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OsBC1L4 encodes a COBRA-like protein that affects cellulose synthesis in rice

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Abstract Plant morphogenesis is highly dependent on the regulation of cell division and expansion. The organization of the cellulose microfibrils in the cell wall is a key determinant of cell expansion. Previously, a dwarf mutant with fewer tillers, *Osbc1l4* (*Oryza sativa brittle culm 1 like 4*), was identified by screening a rice T-DNA insertion mutant library. It is reported here that *OsBC1L4* encodes a COBRA-like protein that exhibits typical structural features of a glycosylphosphatidylinositol-anchor protein. The T-DNA insertion in *OsBC1L4* results in abnormal cell expansion. A decrease in cellulose content but the increase in pectin and starch contents was identified in *Osbc1l4* mutants by measuring the content of wall components. *OsBC1L4* was expressed in all tissues/organs examined, with a low level in leaves. OsBC1L4 protein is mainly located in the cell wall and plasma membrane. Correlation analysis indicated that the expression of *OsBC1L4* was highly correlated to that of several primary wall-forming cellulose synthase genes (*CESA*s). Moreover, the expression level of several cellulose-related genes is increased in *Osbc1l4* mutants, which suggests that a feedback mechanism may exist during cellulose synthesis.

Keywords *OsBC1L4* (*Oryza sativa Brittle Culm 1 Like 4*) · Cellulose synthesis · Cell expansion · COBRA-like protein · Rice

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Abbreviations

BC1L	BRITTLE CULM1-like
GPI	Glycosylphosphatidylinositol
CSLF	Cellulose synthase-like F gene
CCVS domain	Cysteine-rich domain
RT–PCR	Reverse transcription polymerase chain reaction

Introduction

The plant cell wall is a complex and dynamic network that provides fundamental mechanical support for the plant body and sufficient plasticity for cell expansion (Cosgrove 2001, 2005; Crowell et al. 2010). Plant cell walls are of two types: primary and secondary. The primary wall surrounds all cells and appears to be the determinant of cell expansion and final cell shape and size (Carpita and Gibeaut 1993; Cosgrove 1997; Kotilainen et al. 1999; Martin et al. 2001; Smith 2003). The secondary wall, which is laid down after cessation of cell expansion in some specialized cells, provides the main mechanical support for the plant (Campbell and Sederoff 1996). Cellulose, a polymer composed of hydrogen-bonded 1,4-β-glucan chains, is an important component of both primary and secondary cell walls and is responsible for the direction of cell expansion (Somerville 2006; Baskin 2005).

The cell wall of angiosperms can generally be categorized into two types (Carpita and McCann 2000). Type I walls are generally found in dicotyledonous plants, and Type II walls are typically found in grasses (the Poales). In contrast to type I cell walls, the type II cell walls contain mixed-link β-glucans or glucuronoarabinoxylans as the main hemicellulosic polysaccharides, a complex network of phenylpropanoids, and less pectin (Carpita and McCann

2000). Cellulose is the largest component of the two types of cell walls (Somerville 2006). Despite several recent advances in identification of components involved in cellulose deposition, the mechanisms that regulate cellulose synthesis and cell expansion remain to be elucidated.

To identify genes that are involved in the synthesis of cellulose, many mutants defective in cellulose synthesis have been isolated and characterized. For example, the *Arabidopsis rsw1* mutant causes a reduction in cellulose content of cell wall, and results in a dwarf phenotype (Arioli et al. 1998). *RSW1* encodes the catalytic subunit of cellulose synthase (*CESA*) and belongs to a gene family containing 10 members in *Arabidopsis*. *CESA1* (*rsw1*), *CESA3* (*ixr1*), and *CESA6* (*prc1*) are required for cellulose synthesis in primary cell walls (Arioli et al. 1998; Fagard et al. 2000; Scheible et al. 2001), whereas *CESA4* (*irx5*), *CESA7* (*irx3*), and *CESA8* (*irx1*) are required for cellulose synthesis during secondary wall formation (Taylor et al. 1999, 2000). The *CESA* genes with similar function are coexpressed (Tanaka et al. 2003; Taylor et al. 2003). CESA protein has been immunolocalized to the cellulose synthesis complex which forms a rosette structure embedded within the plasma membrane (Kimura et al. 1999; Taylor et al. 2000; Desprez et al. 2007). In addition to *CESA*s, several other components affecting cellulose synthesis have been identified. The mutations in the *KORRIGAN* (*KOR*) gene, which encodes an endo-1,4-β-glucanase, lead to dwarfism, radial swelling of root tips, collapse of xylem vessels, and reduced cellulose content (Nicol et al. 1998; Zuo et al. 2000; Lane et al. 2001; Sato et al. 2001; Peng et al. 2002; Szyjanowicz et al. 2004). Rice cellulose synthase-like *CSLF* genes were reported to mediate the synthesis of cell wall (1,3; 1,4)-β-D-glucans (Burton et al. 2006a, b). Mutations in the *KOBITO* (*KOB*) gene result in dwarf phenotype and cellulose deficiency (Pagant et al. 2002).

Mutants of the *COBRA* gene showed severe growth defect with a change in both cellulose content and orientation (Benfey et al. 1993; Hauser et al. 1995; Schindelman et al. 2001; Roudier et al. 2005). The *COBRA* gene encodes a glycosylphosphatidylinositol (GPI)-anchored plant-specific protein. GPI membrane anchor is an important post-transcriptional modification of some cell wall-localized proteins and several genes required for correct production of GPI anchored protein have been cloned and characterized in *Arabidopsis*, such as *PEANUT1-5* (*PNT*) (Gillmor et al. 2005). *PNT1* encodes the *Arabidopsis* homolog of endoplasmic reticulum–localized mannosyltransferase PIG-M and *pnt1* mutant showed decreased crystalline cellulose, increased pectins, irregular and ectopic deposition of pectins, xyloglucans, and callose, less viable pollen, and radially swollen embryos (Gillmor et al. 2005). COBRA protein is a key regulator of diffuse anisotropic expansion throughout postembryonic development in *Arabidopsis* and follows a typical GPI secretion path, finally reaching the cell periphery through vesicular trafficking (Roudier et al. 2005). The COBRA protein contains an N-terminal signal sequence for secretion, a hydrophilic middle, a highly hydrophobic C terminus, a cysteine-rich CCVS motif, an ω-attachment site for GPI processing, two putative N-glycosylation sites, and two predicted cellulose binding sites (Roudier et al. 2002). COBRA belongs to a gene family that has 12 members in *Arabidopsis*, 11 in rice, and 9 in maize (Roudier et al. 2002; Li et al. 2003; Brady et al. 2007). In addition to COBRA, several other *COBRA*-like genes have been isolated and characterized. For example, *AtCOBL4*, *BC1/CWA1*, and *ZmBK2* are all involved in controlling plant mechanical strength and are required for cellulose synthesis in the secondary cell wall in *Arabidopsis*, rice, and maize, respectively (Li et al. 2003; Brown et al. 2005a, b; Persson et al. 2005; Ching et al. 2006; Sindhu et al. 2007; Sato et al. 2010). *AtCOBL9* and *ZmBk2L1* are required for tip-directed growth in root hair development in *Arabidopsis* and maize, respectively (Parker et al. 2000; Jones et al. 2006; Hochholdinger et al. 2008). The molecular characterization, expression pattern, and phenotype variations of knockout mutants of *OsBC1L* genes have been reported in rice (Dai et al. 2009). Generally, the *Osbc1l4* (*Oryza sativa brittle culm 1 like 4*) mutants exhibited a dwarf phenotype with fewer tillers, while *Osbc1l5* may cause a defect in pollen germination (Dai et al. 2009).

