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OsBLE3, a brassinolide-enhanced gene, is involved in the growth of rice

Guangxiao Yang [a], Hidemitsu Nakamura [a], Hiroaki Ichikawa [a], Hidemi Kitano [b], Setsuko Komatsu [a,c,*]

[a] *National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba 305-8602, Japan*
[b] *BioScience and Biotechnology Center, Nagoya University, Chikusa, Nagoya 464-8601, Japan*
[c] *National Institute of Crop Science, 2-1-18 Kannondai, Tsukuba 305-8518, Japan*

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Abstract

Brassinosteroids (BRs) are a group of plant hormones involved in a wide range of plant growth and developmental processes. To investigate the mechanism of BR action in monocots, a brassinolide (BL) upregulated gene designated *OsBLE3* was identified, cloned and characterized in rice. It was mainly expressed in roots and leaf sheaths with levels of expression directly dependent on the dose of BL. In situ hybridization detected *OsBLE3* mRNA in the shoot apical meristem, organ primordia and vascular tissue. Furthermore, its expression was enhanced by co-treatment with BL and low concentrations of IAA. These results, and the existence of auxin response elements in the 5′-flanking region of the *OsBLE3* gene, indicate that *OsBLE3* expression is under control of both BR and auxin. The GUS reporter gene driven by a 2277 bp *OsBLE3* putative promoter was mainly expressed in vascular tissues, branch root primordia and was responsive to exogenous BL treatment. *OsBLE3* transcript levels were greatly reduced in *brd1* plants, a BL deficient mutant, compared to the wild type control. In *OsBRI1* antisense transgenic rice and *OsBLE3*, the BR-insensitive mutant expression of *OsBLE3* in response to exogenous BL treatment was significantly lower compared to that in control plants transformed with a vacant vector. Reduced *OsBLE3* expression and growth retardation was also observed in *OsBLE3* antisense transgenic rice plants. Internode cell length of the *OsBLE3* antisense transgenic lines was about 70% of that in the vacant vector transformed control lines. These results suggest that OsBLE3 is involved in cell elongation in rice through dual regulation by BL and IAA.

Keywords: Rice; *Oryza sativa*; Graminaceae; Brassinolide; Gene expression; Growth; *OsBLE3*

1. Introduction

Brassinosteroids (BRs) are naturally occurring plant steroids with structural similarities to insect and animal steroid hormones (Mandava, 1988). Exogenous application of BRs to plant tissues evokes a wide range of physiological effects, including promotion of cell elongation and division, tracheary element differentiation, tissue bending, and ethylene biosynthesis (Sasse, 1997). Analyses of mutants defective in BR biosynthesis revealed that these compounds are essential regulators of normal plant growth and development (Clouse and Sasse, 1998; Li and Chory, 1999). Studies on BR-biosynthetic mutants have also provided a better understanding of the biosynthetic pathways and metabolism of BRs (Schumacher and Chory, 2000.). Several important components of the BR signaling pathway have been identified and characterized with the help of BR-insensitive mutants. Perception of BR by BRI1 (BR-INSENSITIVE), a leucine-rich repeat receptor kinase,

Abbreviations: AuxRE, auxin response element; BL, brassinolide; OsBLE3, brassinolide-enhanced gene; BR, brassinosteroid.

* Corresponding author. Tel.: +81 29 838 7446; fax: +81 29 838 7464.
E-mail address: skomatsu@affrc.go.jp (S. Komatsu).

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is hypothesized to initiate a phosphorylation cascade that deactivates the downstream GSK3/SHAGGY-like BIN2 kinase, a negative regulator of BR signaling (Li and Chory, 1997; Li and Nam, 2002). By phosphorylating BES1 and BRZ1, BIN2 might prevent their translocation to the nucleus, where they act as regulators of specific gene expression (Li and Nam, 2002; Yin et al., 2002).

Several genes whose expression is regulated by BR applications have been identified, and some were predicted to function in cell expansion and cell division (Bishop and Koncz, 2002). The first identified BR-regulated gene, termed *BRU1* (*brassinosteroid upregulated*), has extensive homology to xyloglucan endotransglycosylases (XETs), a group of enzymes with cell wall-modifying function (Zurek and Clouse, 1994). Furthermore, recombinant BRU1 protein possesses XET activity (Man-Ho et al., 1998). Multiple XETs from different plant species are found to be induced after BR treatment (Kauschmann et al., 1996; Koka et al., 2000; Uozu et al., 2000). These results, therefore, provide a direct link between BRs and an induced growth response.

Previous studies suggested that the actions of BRs and auxin are related (Mandava, 1988). BRs and auxins synergistically promote tissue elongation and organ bending (Takeno and Pharis, 1982; Yopp et al., 1981). Although numerous physiological studies have addressed interactions between BRs and auxins, little is known about molecular mechanisms underlying the interaction of these two phytohormones. A recent comprehensive expression profiling analysis of *Arabidopsis* in response to IAA and brassinolide (BL) revealed that some genes were upregulated by both IAA and BL (Goda et al., 2004). This study provided us with some insight into the interaction of transcriptional regulation by these two hormones.

Three studies have been reported on microarray analysis of BR-regulated gene expression in *Arabidopsis* using the Affymetrix GeneChip (Goda et al., 2002; Müssig et al., 2002; Yin et al., 2002), but the results obtained were quite different. This discrepancy may be due to differences in experimental conditions and materials. Among the BR-induced genes found in these reports, genes encoding XETs, glucanases and expansin, three genes that have been implicated in cell expansion, were commonly found. BR-repressed genes included the BR biosynthetic enzymes, supporting the concept of negative feedback control for BR biosynthesis.

Although BRs have been identified in monocots, only a few reports have been published on the effects of BR on monocot growth and development. Initially there was some controversy as to whether BRs induce cell elongation in grass plants (He et al., 1991; Yokota and Takahashi, 1986) or whether the role of BRs in monocots and dicots was different. A gene encoding a putative protein kinase with a high similarity to *BRI1* was isolated from a BR-insensitive rice dwarf mutant *d*61 (Yamamuro et al., 2000). Two causative genes for rice BR-deficient dwarf mutants *brd1* (*BR-deficient dwarf*) and *ebisu dwarf* (*d*2), which encode C-6 oxidase and a novel cytochrome P450 respectively, were isolated and characterized (Hong et al., 2002, 2003; Mori et al., 2002). These results demonstrate that endogenous BR does play an important roles in regulating growth and development in monocot plants.

In order to gain insight into the molecular mechanism by which BRs regulate plant growth and development, especially in monocots such as rice, it is necessary to identify and analyze more genes that are controlled by BRs. Two different cDNA microarrays were screened in our previous reports (Yang et al., 2003, 2004; Yang and Komatsu, 2004) Two novel BL-enhanced genes, *OsBLE1* and *OsBLE2*, were identified and characterized in rice. The phenotypic changes in *OsBLE1* and *OsBLE2* antisense transgenic rice revealed that OsBLE1 and OsBLE2 are involved in the growth of rice (Yang et al., 2003; Yang and Komatsu, 2004). In the present study, another novel BL-enhanced gene designated *OsBLE3* that was identified using a cDNA microarray containing 4000 clones randomly selected from a rice cDNA library prepared from seedlings treated with BL (Yang et al., 2004), was characterized.

