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The poplar basic helix-loop-helix transcription factor *BEE3* – Like gene affects biomass production by enhancing proliferation of xylem cells in poplar

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ABSTRACT

Brassinosteroids (BRs) play important roles in many aspects of plant growth and development, including regulation of vascular cambium activities and cell elongation. BR-induced *BEE3* (brassinosteroid enhanced expression 3) is required for a proper BR response. Here, we identified a poplar (*Populus alba* × *Populus glandulosa*) *BEE3*-like gene, *PagBEE3L*, encoding a putative basic helix-loop-helix (bHLH)-type transcription factor. Expression of *PagBEE3L* was induced by brassinolide (BL). Transcripts of *PagBEE3L* were mainly detected in stems, with the internode having a low level of transcription and the node having a relatively higher level. The function of the *PagBEE3L* gene was investigated through phenotypic analyses with *PagBEE3L*-overexpressing (ox) transgenic lines. This work particularly focused on a potential role of *PagBEE3L* in stem growth and development of polar. The *PagBEE3L*-ox poplar showed thicker and longer stems than wild-type plants. The xylem cells from the stems of *PagBEE3L*-ox plants revealed remarkably enhanced proliferation, resulting in an earlier thickening growth than wild-type plants. Therefore, this work suggests that xylem development of poplar is accelerated in *PagBEE3L*-ox plants and *PagBEE3L* plays a role in stem growth by increasing the proliferation of xylem cells to promote the initial thickening growth of poplar stems.

1. Introduction

Populus is a genus of trees characterized by a rapid growth rate, high productivity, and adaption to a wide variety of environments, which makes it one of the most promising bioenergy feedstocks [1–3]. Due to these exceptional qualities, *Populus* have been used extensively for pulp and biofuel, and have been studied as a model tree because they readily undergo transformation [4,5]. Initial studies have focused on increasing biomass in trees of the commercially valuable *Populus*. Plant biomass used for foods, energy, and lumber is primarily derived from vegetative tissues. Therefore, an increase in vegetative growth of plants results in higher production of plant biomass. Traditional breeding has been traditionally used to increase biomass with limited success. However, recent genetic studies have identified a number of factors that regulate processes such as vegetative meristem activities, cell elongation, photosynthetic efficiency, and secondary wall biosynthesis [6], and these factors may be leveraged to promote growth.

Phytohormones play important roles in plant growth and development, including the regulation of vascular cambium activities and cell elongation. Thus, experimental manipulation of these phytohormones may increase biomass yield. Most plant hormones (auxin, cytokinin, gibberellins, ethylene, brassinosteroids, and jasmonate) promote cambial activity [7–10]. Auxin is the primary hormone involved in the regulation of cambial activity and cell differentiation [10], while ABA is as a negative regulator of cambial growth through negative interaction with auxin [11]. Cytokinin regulates vascular cambium activity, which is essential for secondary xylem development [8,12]. Brassinosteroids (BRs) have been shown to positively regulate xylem cell differentiation and development of secondary xylem [7,13,14]. Gibberellic acid (GA) stimulates cambial cell proliferation with auxin and elongation of xylem fibers [15,16].

Although BR and GA have similar effects on a wide range of developmental processes, there are no reports that BR influences plant biomass. BR and GA deficient mutants are characterized by similar phenotypes, including dwarfism, reduced seed germination,

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and delayed flowering [17,18]. GA biosynthesis- and signaling-related genes have been intensively isolated and characterized in *Arabidopsis* and rice [16,19,20]. Growth, biomass production and xylem fiber length were enhanced in *GA2ox1*-overexpressing poplar, suggesting an integral role of GA biosynthesis [16]. Stem elongation was also increased in *Gibberellin Insensitive Dwarf1* (*GID1*) overexpressing aspen [20]. However, the role of BR in biomass production through xylem development has not yet been elucidated.