Here, the morphologies of *Osbc1l4* mutant were characterized in detail. We further confirmed the function of *OsBC1L4* by a complementation experiment. In addition, we described the expression pattern of *OsBC1L4* and subcellular localization of its protein systematically. Cytological and molecular analyses indicate that *OsBC1L4* is involved in cell expansion and cellulose synthesis in rice. Moreover, we found that a putative feedback mechanism might exists which might attempts to compensate for the reduced cellulose production in *Osbc1l4* mutants by increasing the expression of other cellulose-related genes. The identification of *OsBC1L4* and its role in cellulose synthesis in rice is an important step toward understanding the roles of *COBRA*-like genes in cellulose synthesis.

Materials and methods

Plant materials and growth conditions

Rice (*Oryza sativa*) mutant plants were planted in the experimental field (with a planting density of 16.5 cm × 26.4 cm) of Huazhong Agriculture University in Wuhan, China, in the summer (latitude 30.5°N, 15 m above sea

level; average daily temperature approximately 28°C). We transferred plants into pots when taking pictures with a camera (SONY DSC-V1, Japan). The *Osbc1l4* mutant was identified by screening our T-DNA insertion mutants (http://rmd.ncpgr.cn).

Light and scanning microscopy

For light microscopy, hand-cut sections of wild-type and mutant culms were observed with a microscope (DM4000B, Leica, Wetzlar, Germany), and photographed using a camera (DFC480, Leica, Wetzlar, Germany).

For scanning electron microscopy, samples were prepared according to a previously reported method (Mou et al. 2000), with some modifications. In brief, the mature culms were excised with a blade and immediately placed in 70% ethanol, 5% acetic acid, and 3.7% formaldehyde for 24 h. Then samples were critical-point dried, sputter-coated with gold, and observed with a scanning electron microscope (S570, Hitachi, Tokyo, Japan).

Cell wall composition analysis

Cellulose contents were assayed according to the methods described previously (Updegraff 1969). Briefly, the culms were ground into fine powder. The powder was washed in phosphate buffer (50 mM, pH 7.2) three times, extracted twice with 70% ethanol at 70°C for 1 h, and dried under vacuum. The dried cell wall materials were assayed for cellulose content with the anthrone reagent with Whatman 3MM paper as the standard. Pectin and hemicelluloses contents were measured according to a previously reported method (Peng et al. 2000). For the measurement of starch content, the culms were ground in 80% ethanol, washed with water, and extracted three times with 80% $Ca(NO_3)_2$. After mixing with 0.01 N I_2-KI, the light absorption at 620 nm was measured. Lignin contents were measured according to a previously reported method (Li et al. 2009). In brief, the *Osbc1l4* and wild-type culms were ground into fine powder and extracted four times with methanol. After vacuum drying, lignin content was quantified according to the method described by Kirk and Obst (1988). For the quantification of cell wall components, we calculated means ± SE of three independent biological repeats of mutants and wild-type plants.

Genotyping of the *Osbc1l4* plants

The PCR primers that flanked the T-DNA insertion site were designed for genotyping analysis according to the method described by Dai et al. (2009). The PCR was performed in an ABI 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the following cycling profile: 94°C for 5 min, followed by 28 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, and a final 5-min extension at 72°C. Primers for genotyping were listed in Supplemental Table 1.

Genetic complementation test

A 5.96-kb genomic DNA fragment containing the entire *OsBC1L4* coding region, the 1,891-bp upstream sequence, and the 1,214-bp downstream sequence was inserted into the binary vector pCAMBIA2301 to generate the transformation plasmid pCg001 for the complementation test. A control plasmid, the empty pCAMBIA2301 vector, was also transformed. The two binary plasmids were introduced separately into *Agrobacterium tumefaciens* EHA105 by electroporation, and the mutants were transformed according to the methods of our previous study (Wu et al. 2003).

Sequence analyses

The signal peptide was predicted with SignalP Version 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al. 2004), and the hydrophobic profile was made using the Kyte-Doolittle method (Kyte and Doolittle 1982). GPI modification was predicted using Big-PI (http://mendel.imp.ac.at/gpi/gpi_server.html) (Eisenhaber et al. 1998). Prediction of N-glycosylation sites was performed using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). Cellulose-binding domain analysis was performed with SUPERFAMILY 1.69 (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/hmm.html) (Gough et al. 2001).

RT–PCR and real-time PCR

For RT–PCR, the Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used according the manufacturer's instructions to extract total RNA of various tissues from wild-type Zhonghua 11 and *Osbc1l4* plants. Then, RT–PCR was performed according to a previously reported method (Li et al. 2009). PCR conditions included amplification at 94°C for 3 min; 28 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, and a final 5-min extension at 72°C. The primers used for RT–PCR are listed in Supplemental Table 1. PCR amplification was repeated three times, and the rice *GAPDH* gene was used as an internal control.

