genes that encode epidermal cell fate specification and leaf shape in EBB1 transgenics supports this putative function. Furthermore, because L1 interfaces most closely with the ambient environment, it may have a unique role in perception of environmental cues (56), particularly changes in temperature that typically are the sole drivers of bud-break. Consistent with this hypothesis, our microarray analysis indicate significant enrichment in *EBB1* transgenics of gene ontology (GO) categories associated with perception of various environmental cues, including temperature (discussed below).

The precise function of DRN/DRNL in SAM organization is unclear, however, in *Arabidopsis* DRN/DRNL is known to act via the auxin signal transduction pathway and physically interacts with BIM1, a brassinsteroid-regulated bHLH transcription factor (10, 11). Both auxin and brassinosteroid action in the SAM is localized in the L1 layer (22, 46, 56), where we find the highest expression of *EBB1*. The putative positive interaction of EBB1 with auxin and brassinosteroid signaling is also supported by our microarray analysis. We found among the differentially expressed genes a significant enrichment of GO processes involved in

hormone-mediated signaling pathways. These were dominated by genes involved in auxin signaling and brassinosteriod biosynthesis. In addition, two of the putative EBB1 gene targets encode SHORT INTERNODE RELATED SEQUENCES (SRS) transcription factors that have been implicated in regulation of auxin biosynthesis in Arabidopsis through activation of a YUCCA gene (18, 57). In support to our findings, recent evidence suggests that auxin is important for reinitiating growth after dormancy (44). Therefore our work and information about the orthologous gene in Arabidopsis suggest of a possible connection of EBB1 with auxin and brassinosteroid signaling.

Comparative microarray analysis of gene expression in *EBB1-oe* and *amiEBB1* plants showed a large number (971) of genes that displayed significant changes in their expression compared to WT-717 plants (dataset S1). Consistent with EBB1 function in regulation of bud-break, the *EBB1-* transgenics transcriptome shows significant enrichment of biological categories associated with responses to various environmental cues. The EBB1 differentially regulated genes were also significantly enriched in processes typical for actively growing apices, such as these associated with various metabolic processes, meristem growth, and regulation of hormone levels. Consistent with a putative function of EBB1 in SAM regulation we found genes associated with meristem growth in the L1 layer. For example, poplar homologs of ERECTA-LIKE 1, GLABROUS 3, GLABROUS 2, LITTLE ZIPPER 3, and HOMEOBOX 51 were all upregulated by the EBB1 overexpression. *EBB1-oe* plants also showed enhanced expression of genes that maintain meristem identity such as *APETALA2-like*. APETALA2 in conjunction with other transcription factors regulates the stem cell niche in the SAM, and involves interaction with the WUS-CLV3 pathway (62). The seemingly multi–functional role of EBB1 in SAM corresponds well with its self-sufficiency to organize and stimulate meristem activity *de novo* from differentiated tissues in the transgenic poplar plants (Figures S1 and S2).

We also found a striking and significant overlap (more than 1/4 of all regulated genes) of the differentially-expressed genes in EBB1 transgenics with ones that were previously found to be significantly changed in abundance during dormancy induction in the same genetic background (e.g., WT-717) (54). Even more striking are the trends in the expression patterns among the common set of regulated genes. The mean of expression of all genes that were upregulated in *EBB1-oe* and downregulated in amiEBB1 plants showed a declining expression trend during the progression into dormancy. Conversely, genes increased in expression in *amiEBB1* and

downregulated in *EBB1-oe* plants showed an increasing trajectory during the same period. This suggests that EBB1 and/or the pathways it affects, suppress mechanisms that are associated with induction and preparation for dormancy, while at the same time promoting responses that are associated with actively growing apices. For example, the closest poplar ortholog of SVP (one of the DAM genes) (Potri.007G010800, Affy probe PtpAffx.4750.1.S1_at, Table S3; cDNA Probe PU01890, Table S5) was specifically and strongly downregulated in *EBB1-oe* plants but upregulated during dormancy induction （54）. Downregulation of DAM genes is necessary for release from endodormancy （55） and could be linked to the early bud break phenotype of *EBB1-oe* plants.