Brassinosteroids are a class of plant steroid hormone regulating growth and development processes such as stem elongation, leaf development, xylem differentiation, root growth inhibition, pollen tube growth, apical dominance and senescence [21–26]. Mutants deficient in biosynthesis or BR signaling genes consequently display reduced growth, such as decreased cell elongation, resulting in pleiotropic dwarf phenotypes [22,23]. In contrast, overexpression of BR-related genes enhances cell elongation [24,26]. Although BRs are generally considered to be important regulators of cell division and elongation, their role in stem growth by cambial development has not yet been determined. In the last few years, research into BR signaling has linked BR to numerous biological effects.

BR target genes that show two-to three-fold expression changes with changes in BR treatment [27,28]. Among those genes, the most responsive genes to BR are a set of basic helix-loop-helix (bHLH) transcription factor genes. They include *AtBEEs* (brassinosteroid enhanced expression) which were identified due to their early induction by BL in *Arabidopsis*. The *AtBEEs* act downstream of BRI1 in BR signaling and are antagonistically regulated by ABA [29]. Also, *AtBEE1* and *AtBEE3* interact with CESTA as a feedback regulator of BR biosynthesis [30]. However, the exact function of *AtBEEs* has remained elusive in growth and development of plants.

In this study, we identify the *BEE3*-like gene from hybrid poplar (*Populus alba* × *Populus glandulosa*), *PagBEE3L*. Among those tissues analyzed, *PagBEE3L* was expressed at the highest level in stems and its transcript level was enhanced by BL treatment. In addition, our functional analysis with *PagBEE3L* gain-of-function mutants suggests that *PagBEE3L* has a critical role in xylem differentiation of poplar stems.

2. Materials and methods

2.1. Hormone treatment

Hormone treatments were performed with stem cutting-propagated hybrid poplar (*Populus alba* × *P. glandulosa*) *in vitro*. Six-weeks-old wild-type poplars were incubated with the hormone solutions. The concentrations for the treatments were 0 (control), 1, 50, and 200 μM BL (Epibrassinolide, Sigma–Aldrich, St. Louis. MO, USA). BL-treated stems were subjected to *PagBEE3L* expression analysis by quantitative RT-PCR (qRT-PCR).

2.2. Construction and plant transformation

To identify the transcription factors involved in stem development of poplar (*Pouplus alba* × *P. glandulosa*), cDNA microarray hybridization was performed with RNA isolated from a node removed from an apical bud (unpublished). One of the genes induced within 1 h of decapitation was chosen for further investigation. This cDNA clone was homologous to a known gene, *Arabidopsis BEE3*, through NCBI BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Poplar (*Populus alba* × *P. glandulosa*) *BEE3* sequence was determined by PCR using primers based on database for *Poplus trichocarpa* (http://www.phytozome.net/; http://popgenie.org/). For the *PagBEE3L*-ox construct, the full-length poplar *BEE3* cDNA was inserted into the pK2GW7 plasmid (VIB-Ghent University, Ghent, Belgium) using the Gateway system [31].

Poplar (*Populus alba* × *P. glandulosa*) was transformed and regenerated essentially as described by Choi et al. [32,33]. For this experiment, three lines among seven independent lines were selected and propagated by cutting *in vitro*. Six-week-old transgenic poplar were planted in a fertilized soil mixture (Saengsaeng-sangto: perlite, 7:3) maintained moist and then were transferred into pots [20 (diameter) × 25 (height) cm] in a growth chamber. Phenotypic observations were made both when plants were both *in vitro* on MS medium [34] and in pots containing soil.

2.3. Subcellular localization of PagBEE3L

The coding sequence of *PagBEE3L* was fused in-frame to the coding region of smGFP to generate the *PagBEE3L:smGFP* fusion construct under the control of the CaMV 35S promoter. A transient transformation assay was performed as described by *Arabidopsis* mesophyll protoplast transient expression assay protocols [35]. The transfected cells were observed under confocal laser scanning microscope (Leica, TCS SP5, Wetzlar, Germany).