2. Results

2.1. Identification and cloning of OsBLE3, a gene upregulated by BL

Identification of BR-regulated genes in rice using a cDNA microarray containing 1265 unique genes has been reported previously (Yang et al., 2003; Yang and Komatsu, 2004). To identify additional novel BR upregulated genes, a cDNA microarray containing 4000 EST clones randomly selected from a rice cDNA library prepared from seedlings treated with BL was used to monitor changes of gene expression induced by BL. A number of unique clones were found to be BL upregulated after data analysis (Yang et al., 2004). Clone number 2994, designated *OsBLE3*, was selected for detailed characterization because its transcript abundance was highly stimulated by BL treatment and its function was unknown. Since the full-length EST insert was approximately the same size as that detected in a Northern blot, the insert was sequenced and analyzed.[1] The full-length of the insert was 865 bp, including a poly (A) tail (Fig. 1). The deduced protein contains 162 amino acid residues with a predicted molecular mass of 16.8 kDa and pI of 8.24. An in-frame stop codon (TGA) was found 69 bp upstream from the putative initial ATG, indicating that the insert of *OsBLE3* cDNA contains a full-length open reading frame. A rice genome sequence search using RiceBLAST (http://riceblast.dna.affrc.go.jp) revealed that *OsBLE3* is a single copy gene localized on

[1] The nucleotide sequence reported in this paper has been submitted to the DNA Data Bank of Japan (DDBJ) under accession number of AB091471.

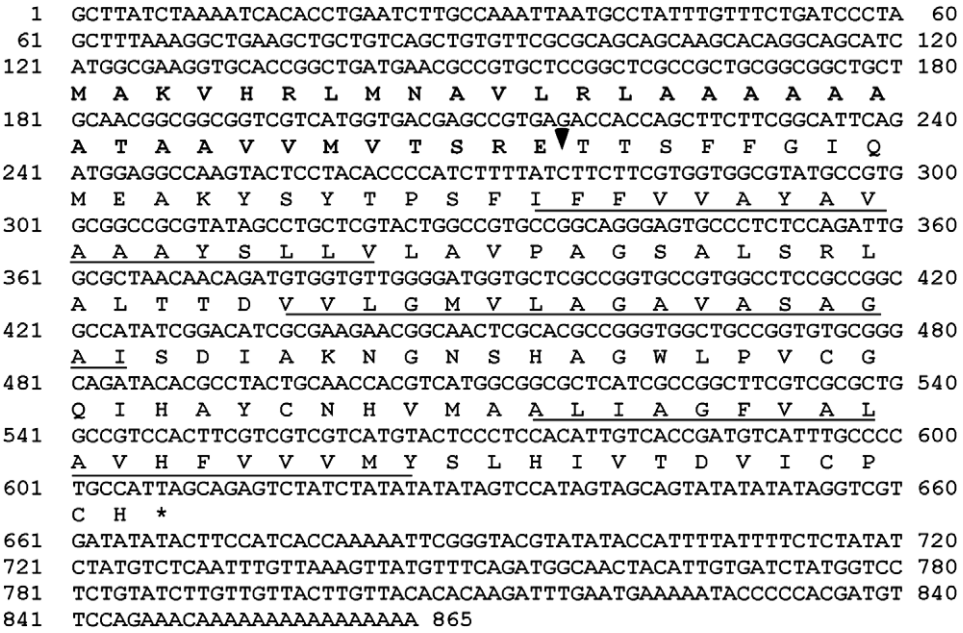

Fig. 1. Nucleotide and predicted amino acid sequence of *OsBLE3*. The full-length cDNA is 865 bp-long, including a poly (A) tail. The ORF has 162 amino acid residues, starting at position 121 with ATG and ending at position 609 with a stop codon (TAG). Three possible mitochondrial transmembrane domains predicted by PSORT are underlined. The putative mitochondrial targeting signal sequence predicted by MitoProt II is in bold face letters. The cleavage site between the putative signal sequence and the N-terminal residue of the mature protein is indicated by an arrowhead.

chromosome 5, and consists of three exons and two introns.

OsBLE3 contains three transmembrane domains (Fig. 1) as predicted by PSORT (Nakai and Kanehisa, 1991), and was classified as a mitochondrial inner membrane protein with 72% probability. The N-terminal mitochondrial targeting region is predicted to be cleaved after 32 amino acids with 93% possibility by the MitoProt II program (Claros and Vincens, 1996), resulting in a mature polypeptide of 13.5 kDa (Fig. 1).

A protein (At4g03540) with 32% identity (49/151) and 47% similarity (71/151) at the amino acid level to OsBLE3 was found in *Arabidopsis* that belongs to an integral membrane family protein. Both OsBLE3 and At4g03540 have a domain of unknown function (DUF588) as predicted by SMART (Schltz et al., 2000). This family of plant proteins contains a domain that may have a catalytic activity. It has a conserved arginine and aspartate that could form an active site. These proteins are predicted to contain three or four transmembrane helices and their exact functions are still unclear.

2.2. *OsBLE3 is expressed in roots and leaf sheaths of rice seedlings*

To examine the differences in expression patterns and expression changes of *OsBLE3* to BL treatment in various organs in rice seedlings, Northern blot analyses were carried out using total RNA extracted from root, leaf sheath and leaf blade of one-week-old seedlings treated with 1 μM BL or from cultured callus treated with 1 μM BL for 24 h. *OsBLE3* was expressed abundantly in root, moderately in leaf sheath and weakly in callus, while no hybridization signal with leaf blade RNA was detected. The expression levels of *OsBLE3* were clearly increased by treating root and leaf sheath with 1 μM BL, but not in the leaf blade or callus (Fig. 2A).

Northern blot analysis was also carried out in order to examine the dose dependency of BL on *OsBLE3* expression. Total RNA was extracted from roots and leaf sheaths of one-week-old seedlings treated with 0.01, 0.1 and 1 μM BL for 24 h. The expression of *OsBLE3* was dose-dependent with BL treatment both in root and leaf sheath with BL concentrations as low as 0.01 μM providing an obvious enhancement on *OsBLE3* expression (Fig. 2B).

In order to determine whether *OsBLE3* mRNA was expressed in specific cells types, an *in situ* hybridization was performed. Longitudinal sections of shoot from three-week-old seedlings were hybridized to Dig-labeled antisense RNA prepared from the *OsBLE3* cDNA (Fig. 3B) with the Dig-labeled sense RNA used as a negative control (Fig. 3A). Higher levels of *OsBLE3* mRNA were expressed mainly in the large vascular bundles, nodal vascular anatomoses, root primordia, and the shoot apical meristem, where the cells are vigorously undergoing division and/or elongation.