Real-time PCR was performed on an ABI PRISM 7500 PCR instrument (Applied Biosystems). *Ubiquitin* gene was used as the endogenous control. Real-time PCR was performed in an optical 96-well plate that included SYBR Premix EX Taq and 0.5 μl of ROX Reference Dye II (Takara, Otsu, Shiga, Japan), 1 μl of the cDNA sample, and 0.2 μM each gene-specific primer, in a final volume of

25 μl. The reactions had the following temperature profile: 95°C for 10 s, 40 cycles of 95°C for 5 s, and 60°C for 40 s. Disassociation curve analysis was performed as follows: 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. Data were collected using the ABI Prism 7500 sequence detection system following the instruction manual. The primers used in real-time PCR are listed in Supplemental Table 2. We calculated the average expression level (means ± SE) using three separate RNA extractions each with three technical repeats and a significant difference ($P < 0.05$) between wild-type and *Osbc1l4* plants was detected by *t* test.

Co-expression analysis

Co-expression data was extracted from the CREP database which calculates all Pearson correlations by selecting each gene against all genes in the microarray and the corresponding gene pairs with correlation coefficients larger than 0.8 or the highest 100 and the lowest 100 are listed (http://crep.ncpgr.cn/crep-cgi/home.pl) (Wang et al. 2010). We examined nine genes which showed highest correlation coefficients (>0.88) with *OsBC1L4*.

Subcellular location

To verify the subcellular localization of the OsBC1L4 protein, onion epidermal cells were transformed with the *Ubi*::*OsBC1L4*:*GFP* vector by gene gun (PDS-1000/He, BIO-RAD), with *Ubi*::*GFP* vector as a control. To avoid it being cleaved away during OsBC1L4 protein modification, the GFP was inserted inside the OsBC1L4 protein at the position 105 amino acids from the N-terminal. The primers used in making *Ubi*::*OsBC1L4*:*GFP* vector are listed in Supplemental Table 1. The PCR fragments were digested with *Kpn*I and *Sma*lI (Takara, Japan), and inserted into the binary vector PU1301 (which contains a maize ubiquitin promoter) in the correct direction (Li et al. 2009). The bombarded cells were incubated in the dark at 22–24°C for 12–24 h to allow transient expression of the protein and then treated with 0.5 M NaCl for 3–10 min to separate cell walls and protoplasts. The GFP location was viewed with a fluorescent microscope (DM4000B, Leica), and photographed using a camera (DFC480, Leica).

Table 1 Quantification of wall components in wild-type, mutant, and complemented plants

Sample	Wild type	*Osbc1l4* mutant	Complemented plant
Cellulose	202.4 ± 5.4	151.1 ± 3.3	203.5 ± 5.5
Pectin	8.02 ± 0.54	12.74 ± 0.15	8.36 ± 0.65
Starch	3.49 ± 0.11	14.22 ± 0.62	3.53 ± 0.06
Hemicelluloses	140.8 ± 1.7	143.3 ± 1.5	
Lignin	147.2 ± 1.1	145.7 ± 1.2	

The results are means ± SE of three independent assays. Each wall component was calculated as mg g^{-1} dry weight

Results

Phenotypic analysis of the *Osbc1l4* mutant

Previously, we produced a T-DNA insertion mutant library with about 120,000 independent transgenic lines and a flanking sequence database that contains about 15,000 flanking sequences of T-DNA (Wu et al. 2003; Zhang et al. 2007). By employing both forward- and reverse-genetics strategies to screen this mutant library, many important agronomic genes have been identified. In this study, a dwarf mutant *Osbc1l4* (*Oryza sativa brittle culm 1 like 4*) from this library was examined in detail.

The mutant *Osbc1l4* showed a typical dwarf phenotype (Fig. 1a, b). Further investigation of the mutant plants indicated that elongation of all the internodes was affected (Fig. 1c, d). However, the dwarf phenotype of the mutant was not a result of a brassinosteroid, auxin, or gibberellin deficiency, because none of these hormones added to the growth medium could rescue the dwarf phenotype (data not shown), indicating the dwarf phenotype was not caused by the mutations in hormone synthetic genes. In addition, compared with wild-type plants, the mutants also had fewer tillers.

Cellular defects in *Osbc1l4* mutants

To investigate the cellular defects of *Osbc1l4* mutants, light microscopy analysis was conducted. Some swollen epidermal cells were seen in the second internode in *Osbc1l4* plants by light microscopy, indicating that cell expansion of *Osbc1l4* plants may be affected (Fig. 2a, b). The structure of cells in the second internode of *Osbc1l4* mutants was compared with that of wild-type counterparts using scanning electron microscopy (SEM). The surface of wild-type stems was smooth, but in contrast, the epidermal cells of *Osbc1l4* mutant stems were swollen, further demonstrating that the *Osbc1l4* mutation affects the cell expansion during plant morphogenesis (Fig. 2c, d).

When we examined the transverse section of culms from mutants and wild-type plants by microscopy, we found that a large amount of starch-like particles were accumulated in the parenchyma cells of the *Osbc1l4* mutants, but not in wild-type plants (Fig. 2e, f). We performed I_2-KI staining in the geometrical center of the second internode in *Osbc1l4* and wild-type plants when internode growth