Using the microarray data coupled with promoter analysis we identified 65 putative EBB1 target genes. Interestingly and in support to the validity of our analysis, we found a significant enrichment among the 65 putative target genes of SHI RELATED SEQUENCE (SRS) genes ($P < 0.001$, Fisher's exact test). In Arabidopsis, SRS genes like SHORT INTERNODES (SHI) and STYLISH (STY) were demonstrated to be direct targets of DRN/ESR1 activation (Eklund et al., 2011). Enrichment of various GO categories among the putative target genes suggests of a broad regulatory context of EBB1 function. For example, the most enriched GO category among the target genes was of nitrogen metabolism. Nitrogen mobilization/remobilization during growth/dormancy cycles is essential for the long term survival of woody perennials （13, 15）. Enrichment of processes linked to development and response to stimulus in the target genes is consistent with EBB1 meristem function and response to environmental/hormonal clues. Finally, enrichment of processes linked to transcription regulation which are mainly represented by transcription factors of various families suggests that EBB1 mediates its response through regulation of other transcription factors. This is supported by work in Arabidopsis showing that SRS transcription factors are direct targets of one the EBB1 Arabidopsis orthologs （17）. Besides transcription factors, the EBB1-mediated regulatory mechanisms likely involve other signaling pathways. For example, the most significant correlation among the target genes with EBB1 expression was found for a gene encoding a Candidate G-Protein Coupled Receptor 1 (CAND1). In Arabidopsis, CAND1 was shown to physically interact with GTP-binding protein alpha subunit1 (GPA1), the only G alpha encoding gene in the Arabidopsis genome （23）. GPA1 is part of abscisic acid, brassinosteroid, gibberellin and sugar signaling and response pathways

(review in (60). Furthermore in maize a GPA1 ortholog regulates meristem activity in CLAVATA dependent manner (6).

The experimental evidence presented here, and the known functions of the close homologs DRN/DRNL in Arabidopsis, suggests that EBB1 has a dual function in the SAM; it appears to both regulate meristem cell proliferation and stem cell maintenance. This type of regulation is unusual, as many other meristem genes have highly specialized roles (e.g., WUS, CLV, STM). Accordingly, the absence of EBB1 during dormancy establishment allows progression through the physiological, developmental, and adaptive changes leading to dormancy, while the expression of EBB1 in specific cell layers prior to bud-break enables re-activation of growth in the SAM and leaf primordial and re-entry into active growth phase.

Vegetative bud dormancy is an important adaptive and economic trait, whose significance is likely to grow as a result of rapid climate change. Most cold injuries in trees occur due to frost damage as a result of either late spring frosts around the time of bud-break, or early fall frosts around the time of growth cessation (16). In conifers—where the adaptive consequences of genetic variation in phenology have been very well studied—late frost damage after bud-break is two- to three-fold more likely to cause damage than is injury due to late bud-set (42, 58). Through analysis of EBB1 and the physiological processes of which it is a part of, it should be possible to gain new insights into control of dormancy release in perennial plants. This will enable novel approaches for population management, molecular breeding, and genetic engineering of dormancy-associate traits.

MATERIALS AND METHODS

Activation tagging, field trial and screening for bud phenology; Plant material and treatments; Plasmid rescue, positioning of the tag and sequence analyses; Expression analyses; Generation of binary vector constructs and transformation; Microscopy and in situ RT-PCR analysis; Microarray hybridization and data analysis are all described in detail in ***SI Materials and Methods***. They are shown in the above-mentioned sequence.

ACKNOWLEDGMENTS

This work was supported in part by grants from the USDA-NRI Plant Genome program (2003-04345), the US Department of Energy, Poplar Genome Based Research for Carbon Sequestration

in Terrestrial Ecosystems (DE-FG02-06ER64185, DE-FG02-05ER64113), the Consortium for Plant Biotechnology Research, Inc. (GO12026-203A), the USDA Biotechnology Risk Assessment Research Grants Program (2004-35300-14687), the USDA McIntire Stennis Fund and industrial members of the Tree Biosafety and Genomics Research Cooperative at Oregon State University. We thank Chamini Illangasinghe for production of binary constructs and RT-PCR analyses and Petio Kotov and Sharon Junttila for help with histological analysis.