2.4. qRT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) and cDNA was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Otsu, Japan) according to the manufacturer's instructions. The real-time PCR analysis was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad). The real-time PCR mixture was prepared with 1 × IQ™SYBR® Supermix (Bio-Rad) in a final reaction volume of 20 μl. To evaluate the data CFX96™ Real-Time System quantification software (Bio-Rad) was used. ΔCt was calculated with *actin* as the endogenous control and relative quantification values (RQ) were obtained by the $2^{-\Delta Ct}$ method ($RQ = 2^{-\Delta Ct}$). The primers used for real-time PCR were as follows: *BEE3*-specific primers (5′-CCACCATTTCCTTTCC-TAGA-3′; 5′-AAACCTTCCGCTAAGTTTCC-3′), *actin*-specific primers (5′-CATCCAGGCTGTCCTTTCCC-3′; 5′-AACGAAGGATGGCGTGTGG-3′).

2.5. Anatomical analysis

The primary stem on each nine-weeks-old plant was collected, and transverse sections of the stems were prepared from samples obtained at 20 cm below the apical meristem. The stem samples were cut by hand into 1.0 μm thick sections. The fresh sections were stained using 0.05% (w/v) toluidine blue (sigma) and were observed under bright-field microscopy (Leica DE/DM R; Camera, PC 300F).

3. Results

3.1. Identification of a BEE3-like gene in poplar

PagBEE3L encodes a putative bHLH (basic helix-loop-helix) transcriptional regulator with a typical bHLH domain. The *PagBEE3L* cDNA (GenBank accession no. KM234273) was 727 nucleotides long and consisted of an open reading frame of 714 nucleotides, encoding a 237 amino acid protein. The sequence alignment showed that all of the BEE3 analyzed are highly conserved at the bHLH domain (Supplementary Fig. 1A).

The phylogenetic analysis showed that PagBEE3L and PtBEE3 were clustered with other BEE3 proteins that belong to the XII subfamily of bHLH transcription factors such as the AtBEE3. A BEE protein from poplar and the other six poplar BEE proteins were clustered within the BEE2 group (Supplementary Fig. 1B).

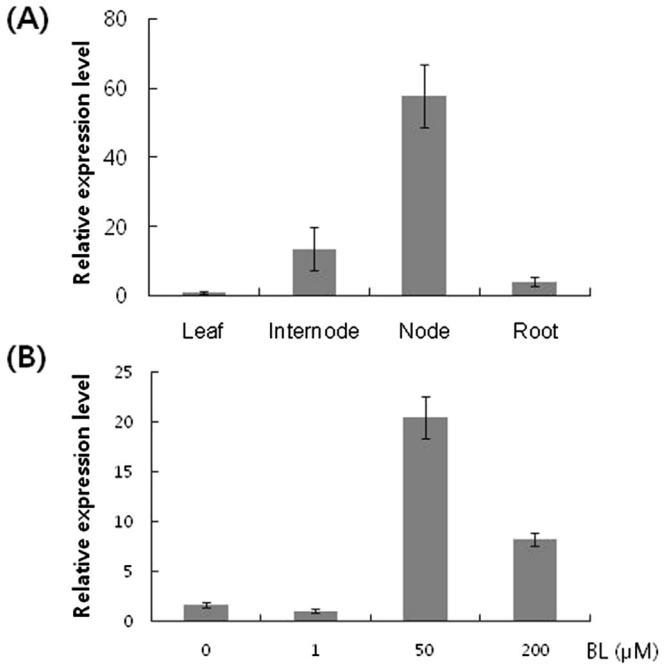

Fig. 1. Expression patterns of *PagBEE3L* (A) The transcript levels of *PagBEE3L* were measured in various tissues. Six-week-old plantlets grown in *in vitro* were used. Total RNA was isolated from various tissues of 6-year-old poplars grown in *in vitro* and was prepared for real-time PCR. (B) Transcript levels are shown after application of exogenous BL for 3 h. Total RNA was extracted from the stems treated with various concentrations of brassinolide (BL). Error bars indicate the standard deviation between three technical replicates measured on stems collected from at least five different poplar plantlets and subsequently pooled for analysis. Real-time PCR data were normalized by the endogenous *actin* gene.

Therefore, this phylogenetic analysis revealed that PagBEE3L belongs to a subfamily including *Arabidopsis* BEE1 and BEE3, which itself is classified as another subfamily of *Arabidopsis* bHLH proteins.