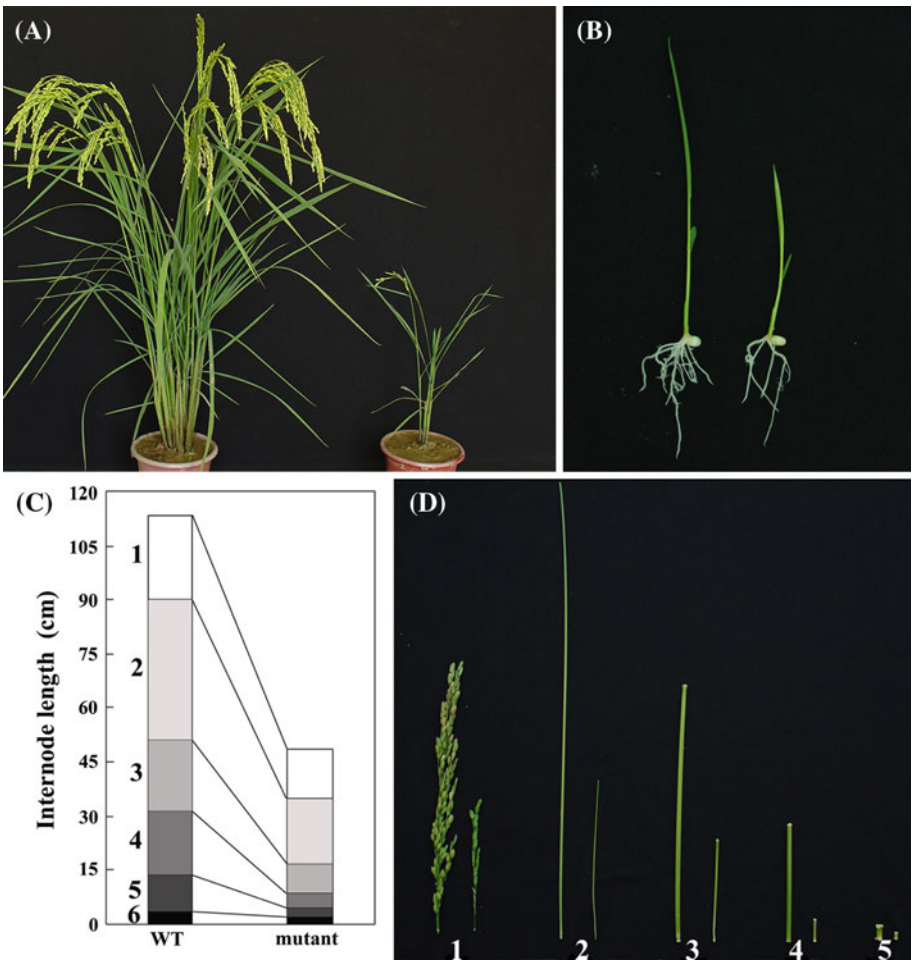

Fig. 1 Phenotype comparison between wild-type and mutant plants. Wild-type (*left*) and mutant plant (*right*) in mature stage (**a**) and seedling stage (**b**). Schematic representation (**c**) showing the average internode length of 5 biological repeats of wild-type (*WT*, *left*) and mutant plants (*right*). **d** Morphologyof wild-type (*WT*, *left*) and mutant internodes (*right*). Each internodes are numbered from the inflorescence to the base

stopped. This result indicated that these particles were indeed starch grains (Fig. 2f, insets). Taken together, these results suggest that *OsBC1L4* may be involved in sugar chain metabolism, which, in its absence, results in accumulation of starch grains in parenchyma, and loosening of the cell wall (causing the swelling observed in epidermal cells), perhaps by altering the sugar content of the cell walls (see below).

The *Osbc1l4* plants have altered cell wall composition

To investigate cell wall composition of *Osbc1l4* mutants, the contents of cellulose, pectin, starch, hemicelluloses, and lignin in the mature internodes from the *Osbc1l4* mutants and wild-type plants were compared. The results demonstrated that the amount of crystalline cellulose in *Osbc1l4* culms was reduced to 75% of those in the wild-type culms (Table 1), suggesting that the mutation in *OsBC1L4* may directly or indirectly affect cellulose synthesis in rice. In contrast, the pectin content was increased by 59% and the starch content was increased by three-fold when compared with those of wild type (Table 1). However, it was not found any obvious difference in the hemicelluloses and Klason lignin contents between the wild-type and *Osbc1l4* plants (Table 1).

The T-DNA insertion in *OsBC1l4* caused the mutant phenotype

The segregation of the self-pollinated progeny of T1 heterozygous plants of this mutant line fitted a 3:1 ratio, with 156 plants of wild-type phenotype and 44 plants of mutant appearance ($\chi^2 = 0.96$), indicating that the mutant phenotype was caused by a single recessive mutation of a single Mendelian locus.

The genomic fragment flanking the T-DNA insertion site was amplified by thermal asymmetric interlaced PCR (TAIL-PCR; Zhang et al. 2007). Blast analysis of the flanking sequence against the Rice Genome Annotation database (http://rice.plantbiology.msu.edu/index.shtml) revealed that the T-DNA was inserted in gene LOC_Os05g32110 located on chromosome 5 in the BAC clone OSJNBa0073E05. The full-length cDNA of this gene was identified as AK070472 in the KOME rice full-length cDNA database (http://cdna01.

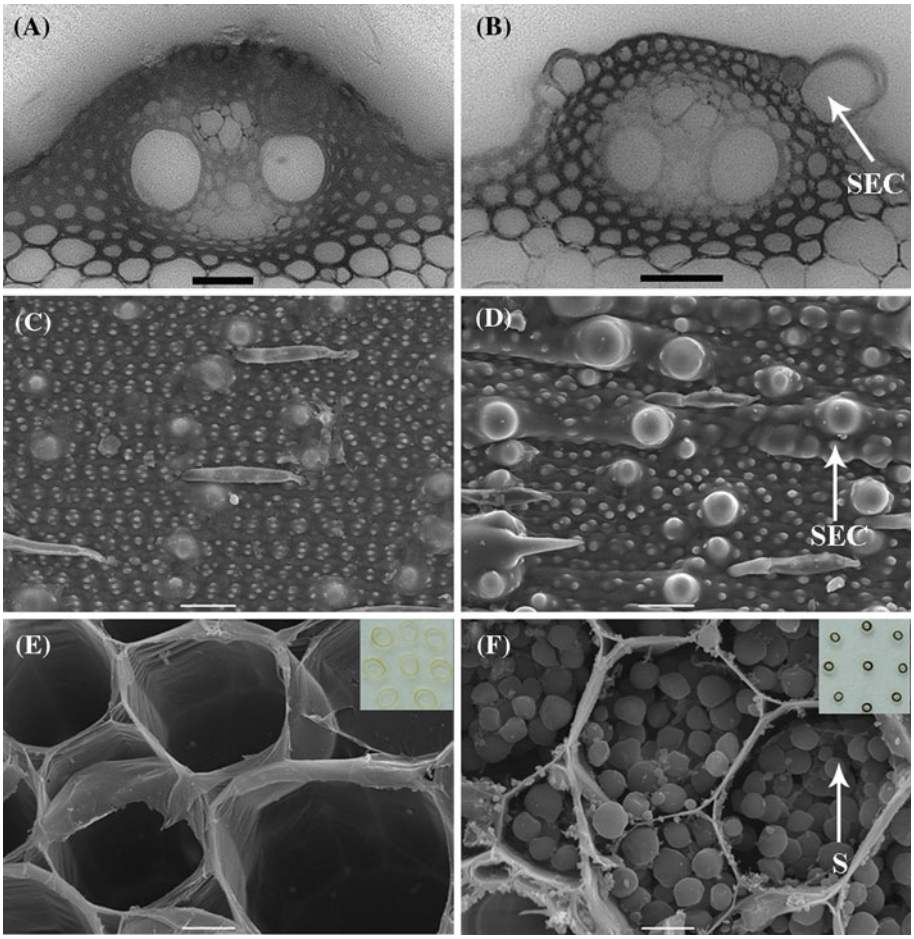

Fig. 2 Cellular analysis of the wild-type and *Osbc1l4* culms. Hand-cut sections of culms of wild-type (**a**) and *Osbc1l4* plants (**b**) showing the abnormal cell expansion. The surface of wild-type (**c**) and *Osbc1l4* culms (**d**). SEC, swollen epidermal cells. The parenchyma cells of wild-type (**e**) and *Osbc1l4* culms (**f**), indicating the accumulation of starch particles in *Osbc1l4* plants. *S* starch. Insets show the culms after I_2-KI staining. *Bars* indicate 20 μm for panels **a**, **b**, **c**, **d** and 10 μm for **e** and **f**