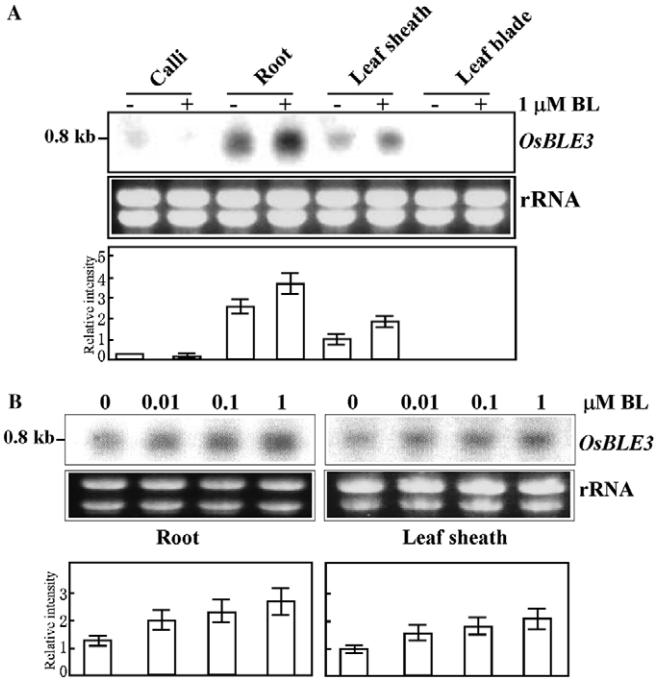

Fig. 2. Organ specificity and BL dose dependence expression of *OsBLE3*. (A) Root, leaf sheath and leaf blade of one-week-old seedlings treated with 1 μM BL or from calli treated with 1 μM BL for 24 h. (B) Root and leaf sheath of one-week-old seedlings treated with 0.01, 0.1 and 1 μM BL through for 24 h. Total RNAs (20 μg per lane) were separated on a denaturing formaldehyde agarose gel, blotted onto a membrane, and probed with PCR-amplified full-length insert of *OsBLE3* cDNA. rRNA stained with ethidium bromide was used as loading control. The signals were normalized using phosphor imager. Relative signal intensities represent the mean values of three experiments ±SE.

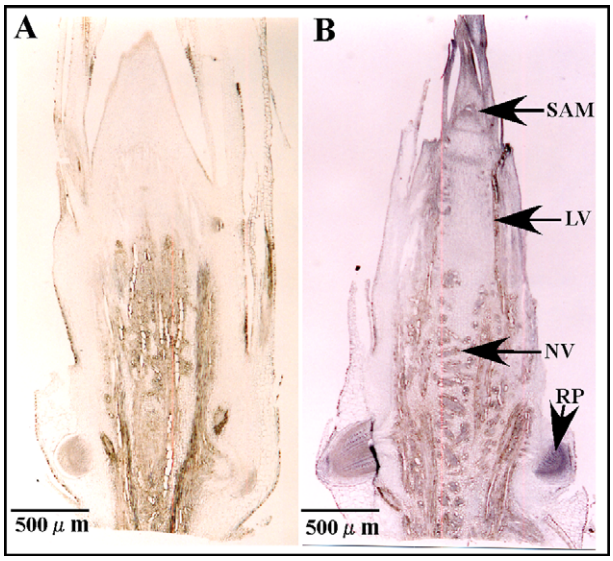

Fig. 3. Localization of *OsBLE3* mRNA. Longitudinal sections of shoot from three-week-old seedlings were hybridized to Dig-labeled antisense RNA prepared from *OsBLE3* cDNA (B). Dig-labeled sense RNA was used as a negative control (A). SAM, shoot apical meristem; LV, large vascular bundle; NV, nodal vascular anatomoses; RP, root primordia.

2.3. OsBLE3 expression is regulated both by BL and auxin

Cross-talk among plant hormone signaling pathways plays an important roles in regulating plant growth and development. Many genes are regulated by more than one kind of hormone. For example, *TCH4*, an Arabidopsis *XET* gene, is upregulated by auxin and BL (Xu et al., 1995). Both BL and zeatin increase the expression of *CycD3* (Hu et al., 2000). *cis*-Element scanning of the 5′ flanking *OsBLE3* genomic sequence by PLACE (Higo et al., 1999) identified several important regulatory motifs within 1 kb of the translation start site. Among them, TGTCTC, a core element of the previously reported auxin response element (auxRE) (Ulmasov et al., 1999) is present at position −343/−338 and −434/−429 (reverse strand) relative to the translation start site (+1) of *OsBLE3* (Fig. 4A). The existence of TGTCTC in the promoter region of *OsBLE3* suggested that *OsBLE3* expression might be regulated not only by BL, but also by auxin. To clarify this possibility, firstly, the influence of BL and IAA on *OsBLE3* expression was compared. Total RNA was extracted from roots and leaf sheaths of one-week-old seedlings treated with a combination of 1 μM BL and 1 μM IAA for 24 h. Similar to that of the BL treatment, IAA also clearly increased *OsBLE3* expression both in root and leaf sheath tissues (Fig. 4B). Next, one-week-old seedlings were treated with 0.1, 1, and 5 μM IAA in the presence or absence of 0.1 μM BL respectively for 24 h. Total RNA was extracted from leaf sheaths and Northern blot analysis was carried out. IAA alone induced *OsBLE3* expression in a dose-dependent manner at the concentrations used, while addition of 0.1 μM BL could enhance *OsBLE3* expression to maximum levels at the lowest IAA concentrations (Fig. 4C).

2.4. OsBLE3 promoter::GUS responds to exogenous BL

Based on the rice genomic DNA sequence information, a 2277 bp fragment flanking the 5′ side of the *OsBLE3* translation initiation site was PCR-amplified and cloned into a binary vector pSMAHdN627-M2GUS (Fig. 5A). The resulting plasmid carrying *OsBLE3 promoter::GUS* fusion was transformed into rice via *Agrobacterium*-mediated transformation. The GUS activity, as reflected by GUS staining, was clearly seen in vascular bundle tissues in the seedlings of transgenic plants, but not in the leaf tissues (Fig. 5B-a and B-b) while GUS activity was observed as scattered spots on the lower part of roots (Fig. 5B-c and B-d). Careful examination showed that *GUS* was expressed in correspondence with branch root primodia (Fig. 5B-e).

Next, *OsBLE3 promoter::GUS* transgenic seedlings grown on MS solid medium were treated with 0.01, 0.1 and 1 μM BL for 24 h, and BL induced GUS activity was examined. Exogenous BL induced GUS activity in a dose-dependent manner between 0.01 and 0.1 μM (Fig. 5C). The GUS activity assays confirm the responsiveness of