3.2. PagBEE3L is up-regulated by exogenous BL in poplar

Arabidopsis BEE genes (*BEE1, BEE2, and BEE3*) are rapidly induced by BL treatment and have redundant roles in BR signaling [28]. The expression profile of *PagBEE3L* was investigated in poplar plantlets (grown for four weeks *in vitro*) under various concentrations of BL. Total RNA was extracted from the BL-treated stems and subjected to the quantitative RT-PCR (qRT-PCR) using *PagBEE3L*-specific primers. The *PagBEE3L* expression was up-regulated in response to BL treatment. The transcript level of *PagBEE3L* was not changed at 1 μM BL treatment, but increased at 50 μM BL treatment. At 200 μM BL treatment, however, its expression was again decreased (Fig. 1A). These results indicate that the exogenous BL increases the transcript levels of *PagBEE3L*.

The expression pattern of *PagBEE3L* was also investigated in various tissues (leaf, stem [node and internode] and root) grown *in vitro* for six weeks. The *PagBEE3L* transcript was mainly detected in stems containing internode and node, but still occurred in leaf and root tissues at low levels (Fig. 1B). In addition, *PagBEE3L* may play a role in shoots branching from nodes, because the expression level of *PagBEE3L* is the highest in node tissues (Fig. 1B). These results suggest that *PagBEE3L* may have a function in stem development of poplar.

3.3. Subcellular localization of PagBEE3L

To determine the subcellular localization of PagBEE3L, the *PagBEE3L* cDNA fused to green fluorescent protein (GFP) was introduced into protoplasts of Arabidopsis leaves and GFP signals were

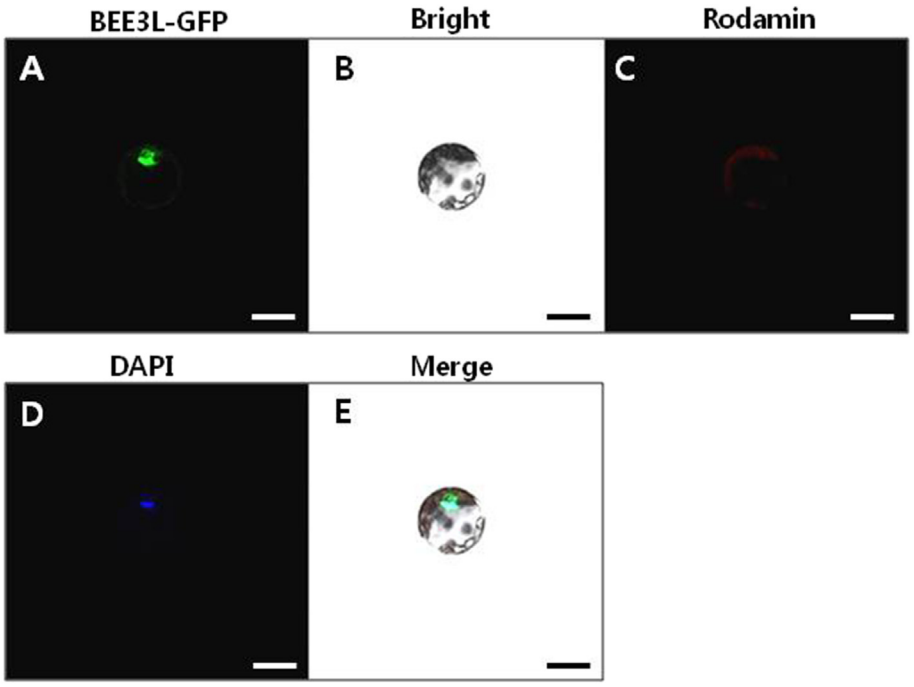

Fig. 2. Subcellular localization of PagBEE3L in *Arabidopsis thaliana* mesophyll cells This figures (A–E) PagBEE3L:GFP fusion protein, (A, C, D) fluorescent images, and (B, E) visible light images. (D) *Arabidopsis* protoplast was stained with the DNA binding dye DAPI. (E) Merged image of GFP with DAPI signal. The scale bars represent 20 μm. GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

examined. As Fig. 2 was shown, PagBEE3L was clearly localized into nuclei. This suggests that PagBEE3L, like three *Arabidopsis* BEE proteins, is a nuclear protein which is a typical feature of transcription factors.