dna.affrc.go.jp/cDNA). The gene is composed of six exons and five introns, with a coding sequence of 1,374 bp (Fig. 3a). The T-DNA was inserted in the fourth exon, 1,955 bp downstream of the ATG start codon (Fig. 3a). The predicted protein encoded by this gene contains 457 amino acid residues. BLAST analysis indicated that the protein belonged to the *COBRA* gene family and was previously named *OsBC1L4* (Li et al. 2003; Dai et al. 2009).

To confirm that the mutant phenotype was caused by the T-DNA interruption in *OsBC1L4*, transgenic plants were subsequently analyzed in the T1 generation for cosegregation between the mutant phenotype and the T-DNA insertion in *OsBC1L4*. A population of 20 T1 plants including four mutant plants was analyzed by PCR. Among 20 plants, four which were homozygous for the T-DNA insertion (Fig. 3b) exhibited mutant phenotype, whereas the remaining 16 plants, being either heterozygous or wild-type segregants, showed the wild-type phenotype. This result suggests that the T-DNA insertion in *OsBC1L4* co-segregates with the mutant phenotype.

To further confirm that the mutant phenotype was caused by the loss of *OsBC1L4* function, a functional complementation experiment was performed. A plasmid pCg001, carrying a 6.0-kb wild-type genomic fragment containing the entire *OsBC1L4* coding sequence, 1.9-kb upstream region, and 1.2-kb downstream region was transformed into the mutant, with an empty pCAMBIA2301 vector transformed as a control (Fig. 3c). The mutant phenotype was rescued in 55 of 57 independent transgenic plants with pCg001 vector, whereas all the 30 plants with the empty vector showed the mutant phenotype (Fig. 3d). The progeny plants from the rescued single-copy transformants also demonstrated the cosegregation of wild-type phenotype with the *OsBC1L4* gene. Moreover, complemented plants showed normal cell expansion like that of wild type, as well as similar wall composition with that of wild type (Table 1). The result strongly demonstrated that the mutant phenotype is caused by the T-DNA insertion in the *OsBC1L4* gene.

OsBC1L4 encodes a putative GPI-anchored COBRA-like protein

As a member of the COBRA family, OsBC1L4 protein contains all features of a putative GPI-anchor protein (Dai

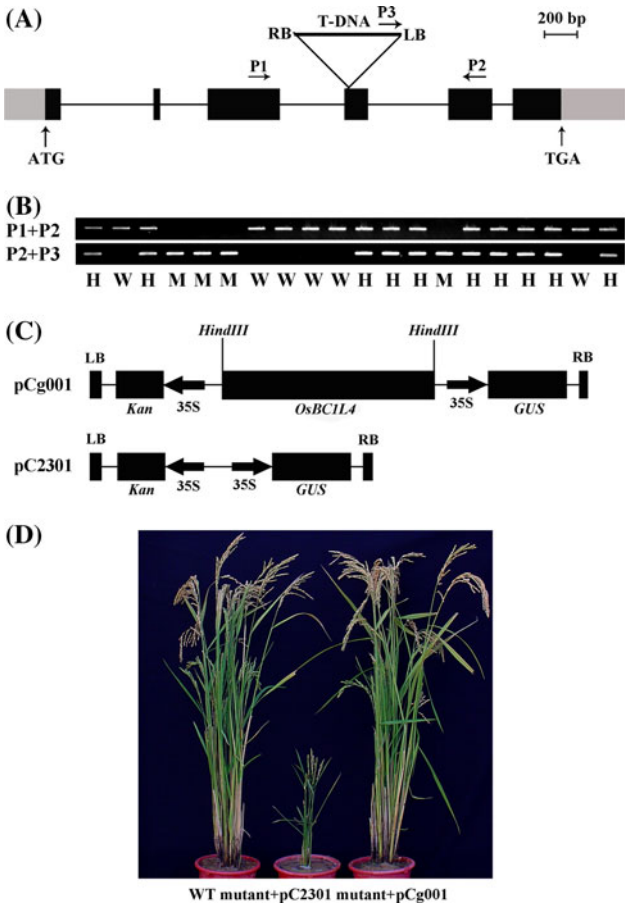

Fig. 3 Identification of the *OsBC1L4* gene. **a** Schematic representation of the intron–exon organization of *OsBC1L4*. Exons are indicated with *boxes*. *P1* reverse primer in *OsBC1L4*; *P2* forward primer in *OsBC1L4*; *P3* vector primer in T-DNA. **b** PCR analysis of the 20 T1 plants, indicating the cosegregation of the T-DNA insertion in *OsBC1L4* with the mutant phenotype. *M* homozygous for the T-DNA insertion; *W* wild type; *H* heterozygous for the T-DNA insertion. **c** Structures of pCg001 (complementation vector) and pC2301 vectors (empty vector). **d** The plant phenotypes transformed with different vectors, indicating the mutant plants transformed with the pCg001 vector were restored to normal phenotype