FIGURE LEGENDS

Fig. 1. Isolation and molecular characterization of *early bud-break 1 dominant* (*ebb1D*) poplar mutant. **(A)** Precocious bud-break of the *ebb1D* in the field during the start of the second growing season. Mutant plants showed advanced bud- break compared to neighboring transgenic and WT-717 (wild-type) trees. Arrows point to two *ebb1D* ramets that show accelerated bud-break compared to majority of neighboring other activation tagging events and WT- 717 plants. (**B**) Precocious bud-break of *ebb1D* mutant (left) compared to WT-717 (right) plants after growth chamber photoperiodic induction of dormancy followed by 11 weeks of chilling. (**C**) Average number of days to bud-break in WT-717 and *ebb1D* (see Materials and methods for detailed description of inductive treatments). Bars show one standard error over means of at least 10 ramets per genotype. Significance of differences tested by Student t-test (*** - $P<0.001$). **(D)** Genome position of activation tag insertion in *ebb1D,* Enh – Enhancer derived from the CaMV 35S promoter. **(E)** Expression of AP2/ERF tagged gene in WT-717 and *ebb1D* mutant plants. **(F)** Unrooted neighbor-joining tree of proteins from *Arabidopsis*, poplar, rice and maize that belong to the same AP2/ERF gene subfamily. Numbers in the branch nodes indicate % bootstrap support of 1,000 iterations. Poptr= *Populus trichocarpa*; FZP = FRIZZLE PANICLE (rice); BD1= BRANCHED SILKLESS 1 (maize).

Fig. 2. EBB1 is a positive regulator of bud-break. **(A)** to **(C)** Precocious bud-break in transgenics with upregulated *EBB1* expression (*EBB1-oe*). **(D)** to **(F)** Delayed bud-break in transgenic plants with suppressed *EBB1* expression (*amiEBB1*). **(A)** and **(D)** Dynamics of bud-break in *EBB1-oe* (**A**) and *amiEBB1* (**B**) compared to WT-717. **(B)** and **(D)** Average number of days to bud-break in *EBB1-oe* (**B**) and *amiEBB1* (**D**) compared to WT-717. **(C)** and **(F)** Bud-break in a typical

EBB1-oe (**C**) and *amiEBB1* (**F**) plants after 1 week (**C**) or 2 weeks (**F**) in long days and high temperatures following chilling treatment (see Materials and Methods for details). Bars in **(B)** and **(D)** show one standard error over genotypes' means (n= 10-15 in (**A**) and (B), n=7-12 in (**D**) and (**E**)). Significance of differences tested by Fisher's Exact Test in (**A**) and (**B**) or Student t-test in (**B**) and (**E**), (**- P<0.01, ***- P<0.001).

Fig. 3. Bud and apex morphology of *EBB1-oe* transgenics. Dormant bud (**A, B, D, E**) and actively growing vegetative SAM (**C**, **F**) in WT-717 (**A**, **B**, **C**) and transgenic *EBB1-oe* (**D**, **E**, **F**) plants. Note the difference in scales' shape in transgenic line, which form more open area around meristem. In wild-type buds, meristem is more compactly surrounded by buds scales. **(B)** and **(E)** represent close-up magnification of the same sections shown on **(A)** and **(D)**. Scale bars = 500µm (**A** and **D**) and 100 µm (**B, C**, **E** and **F**).

Fig. 4. *EBB1* expression and localization. **(A)** *EBB1* expression in various organs. Tissues were collected from WT-717 plants at the same time of the day and correspond to as follows: 1 cm roots tips (Roots); 2-3mm apical shoot including meristem and subtending leaf primordia (Apex); unexpanded young LPI 1-2 leaves (YL); fully-expanded LPI 5-10 leaves (Leaves); petioles of fully-expanded leaves (Petioles); whole stem collected from LPI5-10 (Stem). **(B)** *In situ* RT-PCR localization of *EBB1* transcript in actively growing apices of WT-717 (left), *EBB1-oe* plants (middle). Negative -RT control was performed on *EBB1-oe* apices (right). Arrows indicate the localization of the EBB1 transcript in the L1\L2 layers of the meristem and leaf primordia. Scale bars = 50µm **(C)** *EBB1* is induced by a combination of cytokinin and auxin treatment (see Materials and Methods for more details). (**D**) Expression of *EBB1* in vegetative buds of wild aspen (*Populus tremuloides*) trees**.** Relative expression for all experiments was normalized for loading differences using ubiquitin gene (UBI). Bars and data points show means ± one standard error of at least three independent biological replicates for all experiments except for (**D**) where two individual trees were used as biological replications.