3.4. Overexpression of PagBEE3L promotes vegetative growth of poplar

To identify the function of *PagBEE3L* gene in poplar, *PagBEE3L*-overexpressing transgenic poplar lines were generated under control of *35S* promoter and their phenotypes were observed during poplar growth and development. Positive transformants were selected after PCR analysis of genomic DNA from each transgenic plant (Supplementary Fig. 2). Six independent *PagBEE3L-ox* transgenic lines were obtained and they all contained increased levels of *PagBEE3L* transcript (Supplementary Fig. 3). Accumulation of *PagBEE3L* transcript in the *PagBEE3L*-ox transgenic lines were moderately elevated in lines 2, 4, and 6, and strongly elevated in lines 1, 3, and 5. Line 1, 2 and 3 were selected for further phenotypic analysis.

PagBEE3L-ox lines bearing apical meristem were planted on soil and observed 9 weeks after planting (Fig. 3A). The *PagBEE3L*-ox lines grew faster than the wild-type, being 30 cm taller after 9 weeks (Fig. 3B). Other phenotypic characteristics including larger leaves and thicker stems were observed in *PagBEE3L*-ox poplars (Fig. 3C). Significant differences in the number of node were not observed (data not shown). Therefore, the increase in height is unlikely to be due to an increased number of nodes; instead, it seems to be due to longer internodes. Also, petiole and epidermal cell length in *PagBEE3L*-ox poplars was longer than in wild-type poplars (Fig. 3D, Supplementary Fig. 3). Additionally, the leaf area of *PagBEE3L*-ox poplars was larger than that of wild-type plants (Fig. 3E). These results indicate that *PagBEE3L* plays an important role as a positive regulator in growth and development in poplar.

3.5. PagBEE3L over-expressing poplar plants exhibit enhanced xylem cells in stem

To investigate the exact causes of enhanced growth by poplar *BEE3*, anatomical cross-sections of stems were examined. Plantlets grown on soil for 6 weeks were cross-sectioned at the 20 cm below the SAM of the stem, and then sections were mounted on slides observed under light microscope (Fig. 4). Although the overall vascular morphology of transgenic plants was similar to that of wild-type, a notable increase in the xylem formation was examined in transgenic plants (Fig. 4). However, there was little difference in the formation of other tissues, including phloem, vascular cambium, and outer bark in *PagBEE3L*-ox plants. The length of epidermal cells was not changed in the stem of *PagBEE3L*-ox poplar