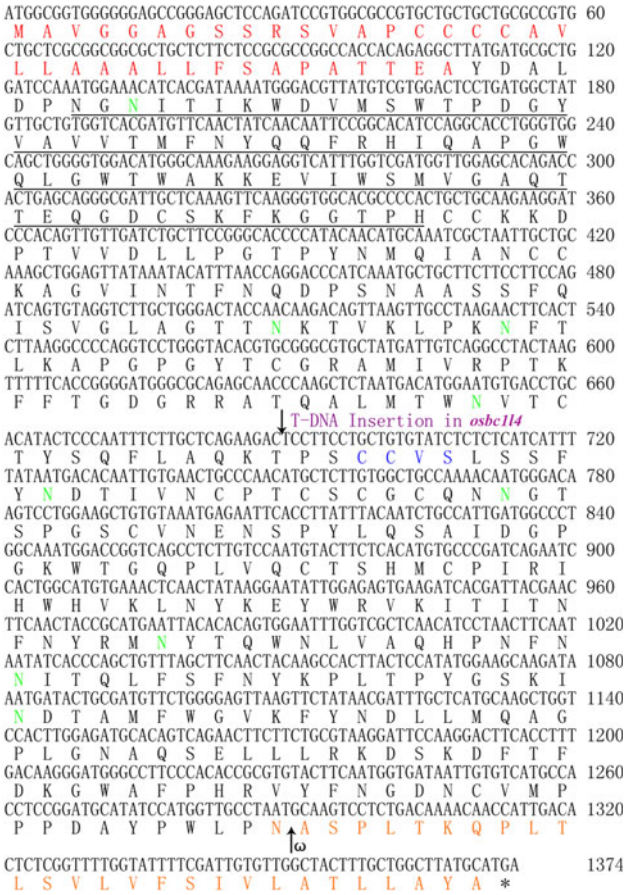

Fig. 4 *OsBC1L4* cDNA and predicted amino acid sequences. Numbers at *right* refer to the positions of nucleotides. *Red letters* indicate *N*-terminal signal peptide; *orange letters* indicate the *C* terminus, including the predicted ω-site and the hydrophobic tail; *blue letters* indicate conserved CCVS motif; *green letters* indicate the potential *N*-glycosylation sites; *purple letters* indicate the T-DNA insertion in *Osbc1l4*, with inserted site marked by an *arrowhead*. The predicted cellulose binding domain is *underlined*

et al. 2009). These features include a central hydrophilic portion located between two hydrophobic regions, an N-terminal signal peptide for secretion (amino acids 1–31), and the specific features around the ω-cleavage site for GPI processing (Fig. 4). OsBC1L4 protein also contains a central cysteine-rich domain (CCVS domain), which is conserved in the COBRA family (Fig. 4). Moreover, OsBC1L4 contains nine potential N-glycosylation sites (Fig. 4), which is a kind of common posttranslational modification of many GPI-anchored proteins and extracellular proteins (Roudier et al. 2002). Furthermore, one putative cellulose binding domain II was identified between amino acid residues 43 and 115 (E value = 0.0086; Fig. 4).

Expression pattern of *OsBC1L4*

To examine the expression pattern of *OsBC1L4* gene, RT-PCR analysis was performed with total RNA prepared from roots (tillering stage), leaves (tillering stage), sheaths (tillering stage), stems (heading stage), and panicles (heading stage) of wild-type rice plants (*Oryza sativa* L. ssp. *japonica* cv. Zhonghua 11). The analysis revealed high expression levels of *OsBC1L4* transcript in roots, stems, and panicles; relatively low expression in sheaths; and barely detectable level in leaves (Fig. 5). In addition, we did not detect the expression of *OsBC1L4* in the *Osbc1l4* mutants, indicating that *Osbc1l4* might be a null mutant (Fig. 5).

Subcellular localization of OsBC1L4

To investigate the subcellular localization of OsBC1L4 protein, a vector expressing a translational fusion between

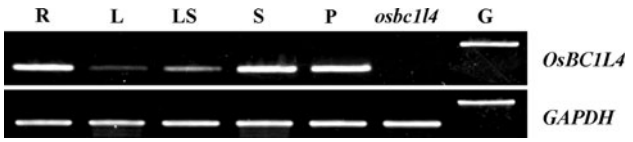

Fig. 5 RT-PCR analysis of *OsBC1L4* expression in wild-type and *Osbc1l4* mutant plants. Total RNA samples were isolated from roots (*R, tillering stage*), leaves (*L, tillering stage*), leaf sheaths (*LS, tillering stage*), stems (*S, heading stage*), panicles (*P, heading stage*) of wild-type plants and stems of *osbc1l4* mutants at the heading stage. Genomic DNA (*G*) was used as a control. *GAPDH* was used as a control for mRNA level

green fluorescent protein (GFP) and OsBC1L4 under the control of the ubiquitin promoter of maize was introduced into onion epidermal cells by gene gun. There is a putative signal peptide near the N-terminal of OsBC1L4 and a GPI-anchor site at the C-terminal, both of which might be cleaved away during the protein modification process. Therefore, the *GFP* was placed between the two putative signals, at the position of 105 amino acids from the N-terminal of OsBC1L4 (Fig. 6a). A vector that contained *GFP* alone under the control of the ubiquitin promoter was used as the control. In the onion epidermal cells bombarded with the *Ubi*::*GFP* vector, the GFP signal was observed universally throughout the cells (Fig. 6b-ii). But in the cells bombarded with the *Ubi*::*OsBC1L4*:*GFP* vector, GFP signal was found in the cell wall and plasma membrane (Fig. 6b-i). This result indicated that the OsBC1L4 protein might follow a GPI-anchored secretion path and finally localize at the cell periphery, which is similar with that of COBRA proteins (Roudier et al. 2005).

Expression association analysis of *OsBC1L4* with other genes

Using the data from the CREP database (http://crep.ncpgr.cn/crep-cgi/home.pl) (Wang et al. 2010), the correlation of the expression levels of *OsBC1L4* with that of other rice genes was examined. As shown in Table 2, *OsBC1L4* shows a high correlation coefficient (>0.88) with nine genes, five of which (*OsCESA1*, *OsCESA8*, *OsCEL1*, *OsCESA*3, and *OsCSLF6*) or their orthologs from *Arabidopsis*, barley, and maize have been implicated in cellulose synthesis (Burton et al. 2004a, b; Zhou et al. 2006; Burton et al. 2008). The correlated expression pattern of *OsBC1L4* and these cellulose-related genes further supports a role of *OsBC1L4* in cellulose synthesis in rice.