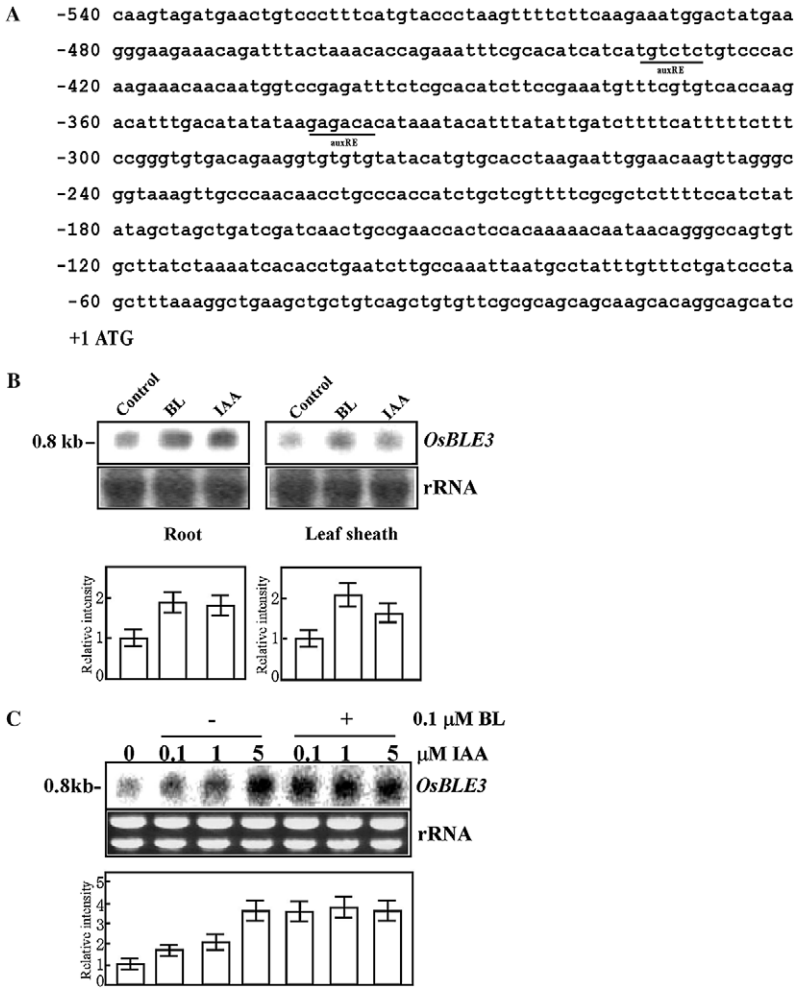

Fig. 4. 5′ *OsBLE3* genomic sequence and effect of BL and IAA on *OsBLE3* expression. (A) 540-bp of 5′ *OsBLE3* genomic sequence. Numbering refers to distance from translational start site designated as +1. auxRE represents auxin response element. (B) Comparison of BL and IAA on *OsBLE3* expression. Root and leaf sheath of one-week-old seedlings were treated with either 1 μM BL or 1 μM IAA respectively for 24 h through. (C) Combinational effect of BL and IAA on *OsBLE3* expression. Leaf sheath of one-week-old seedlings were treated with 0.1, 1, and 5 μM IAA in the presence or absence of 0.1 μM BL, respectively for 24 h through. Northern blot analysis was described in Fig. 2.

OsBLE3 to BL and also shows that the 2277-bp *OsBLE3* promoter fragment is sufficient to confer BL enhanced reporter gene expression.

2.5. OsBLE3 expression is greatly reduced in the BR deficient mutant brd1 and in OsBRI1, an antisense transgenic rice, in response to exogenous BL treatment

A basal level of *OsBLE3* expression occurs in rice seedling root and leaf sheath tissues even without BL treatment. To examine if *OsBLE3* is regulated by endogenous BL, the expression of *OsBLE3* in a BR deficient mutant *brd1* and its wild type counterpart was compared. The mutant *brd1*, defective in the BR biosynthetic enzyme C-6 oxidase (Hong et al. 2002), showed a range of abnormalities in organ development and growth, the most striking of which were reduced stem and leaf elongation (Hong et al. 2002). Northern blot analysis was carried out using total RNA extracted from roots and leaf sheaths of two-week-old plantlets. Compared with *OsBLE3* expression in root and leaf sheath tissues of wild type rice seedlings, *brd1* had only one-fifth the expression level of *OsBLE3* in roots and one-half the expression level leaf sheath (Fig. 6B). This result confirms that *OsBLE3* expression is controlled by endogenous BL.

To investigate if the BL signal leading to enhanced expression of *OsBLE3* is mediated through OsBRI1 or not, expression of *OsBLE3* in response to exogenous BL treatment were compared between antisense *OsBRI1* transgenic rice and control plants transformed with a vacant vector. Antisense *OsBRI1* transgenic plants produced erect leaves and had a dwarfed phenotype varying in severity (Yamamuro et al., 2000). The expression of *OsBLE3* was increased by about 110% with 1 μM BL treatment for 24 h in the leaf sheath tissues in vacant vector transformed control rice, but this increase was not obvious in the *OsBRI1* antisense transgenic rice after BL treatment. This result strongly suggests that enhanced expression of