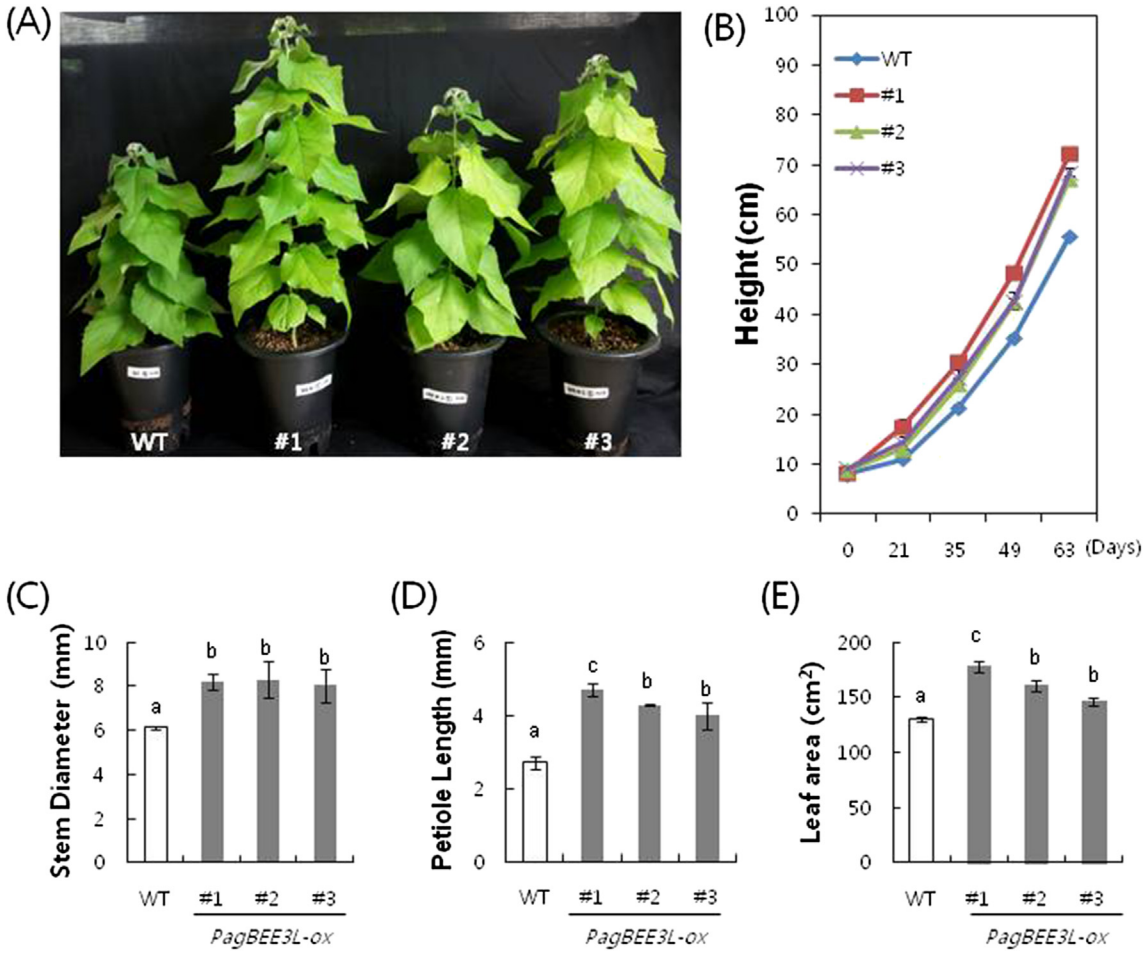

Fig. 3. Phenotypic analysis of *PagBEE3L*-ox transgenic poplars (A) Growth of *PagBEE3L*-ox transgenic poplars at seven weeks after planting in pots is shown, as well as (B) cumulative shoot elongation of the wild-type and transgenic lines grown in the growth chamber for 7 weeks after planting in pots. Number#1-#3 represent *PagBEE3L*-overexpressing poplar lines #1-#3, respectively. (C)–(E) Growth of *PagBEE3L*-overexpressing poplars grown in soil is given in terms of stem diameter, petiole length, and leaf area as measured in 9-weeks-old wild-type and transgenic poplars. Data are the means ± SD from 3 separate measurements of six individual plants. Different letters above the bars indicate significantly different means ($P < 0.05$) as analyzed by Duncan's multiple range rest using the SPSS 12.0 program.

(Supplementary Fig. S4), although stem length was increased (Fig. 3B). These results suggest that overexpression of *PagBEE3L* enhances secondary woody growth by improving xylem development in poplar.

3.6. Overexpression of PagBEE3L increases vegetative biomass in poplar

The growth and development of plants accelerated by hormonal regulation contributes to vegetative biomass yield (Eriksson et al., 2000; Demura and Ye. 2010). As shown in figure 3, *PagBEE3L*-ox poplar exhibited a dramatic phenotypic change relative to wild-type. To verify the effect of *PagBEE3L* overexpression on biomass yield, we measured fresh and dry weight in transgenic and wild-type poplar. Nine weeks after planting, wild-type and *PagBEE3L*-ox poplar were harvested and biomass of shoot and root was measured (Table 1). Relative to the wild-type poplar, *PagBEE3L*-ox poplar exhibited a significantly increased total weight of stems and roots. Transgenic poplar had a 1.24–1.45 fold higher fresh or dry weigh of stems than the wild-type and had a 1.22–1.86 fold higher fresh weight of roots than the wild-type (Table 1). However, there was no significant difference between transgenic and wild-type poplar in a fresh leaf weight.