To investigate whether the expression of other cellulose-related genes is affected in *Osbc1l4* mutant plants, the expression levels of nine genes, including five cellulose-related genes with high expression correlation with *OsBC1L4* (*OsCESA1*, *OsCESA8*, *OsCEL1*, *OsCESA*3, and *OsCSLF6*), three functionally characterized *CESA* genes (*OsCESA4, OsCESA7*, and *OsCESA9*), and one starch synthase gene (*OsGBSSII*), were monitored by real-time PCR. The expression of *OsCESA1* and *OsCESA9* was significantly increased in the *Osbc1l4* mutants compared to wild-type plants ($P < 0.05$; Fig. 7a). Although the expression of several other cellulose-related genes was not significantly affected in the *Osbc1l4* mutants, all of these genes tended to have a higher expression level. The increased expression of cellulose-related genes in the *Osbc1l4* mutants might suggest a feedback mechanism during cellulose synthesis,

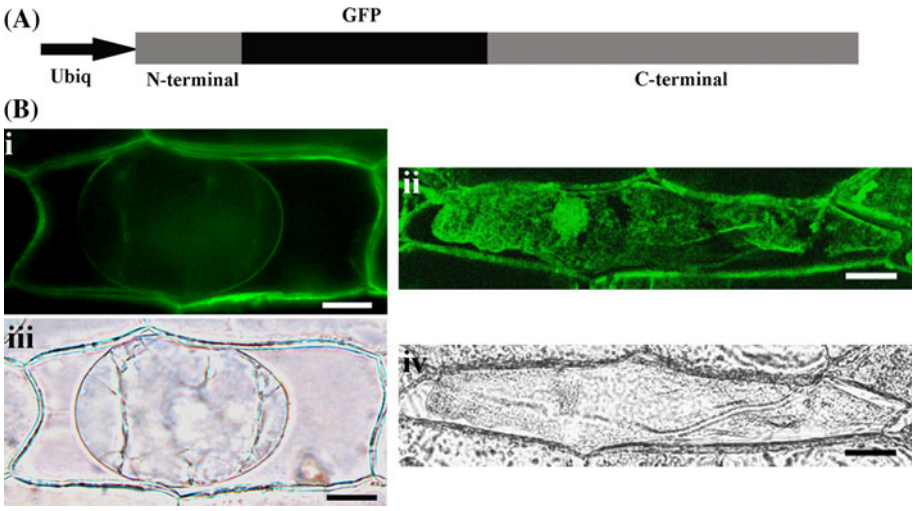

Fig. 6 Subcellular localization of OsBC1L4 protein. **a** The structure of the *Ubi*::*OsBC1L4*:*GFP* vector in which the *GFP* was inserted inside the *OsBC1L4* gene, at the position of 105 amino acids from the *N*-terminal. **b** Subcellular localization of OsBC1L4. Onion cells were treated with 0.5 M NaCl to separate cell walls and protoplasts. The *Ubi*::*OsBC1L4*:*GFP* was transiently expressed in onion epidermal cells and visualized by fluorescence microscopy **b-i**. *Ubi*::*GFP* was used as a negative control **b-ii**. Panels **b-iii** and **b-iv** are photographs of **b-i** and **b-ii**, respectively, without UV light. *Bars* indicate 50 μm

Table 2 Correlation of OsBC1L4 expression pattern with that of other genes in the rice genome

Gene Locus[a]	Correlation[b]	Annotation
LOC_Os05g08370	0.92	OsCESA1
LOC_Os07g10770	0.92	OsCESA8
LOC_Os05g04380	0.90	Peroxidase 1 precursor
LOC_Os03g52630	0.90	Endo-1,4-beta-glucanase OsCEL1
LOC_Os02g33550	0.90	VAMP protein OsSEC22
LOC_Os07g24190	0.90	OsCESA3
LOC_Os04g47520	0.89	Auxin-independent growth promoter
LOC_Os08g06380	0.89	OsCSLF6
LOC_Os06g03640	0.89	Protein binding protein

[a] Gene locus was adopted from the Rice Genome Annotation database (http://rice.plantbiology.msu.edu/)

[b] Those genes with correlation coefficient >0.88 are shown

which might be attempting to compensate for the reduced cellulose production in *Osbc1l4* mutants by increasing the expression of other cellulose-related genes. In addition, the starch synthase gene *OsGBSSII* had a trend toward lower expression in the mutants, which might be caused by the increased starch accumulation in the *Osbc1l4* mutants by another feedback pathway during starch synthesis.

Many *COBRA*-like genes have been shown to be involved in the cellulose synthesis (Roudier et al. 2002, 2005; Li et al. 2003; Brown et al. 2005a, b; Persson et al. 2005; Ching et al. 2006; Sindhu et al. 2007). The expression level of *OsBC1L1* was significantly decreased in *Osbc1l4* mutants ($P < 0.05$; Fig. 7b), whereas other *OsBC1L* genes had no significant change in expression level. However, *OsBC1*, *OsBC1L3*, *OsBC1L7*, and *OsBC1L9* tended to have a higher expression level, while *OsBC1L6* had a trend toward lower expression in the mutants (Fig. 7b).

Discussion

Mutation in OsBC1L4 caused a crystalline cellulose-deficient dwarf phenotype

Dwarf mutants have been studied in an attempt to characterize cell wall synthesis (Höfte and Staehelin 2000). Many cell wall–related dwarf mutants, such as *rsw1*, have been isolated and characterized from *Arabidopsis* (Arioli et al. 1998). The mutations of cellulose-related *KORRIGAN*, *KOBITO*, and *COBRA* genes also result in dwarfism characteristics (Nicol et al. 1998; Pagant et al. 2002; Roudier et al. 2005). In this study, *Osbc1l4* was characterized as a dwarf mutant. By measuring the crystalline cellulose content of *Osbc1l4*, we found a 25% decrease of crystalline cellulose content in *Osbc1l4* mutant plants.

OsBC1L4 is predicted to encode a GPI-anchored COBRA-like protein. The mutants of some genes required for the synthesis of GPI anchor also showed abnormal cell expansion and decreased crystalline cellulose, such as *pnt* (Gillmor et al. 2005), which might affect the formation of cellulose indirectly by participating in the posttranslational modification of GPI-anchored proteins. So far, several members of the *COBRA* gene family have been found to be involved in the synthesis of cell wall and cellulose. For example, COBRA is required in cellulose synthesis of the primary wall. Roudier et al. (2005) reported that the *COBRA* gene was required for the orientation and the synthesis of cellulose microfibrils. Several other *COBRA* family members, *AtCOBL4*, *OsBC1*, and *ZmBK2*, are required for cellulose synthesis of the secondary cell wall in *Arabidopsis*, rice, and maize, respectively (Li et al. 2003; Brown et al. 2005a, b; Persson et al. 2005; Ching et al. 2006; Sindhu et al. 2007).