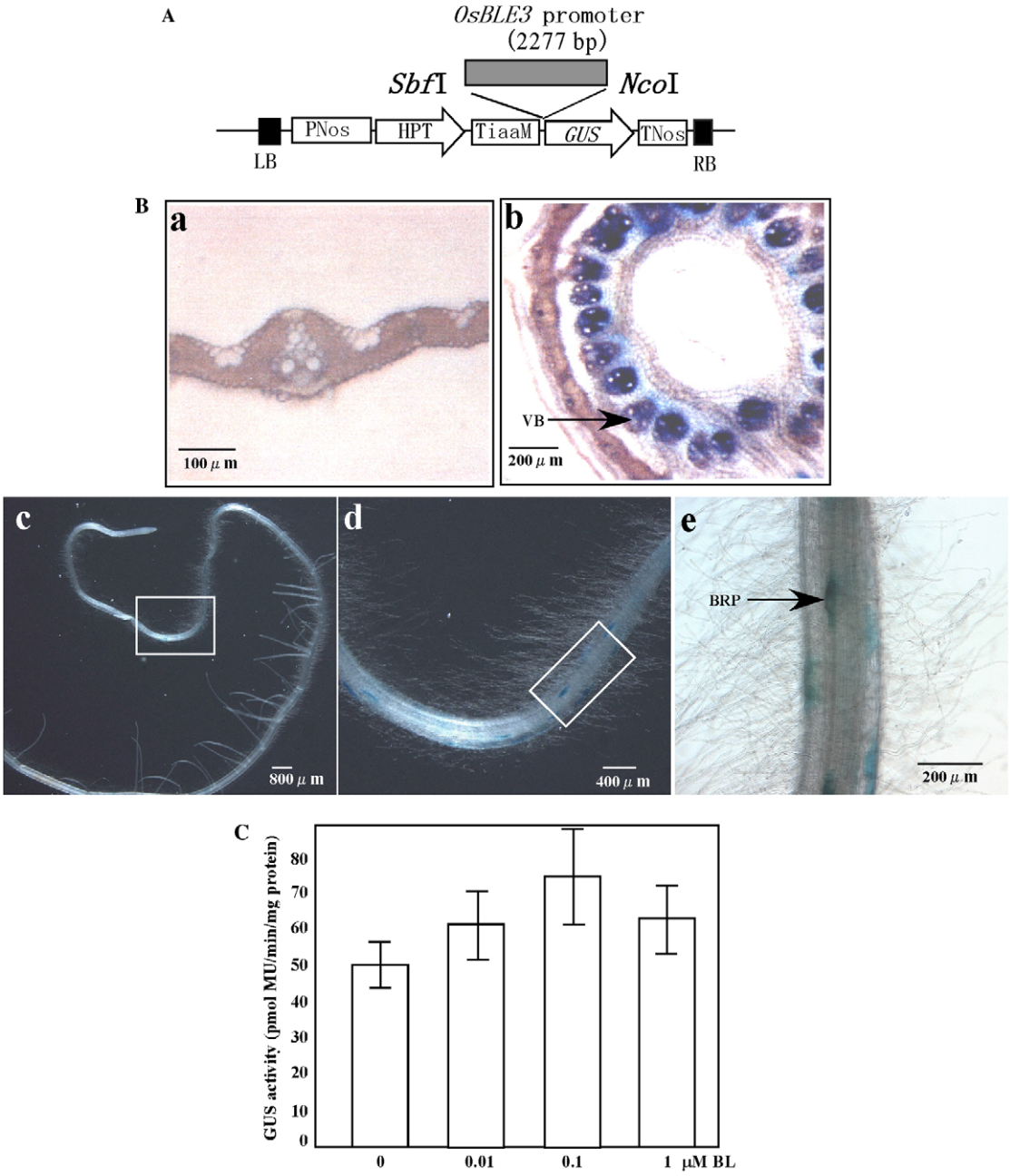

Fig. 5. *OsBLE3 promoter::GUS* fusion analysis. A 2277 bp *OsBLE3* promoter fragment was amplified by PCR from rice genomic DNA and cloned into a binary vector pSMAHdN627-M2GUS (A). Leaf (B-a), culm (B-b) and root (B-c, B-d and B-e) of transgenic rice seedling were stained in GUS staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. VB represents vascular bundle and BRP represents branch root primordia. Bd is enlarged view of Bc's square part and Be is enlarged view of Bd's square part. Reporter *GUS* gene expression in P_{OsBLE3}::*GUS* transgenic rice seedlings in response to BL treatment was measured (C). Ten-day-old seedlings were treated with 0.01, 0.1 and 1 μM BL for 24 h. GUS activity was measured by fluorescent method as described in methods. Values are the average of eight plants, and the assay was done twice using different transgenic lines. Relative signal intensities represent the mean values of three experiments ±SE.

OsBLE3 by BL is mediated, at least in part, through OsBRI1.

2.6. OsBLE3 affects rice plant growth

To assess the effect of loosing *OsBLE3* gene function on rice growth and development, antisense *OsBLE3* transgenic rice plants were produced. The full-length cDNA in reverse orientation was introduced into rice plants under the control of the *CaMV 35S* promoter in a pIG121-Hm binary vector (Fig. 7A) by means of *Agrobacterium*-mediated transformation. Vacant vector transformed rice was used as a control. A total of 12 antisense transgenic lines out of 40 regenerated plants were obtained. Total RNA was isolated from four representative antisense transgenic lines and a control lines and assessed for their levels

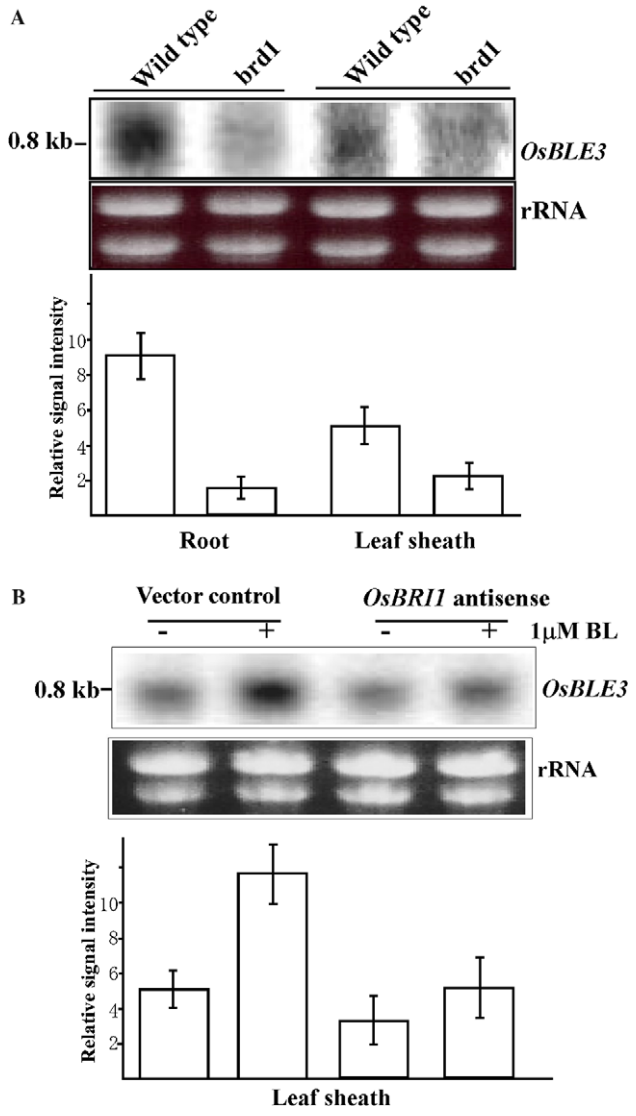

Fig. 6. Comparison of *OsBLE3* transcript in a BL deficient rice mutant *brd1* and its wild type and expression changes in response to exogenous BL treatment in *OsBRI1* antisense transgenic and control rice. (A) Root and leaf sheath of two-week-old seedlings of *brd1* and its wild type. (B) Leaf sheath of two-week-old seedlings of *OsBRI1* antisense transgenic and vacant vector transformed control rice treated with 1 mM BL for 24 h. Northern blot analysis was described in Fig. 2.

of *OsBLE3* transcript expression (Fig. 7B upper). Antisense lines A7, A11 and A12 had greatly reduced levels of *OsBLE3* expression when compared to vacant vector transformed control rice, and their growth was also repressed (Fig. 7B lower). *OsBLE3* mRNA levels in the line A6 were not efficiently inhibited, and its growth showed no difference from that of the vector control line (Fig. 7B). The growth of some antisense transgenic plants was obviously repressed (Fig. 7B and C), displaying a semi-dwarf phenotype, shorter root length and less branching, while no other abnormal changes in morphology were observed (Fig. 7C). All the internodes of antisense transgenic rice were shortened compared to the vector control plant (Fig. 7). Rice internode elongation results from cell division followed by cell elongation. Therefore, reduced internode length of transgenic rice could be a defect in one or both of these two processes. To clarify this point, sections of the first, second and third internode of adult plants were selected and examined under the microscope. The cell morphology in antisense transgenic rice was normal, but the average cell length was reduced when compared with that of control rice (Fig. 7D). The average length of cells in antisense transgenic lines was 65–75% of that in the vector control line, a value comparable to the difference in plant heights between control and antisense transgenic rice (Fig. 7D).

The biological relationship between OsBLE3 and BL *in vivo* was examined using antisense OsBLE3 transgenic rice (Fig. 8). Seeds of vector control and antisense OsBLE3 transgenic rice were germinated on filter paper in the absence or presence of 0.01 or 1 μM BL. Seedlings were examined two days after germination. The vector control plants grew normally in the absence of exogenous BL, whereas in the presence of BL, roots assumed a twisted shape (Fig. 8A). The root length of antisense OsBLE3 transgenic rice was less than the control; however, antisense OsBLE3 transgenic rice was also sensitive to exogenous BL similar to the control (Fig. 8A). We also tested the sensitivity of antisense transgenic rice to BL with a more quantitative method. Typical responses of second leaf lamina joints were measured in the absence or presence of 0.01 or 1 μM BL (Fig. 8B). When vector control rice plants were used for the lamina joint test, the leaf blade was bent almost at right angles to the axis of the leaf sheath in the absence of exogenous BL, becoming even more bent in the presence of increasing concentrations of BL (Fig. 8B). When the antisense OsBLE3 transgenic rice plants were tested, the degree of bending also increased with higher concentrations of BL, similar to what was observed in the vector control plants. The extent of bending of the leaves from the antisense OsBLE3 transgenic rice, however, was much less than that of the vector control plants under the same conditions (Fig. 8B).