Fig. 5. Dormancy induction and EBB1 share common and opposing regulons. **(A)** Venn diagram of common gene set between differentially expressed genes in *EBB1* transgenic apices and genes that are differentially expressed in apices of the same genotype during SD-induced dormancy

(Ruttink et al. 2007). **(B)** Trends in the expression of the common gene set during the 5 weeks of the dormancy induction period. Data points and error bars represent the mean and stand error over the averaged expression of all upregulated and downregulated genes. R^2 represents coefficient of determination for goodness of fit for the calculated linear trendline, linear regression significances are denoted as *- P<0.05, and **- P<0.01.

Figure S1. *EBB1* growth and morphology in the field. Height (**A**) and diameter (**B**) of 3-year-old field-grown *EBB1* and WT-717 plants. Error bars represent one standard error over ramet values. * - indicates P<0.05 as determined by Student's t-test. Adaxial (**C**) and abaxial (**D**) sides of leaves from field-grown *EBB1* (left) and WT (right) plants. **E**. Whole-plant view of *EBB1* trees in the field (in front of light colored cloth screen).

Figure S2. Overexpression of *EBB1* causes a range of phenotypic changes. **A.** Leaf sizes and form observed in *EBB1-oe* transgenic plants. Branching and size of WT (**B**) and *EBB1-oe* transgenic event *(***C**) grown in a greenhouse for 4 months.

Figure S3. Shoot regeneration from leaf segments. Leaf segments from WT-717 and *EBB1-oe* events (+3) ware cultivated on callus induction media (CIM) media (Han, K. H. et al. 2000) for three weeks, transferred on shoot induction media (SIM) media for four weeks, and number of regenerated shoots recorded. Bars represent mean and standard errors from five biological replicates with 20 explants each. * indicates P<0.05 as determined by Student's t-test.

Figure S4. Spontaneous shoot regeneration from cambium-derived callus in *EBB1-oe* plants. **(A)** WT-717 plants. **(B)** and **(C)** *EBB1-oe* plants. Stems were cut approximately a foot from the soil and photos taken three weeks after cutting. Similar responses were seen in approximately half of the *EBB1-oe* events.

Figure S5. Increased cell division rate in the apex of *EBB-oe* transgenics. Cells in meta-, ana- and telophase were counted as dividing. The graph presents mean and standard errors of 10- 12 acid-carmine-stained apices and approximately 2,000 cells. * indicates P<0.05 as determined by Student's t-test

Figure S6. Suppression of EBB1 in 4 independent *amiEBB1* lines. Bars show one standard error over genotypes' means (n= 3). Significance of differences tested by Student t-test (* - $P<0.05$).

Figure S7. Validation of microarray results. Bars represent mean and standard errors over three independent biological replications. Abbreviations used in the figure correspond to the names and gene models as specified in Table S2. All expression estimates were normalized using ubiquitin gene expression as described above.

SUPPLEMENTAL INFORMATION

SI Materials and Methods

Supplementary tables

Table S1. *EBB1* gene subfamily in *Populus trichocarpa* genome.

Table S2. Primers used for validation of microarray results.

Datasets S1. Differentially-expressed genes in *EBB1-oe* and *amiEBB1* apices.

Datasets S2. Gene Ontology classification of differentially-expressed genes in *EBB1-oe* apices.

Datasets S3. Common gene set between genes regulated in EBB1 trasgenics (Dataset S1) and differentially-expressed genes during dormancy induction period（54）.

Datasets S4. Gene Ontology classification of the common gene set in Datasets S3.

Datasets S5. EBB1 putative target genes.

Datasets S6. Enriched GO categories in dataset in Dataset S5.

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