4. Discussion

A number of studies indicate that growth factors such as plant hormone signaling molecules may be essential for increasing plant biomass, especially for the enhancement of vegetative growth in trees. Although some reports have suggested that plant hormone signaling components increase plant biomass [6,16], the roles of BR signaling in regulating plant biomass was not clearly addressed. Here, we showed that *PagBEE3L* is a putative bHLH transcription factor, similar to *Arabidopsis thaliana* BEE3 (*AtBEE3*) and overexpression of *PagBEE3L* causes various phenotypic changes during poplar growth and development. In this study, BEE containing bHLH domain named due to a rapid induction by BL. Indeed, their expression was induced by BL within 30 min of treatment in *Arabidopsis* [29]; *PagBEE3L* was enhanced twenty-fold by 50 μM BL treatment in poplar as (Fig. 1A).

To date, numerous bHLH transcription factor genes have been identified from various plants, but *BEE* genes were only studied in *Arabidopsis*. Accordingly, this study with poplar *BEE3* may give a chance to elucidate a new function of plant *BEE* genes. To address the functional role of *PagBEE3L* in stem growth of poplar, *PagBEE3L*-ox poplar were generated and subsequently examined for stem development phenotypes. The *PagBEE3L*-ox poplar produced thicker and taller stem than the wild-type (Fig. 3). Xylem differentiation and elongation is a very important process during the thickening growth of stems in trees. Secondary xylem (wood) is produced seasonally by the vascular cambium, and cell division within the vascular cambium is a first step during the developmental process of wood formation [36]. The wood cells of tree originate from vacular cambium activity, and the differentiation of xylem cells increases stem diameter [37]. Numerous reports have shown that other hormones (auxin, GAs, cytokinins, *etc.*) affect the differentiation and elongation of cambial cells. Application of IAA (indole-3-acetic acid) and GAs can increase cambial activity in trees [38]. Overexpression of GA 20-oxidase and GA biosynthetic enzyme which affect cell length, cell elongation, and xylem fiber, caused longer and thicker stem [16]. Cytokinin biosynthetic genes positively affect the vascular cambium activity and secondary