3. Discussion

Molecular genetic approaches have revealed that in addition to GA, BRs are also important factors in determining plant height including the monocot rice. To gain insight into the molecular mechanisms by which BRs regulate plant growth, especially for rice, it is necessary to identify and analyze more genes that are controlled by BRs. While microarray analyses in Arabidopsis have provided us with an overall view of BR-regulated gene expression (Goda et al., 2002; Müssig et al., 2002; Yin et al., 2002), characterization of individual BR-regulated gene is not only important for assigning function of unknown genes, but it is also helpful for understanding the molecular mechanisms of BR action in detail. In the present study, a novel BR upregulated gene OsBLE3, whose product might be a mitochondrial inner membrane protein (Fig. 1), was

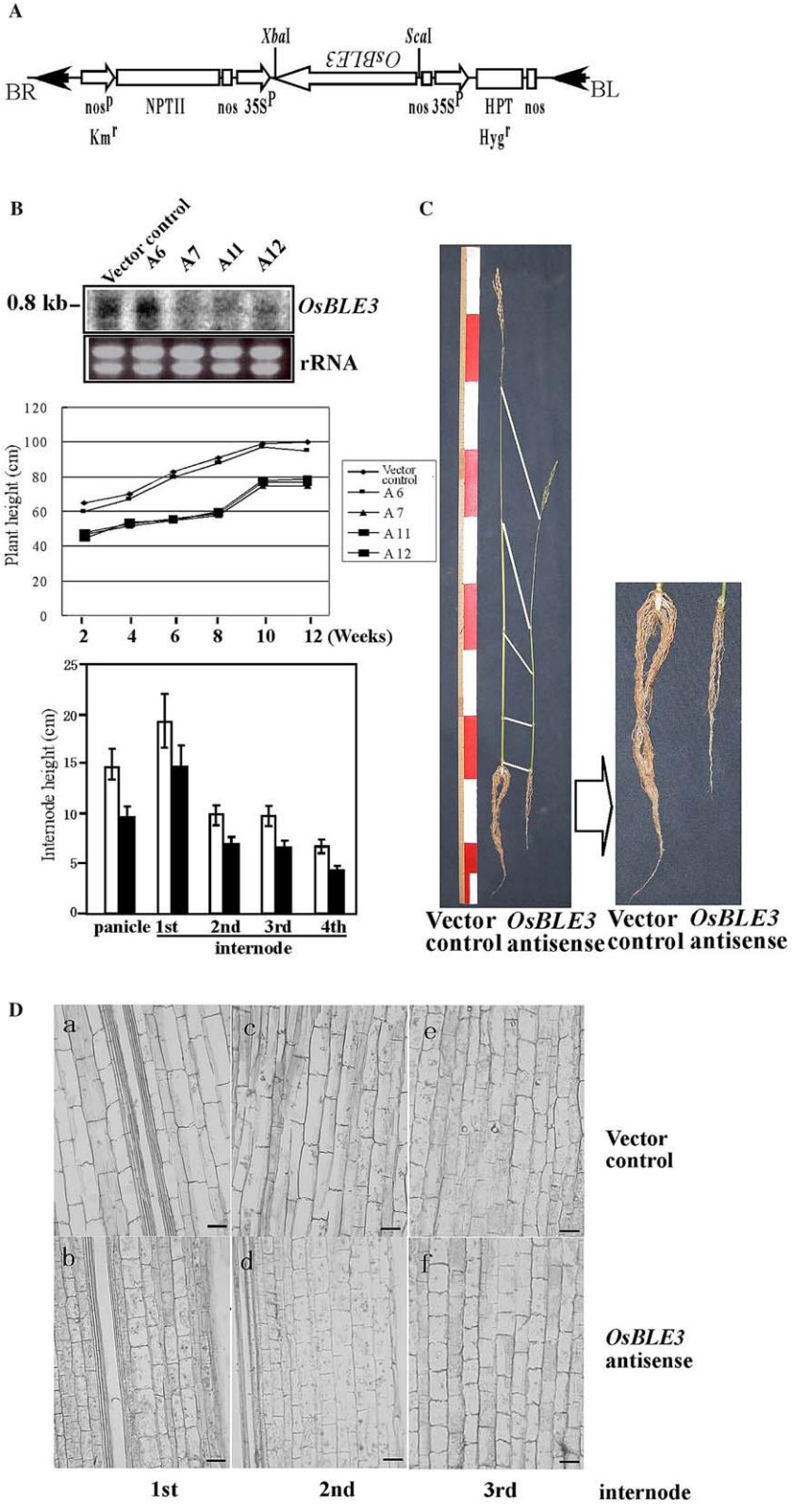

Fig. 7. Phenotypes of antisense *OsBLE3* transgenic rice. Full-length cDNA of *OsBLE3* was amplified by PCR and cloned in antisense orientation into a binary vector pIG121-Hm (A). Expression levels of *OsBLE3* in transgenic lines were analyzed by Northern blot (B upper). Growth of the *OsBLE3* antisense and vector control transgenic plants was recorded (B middle), the length of the up-most three internodes and panicle was compared (B lower). Open bar represents vector control lines and solid bar represents antisense transgenic lines. Data are average of five individual control and antisense transgenic lines ±SD. The culms and roots of a representative vector control and an antisense transgenic plant were shown (C). Longitudinal sections of internode cell in the vector control and *OsBLE3* antisense transgenic rice were observed (D). Longitudinal sections of the first (a and b), second (c and d), and third (e and f) internodes from vector control (a, c and e) and *OsBLE3* antisense (b, d and f) transgenic rice were shown. Scale bar in E equals 100 μm.

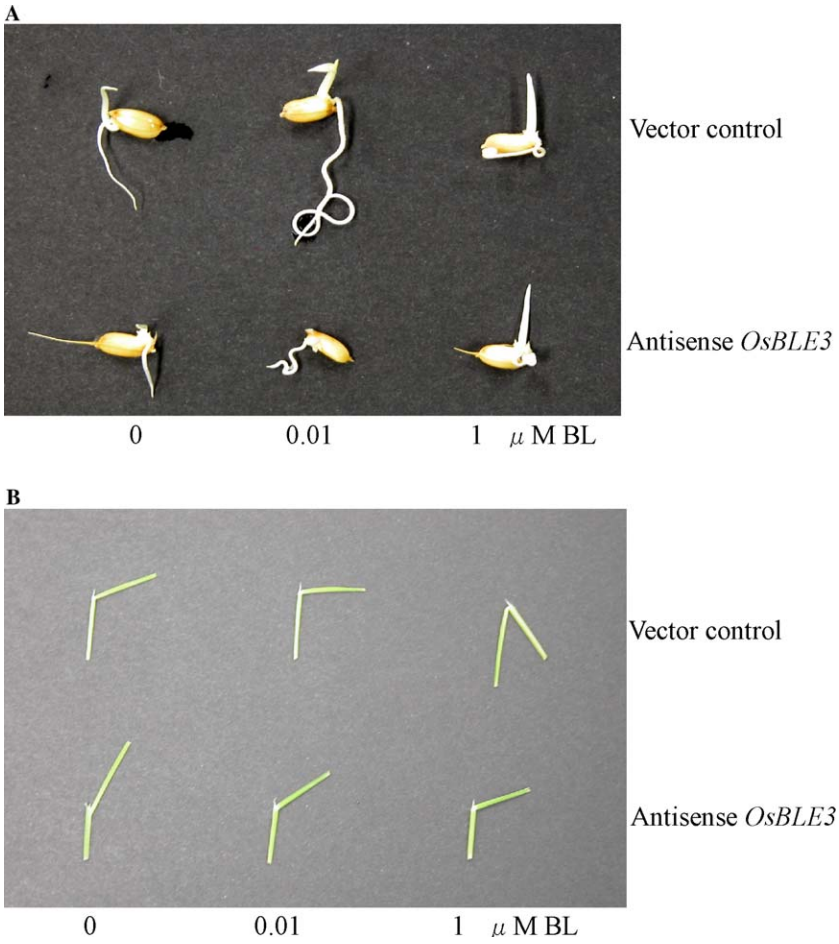

Fig. 8. Effect of BL on the seedling and the degree of inclination of leaf lamina in antisense OsBLE3 transgenic rice. (A) Seeds of vector control and antisense OsBLE3 transgenic rice were germinated on 3MM papers in the absence or presence of 0.01 or 1 μM BL. Seedlings were examined 2 days after germination. (B) Typical response of second leaf lamina joint were measured in the absence or presence of 0.01 or 1 μM BL.

identified in rice by microarray analysis and characterized. OsBLE3 was mainly expressed in root and leaf sheath tissue and was upregulated by BL in a dose-dependent manner (Fig. 2).

In situ hybridization analysis showed that *OsBLE3* mRNA was localized mainly in vascular bundles, shoot apical meristems and root primordia, where cells are actively undergoing division, elongation and differentiation (Fig. 3). These results indicate the possible involvement of *OsBLE3* in cell differentiation, expansion, elongation, and/or division processes regulated by BL. Several lines of evidence indicate that BL might play a significant role in vascular differentiation. In *Helianthus tuberosus* explants, exogenous BL increased the differentiation of tracheary elements tenfold after 24 h treatment (Clouse and Zurek, 1991). Uniconazole, an inhibitor of both GA and BR biosynthesis, prevented the differentiation of *Zinnia elegans* mesophyll cells into tracheary elements, and this inhibition was overcome by BR (Iwasaki and Shibaoka, 1991). In the present study, *OsBLE3* expression in the vascular bundles of rice also suggests the involvement of OsBLE3 expression in vascular differentiation.

The biological activities of BRs sometimes resemble those of auxin or GA. BRs are also known to interact with other plant hormones in very complicated ways interacting additively with GAs and synergistically with auxin (Mandava, 1988). Some evidence shows that many genes are regulated by more than one kind of hormone (Hu et al., 2000; Xu et al., 1995). Recent microarray analysis of BR-regulated gene expression in Arabidopsis revealed that several IAA-responsive genes were also up regulated by BL (Goda et al., 2002; Müssig et al., 2002). Our previously identified BL enhanced gene OsBLE2 was enhanced much more by GA_3 and IAA than by BL (Yang et al., 2003). In this study, OsBLE3 expression was found to be increased both by BL and IAA treatment in the leaf sheath and root of rice seedlings (Fig. 4).

Although BR-response elements in promoters of BR-regulated genes have not been precisely defined, some research indicates that BR might share some common *cis* elements with other signals such as from IAA or some environmental stresses (Iliev et al., 2002). It was shown that BL could also induce the expression of two early auxin inducible genes *IAA5* and *IAA19* in *Arabidopsis* (Goda et al.,

2002). The expression of the *GUS* gene fused with DR5, a synthetic auxin response element, was induced by IAA and BL treatment with similar kinetics to those of *IAA5* and *IAA19* (Nakamura et al., 2003). A recent comparative analysis of auxin- and BL-regulated genes in *Arabidopsis* revealed that the sequence TGTCTC, a core element of a previously reported auxin response element, was abundant in the 5′-flanking region of genes upregulated by both IAA and BL (Goda et al., 2004). This result leads to the hypothesis that genes upregulated by IAA and BL are regulated by a common *cis*-regulating element, which includes TGTCTC (Goda et al., 2004). In fact, two copies of this element are also present within 440 bp of the *OsBLE3* promoter (Fig. 4). IAA alone induced *OsBLE3* expression in a dose-dependent manner, while addition of BL could further enhance *OsBLE3* expression at lower IAA concentrations (Fig. 4). This result indicates that *OsBLE3* expression was regulated by BL and IAA possibly through cross-talk, and the auxRE in the promoter region might be one of the cross-talk points between the BL and IAA signaling pathways. In this study, the 2277-kb *OsBLE3* promoter fragment conferred BL responsiveness to reporter *GUS* gene expression (Fig. 5). *cis*-Element signal scanning using PLACE (Higo et al., 1999) also found several other regulatory motifs in the *OsBLE3* promoter indicating that *OsBLE3* expression might be regulated by multiple signal cues.

BR-deficient rice *brd1* is a mutant with a severe phenotype; elongation of its leaf, stem and root were all greatly repressed (Hong et al. 2002). *OsBLE3* expression in roots and leaf sheaths of *brd1* was greatly reduced when compared to levels in its wild type counterpart (Fig. 6), suggesting that *OsBLE3* expression is regulated by endogenous BL and might be involved in BL-regulated growth processes including shoot or internode elongation. The fact that no obvious increase in *OsBLE3* expression is observed in the *OsBRI1* antisense transgenic plant treated with BL suggests that enhanced expression of *OsBLE3* by BL is mediated, at least in part, through the BRs receptor, OsBRI1.

Finally, to investigate how OsBLE3 affects growth processes in rice, antisense transgenic rice plants were constructed. The growth of *OsBLE3* antisense transgenic rice was repressed and internodes were uniformly shortened. This phenotypic change was different from that of a weak allele of the BR-insensitive dwarf mutant *d*61 (Yamamuro et al., 2000). In contrast to the disorganized internode cells of the *d*61 mutant, the internode cell morphology of *OsBLE3* antisense transgenic rice was normal and well organized in longitudinal files. The cell lengths of *OsBLE3* antisense transgenic rice were about 65–75% of that in control rice, which accounts for the difference in total plant height between antisense transgenic and control rice (Fig. 7). These results indicate that the dwarfed phenotype of antisense transgenic rice may result mainly from repressed cell elongation, although the possibility that cell division was also partially involved in this process could not completely excluded.

In conclusion, the novel BL upregulated gene *OsBLE3* identified in this study is expressed mainly in root and leaf sheath tissues of rice. Expression analyses revealed that the *OsBLE3* is under the control of both BL and IAA. Analysis of antisense transgenic plants suggests that OsBLE3 might be involved in regulating rice growth by affecting the cell elongation process in rice.