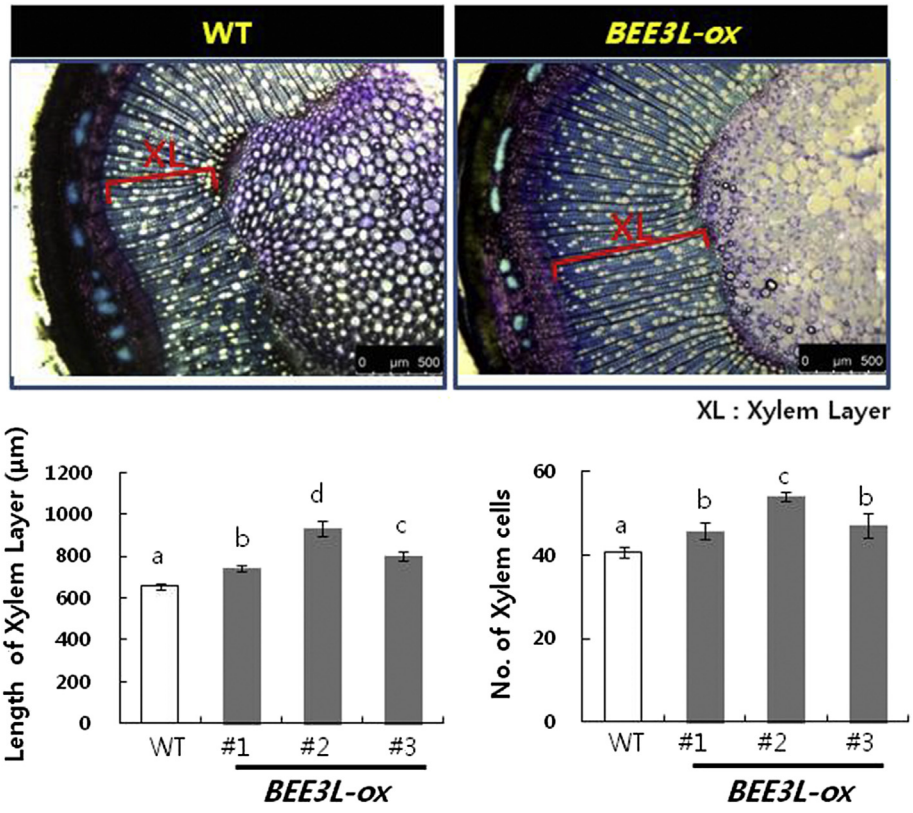

Fig. 4. Cellular phenotype of *PagBEE3L-ox* transgenic poplars Wild-type and transgenic plants were grown in pot for 9 weeks, after planting, and were used for sections. Cross sections of stems were prepared from pieces at 15th internode from the shoot apical meristem. Red lines indicate length of MX layer. At least three individual plants were measured. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Analysis of biomass of wild-type (WT) and *PagBEE3L* overexpressing (ox) transgenic poplars. Data are means ± SD from five individual plants grown in the soil for 9 weeks.

Genotype	Fresh weight (g)[a]						Dry weight (g)[b]	
	Leaf	(Rate)[c]	**Stem**	(Rate)	**Root**	(Rate)	**Stem**	(Rate)
WT	106.3 ± 8.5		39.2 ± 7.3		92.8 ± 3.9		13.1 ± 2.3	
OX-1	135.0 ± 9.2	(1.27)	56.8 ± 8.1	(1.45)	173.0 ± 6.4	(1.86)	17.1 ± 2.1	(1.31)
OX-# 2	100.8 ± 12.9	(0.95)	48.7 ± 5.3	(1.24)	113.5 ± 1.4	(1.22)	16.4 ± 4.6	(1.25)
OX-# 3	126.5 ± 33	(1.19)	55.5 ± 1	(1.41)	170.5 ± 6.4	(1.84)	17.1 ± 5.4	(1.30)

[a] Total weight of leaf, stem and root per plant.
[b] Total dry weight of stem per plant.
[c] Increasing rates (fold) relative to that of WT.

xylem in *Arabidopsis* [8]. Conversely, overexpression of cytokinin catabolic genes in poplar caused a reduced cytokinin level and decreased wood development [12]. However, only a few studies have reported that BRs affect the vascular cells. Mutants of BRL1 and BRL3, BRs signaling molecules, caused abnormal vascular differentiation and cell growth in *Arabidopsis* [7]. The proposed role of BRs was not verified in xylem differentiation of tree, but our data support the hypothesis that BRs contribute to cell divisions in the cambial cells.

Biomass measurements in transgenic poplar revealed that overexpression of *PagBEE3L* leads increases in growth, especially in stems. BR is a major growth regulator in various aspects of plant growth and development and has various roles in plant stem development [7,21,22]. However, little is known about the role BR plays in plant biomass formation. Several studies have attempted to promote growth and vegetative biomass of trees using hormone signaling molecules or hormone biosynthesis-related genes [16,33]. When bacterial trans-zeatin secretion (*tzs*) gene was transformed by the *aux* promoter in poplar (*Populus alba* × *P. tremula* var. *grandulosa*), pAUX-*tzs* transgenic poplars have more shoot biomass than wild-type poplars, but vegetative biomass has not increased [33]. However, GA 20-oxidase overexpressing poplars exhibit a dramatic phenotype by increased biomass [16].

Researchers have reported that transgenic plants, through the modification of genes related to hormone biosynthesis or signaling, showed morphological changes of growth and biomass. In this report, we studied a putative poplar *BEE3* transcription factor which is involved in BR signaling. The *PagBEE3L* transgenic plants reveal that they grow faster and produce more biomass by increasing xylem cell proliferation in stem tissue.

Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.109.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.109.

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