4. Experimental

4.1. Plant materials and treatment

Wild type rice (*Oryza sativa* L. cv Nipponbare) and a BL deficient mutant *brd1* were grown in the granulated nutrient soil (Kureha Chemical, Tokyo, Japan) under white fluorescent light (200 μmol m^{-2} s^{-1}, 12 h light period/day) at 25 °C and 75% relative humidity in a growth chamber. The leaf blade, leaf sheath and root segments were prepared from one-week-old seedlings. Callus was cultured in N_6 liquid medium (Murashige and Skoog, 1962) and subcultured every other week. Plant hormones were applied to the soil for intact plant or added in the N_6 liquid medium for callus. BL (Daiichi Fine Chemical, Toyama, Japan) and IAA (Wako Pure Chemical, Osaka, Japan) stock solutions were made with dimethyl sulfoxide and control treatment contains the same amount of dimethyl sulfoxide (0.1% final concentration).

4.2. RNA extraction and Northern blot analysis

Tissue samples were quick-frozen in liquid nitrogen. Samples were ground to powder using a mortar and pestle, and total RNAs were isolated according to the procedure described Chomczynski and Sacchi (1987). Total RNA (20 μg) was separated on 1.2% agarose containing 6% formaldehyde and transferred to Hybond™-N^+ nylon membrane (Amersham Biosciences). Loading of equal amounts of total RNA for Northern blots was determined by visualization of ethidium bromide-stained rRNA bands. Probe was amplified by PCR using T3/T7 primer pair, purified from agarose gel (Qiagen), and radio-labeled using [α-^{32}P] dCTP (Amersham Biosciences) and the random prime labeling system (Rediprime™ II, Amersham Biosciences). Hybridization was performed at 42 °C in an ultra-sensitive hybridization buffer (ULTRAhyb™, Ambion, Austin, TX, USA) overnight. The blots were washed twice first in 2× SSC, 0.1% SDS at 42 °C for 5 min, then in 0.1× SSC, 0.1% SDS at 68 °C for 15 min, and finally analyzed by phosphor image program using Typhoon 8600 k variable imager (Amersham Biosciences).

4.3. Promoter analysis

A 2277 bp long OsBLE3 promoter fragment was PCR amplified from rice genomic DNA with primer pairs of

5′-ATGCCCCTGCAGGGAGGGAGTAGTAGCTAGC-TTGAG-3′ (5′ side, *Sbf*I site is underlined in the adaptor sequence) and 5′-TTCGCCATGGCTACTG-TACTTGCTTG-3′ (3′ side, *Nco*I site is underlined in the adopter sequence) using KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan) under the PCR conditions; 94 °C for 2 min (1 cycle), 94 °C for 15 s, 63 °C for 30 s, 68 °C for 2 min (30 cycles), 68 °C for 7 min (1 cycle). The 3′ side primer was designed to give the start codon of OsBLE3 on cutting with *Nco*I. The amplified fragment was gel-purified using Wizard SV Gel and PCR Clean-up System according to the supplier's protocol (Promega, Madison, WI, USA). The purified product was incubated with dATPs and Ex Taq (Takara-Bio) at 72 °C for 5 min. The purified fragment was cloned by pGEM-T Easy Vector System (Promega) and confirmed by sequencing. OsBLE3 promoter fragment was released by digesting with *Sbf*I and *Nco*I and cloned into a binary vector pSMAHdN627-M2GUS (Nakamura et al., unpublished) (Fig. 5A). The resulting plasmid carrying OsBLE3 promoter:GUS fusion was transformed into rice via *Agrobacterium*-mediated transformation.

4.4. GUS activity assay

GUS staining was conducted according to Jefferson (1987). Seedlings of transgenic plants were incubated in 50 mM sodium phosphate buffer (pH 7.2) containing 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide and MeOH–H_2O (5:95) at 37 °C for 24 h. The reaction was stopped by incubation in EtOH–H_2O (1:1) for 5 min and EtOH–H_2O (3:1) for another 5 min, and the stained plants were finally kept in 99.5% EtOH.

For measuring GUS enzyme activity, 10-day-old *OsBLE3 promoter::GUS* transgenic rice seedlings were treated with 0.01, 0.1 and 1 μM BL for 24 h. Plants were homogenized in extraction buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% (v/v) sodium *n*-lauroylsarcosine and 0.1% (v/v) Triton X-100. To assay GUS activity, 150 μL of extract was mixed with 200 μL of 1 mM 4-methylumbelliferyl β-D-glucuronide in extraction buffer prewarmed to 37 °C. The mixture was incubated at 37 °C for 30 min, and 100 μL mixture was mixed with 2 mL of stop solution (0.2 M sodium carbonate). The resulting fluorescence was measured with MTP-100F microplate reader (Corona Electric, Ibaraki, Japan) at 365 (excitation) and 465 (emission) nm. Protein content was determined according to Bradford method (Bradford, 1976) to normalize GUS activity.

4.5. In situ hybridization

Shoot meristem tissues taken from three-week-old seedlings were fixed in 4% formaldehyde and 0.25% glutaraldehyde under a vacuum. Fixed samples were then dehydrated through a graded ethanol series followed by a *t*-butanol series, and finally embedded in paraplast. Microtome sections (10 μm thick) were mounted on silicon-coated glass slides (Matsunami Co., Hamamatsu, Japan). Paraplast was removed through a graded ethanol series. Probes for *in situ* hybridization were labeled with digoxygenin-11-UTP (Roche, Mannheim, Germany). pBluescript SK plasmid containing full-length *OsBLE3* cDNA was either treated with *Bam*HI and transcribed with T7 RNA polymerase (Stratagene, Cedar Creek, TX, USA) (antisene probe) or digested with *Xho*I and transcribed with T3 RNA polymerase (Stratagene) (sense probe). Immunological detection was done with an anti-digoxigenin-AP conjugate and 4-nitroblue tetrazolium (Roche) (Kouchi and Hata, 1993).

4.6. Construction of OsBLE3 antisense transgenic rice

For constructing *OsBLE3* antisense transgenic rice, the full-length *OsBLE3* cDNA sequence in the pBluescript SK + plasmid was amplified by PCR using primer pairs of 5′-GCTCTAGACTGGAACATCGTGGGGGT-ATT-3′ (5′-side, underlining *Xba*I site as a linker) and 5′-CGCGAGCTCGCTTATCTAAAATCACACCT-GA (3′-side, underlining *Sac*I site as a linker). The resulting PCR product was cut, purified and ligated between the *CaMV 35S* promoter and nopaline synthase terminator in the binary vector pIG121-Hm by replacing the GUS coding region (Ohta et al., 1990) (Fig. 7A). The binary vector constructs were then transferred into *Agrobacterium tumefacien* strain EHA101 (Hood et al., 1986) and transformed into rice as described (Toki, 1997). Transgenic plants were selected on medium containing hygromycin. The Hygromycin-resistant plants were transplanted to soil and grown to maturity at 30 °C in 16 h light/8 h dark cycle in an isolation greenhouse.

4.7. Preparation of sections for microscopic analysis

Developed culms were fixed in formaldehyde:AcOH:EtOH (1:1:18) and dehydrated in a graded ethanol series. The samples were embedded in the paraplast and cut into 15 μm-thick sections that were stained with Fast Green FCF (Wako Pure Chemical) and examined under the light microscope.

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