The *COBRA*-like gene family is divided into two subgroups that are distinguished by an N-terminal sequence of 170 amino acids (Roudier et al. 2002). *COBRA*, *AtCOBL4*, *ZmBK2*, *OsBC1*, and *OsBC1L4* all belong to the same subgroup. Among the four functionally characterized *COBRA*-like genes in this subgroup, *OsBC1L4* showed the highest identity with *COBRA* (75% identity at the protein level). The *cobra* mutant of *Arabidopsis* involved defects in growth anisotropy (Schindelman et al. 2001; Roudier et al. 2005). In the *Osbc1l4* mutants, some epidermal cells are also swollen, which might indicate a loss of growth anisotropy, at least locally. However, *OsBC1L4* does not appear to be a functional homolog of *Arabidopsis COBRA* based on their different mutant phenotype and expression profiles.

OsBC1L4 is required for formation of primary wall

The primary wall is the determinant of cell expansion and the final cell shape and size (Kotilainen et al. 1999; Martin et al. 2001; Smith 2003). Mutations in primary wall–forming genes often lead to severe alterations in plant form (Arioli et al. 1998). Our results demonstrated that the *Osbc1l4* mutant caused abnormal cell expansion which is defined by primary cell walls and also exhibited a severe growth defect (Fig. 1). Therefore, we speculate that the *OsBC1L4* gene might be required for formation of the primary wall.

CESA genes of similar function tend to be coexpressed in the same cells (Tanaka et al. 2003; Taylor et al. 2003). Using genome-wide microarray analysis of gene expression, genes that were coexpressed with different classes of *CESA* genes were identified in *Arabidopsis* (Brown et al.

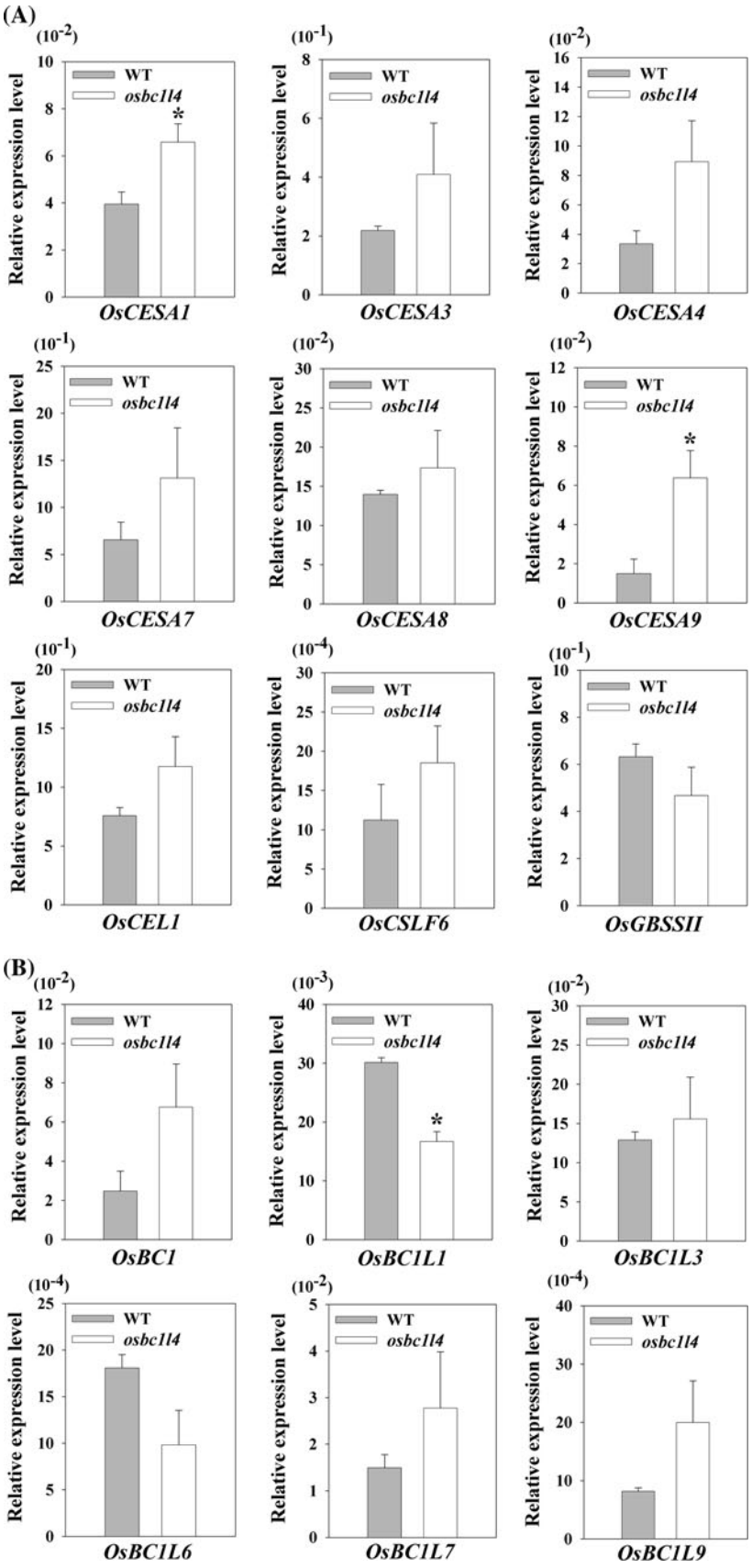

Fig. 7 The expression level of several cellulose-related and *OsBC1L* genes in the culms of wild-type and *Osbc1l4* plants. **a** Expression of several cellulose-related genes and one starch synthase gene (*OsGBSSII*) in wild-type and *Osbc1l4* plants. *OsCESA1*, *OsCESA3*, and *OsCESA8* had been implicated in primary wall synthesis, while *OsCESA4*, *OsCESA7*, and *OsCESA9* are found to participate in the synthesis of secondary wall in rice. **b** Expression level of six *OsBC1L* genes in wild-type and *Osbc1l4* plants. The average values (means ± SE) are based on three separate RNA extractions each with three technical repeats. *Asterisks* indicate that a significant difference ($P < 0.05$) was detected between wild-type and *Osbc1l4* plants

2005a, b; Persson et al. 2005). The expression of *COBRA*, which was involved in primary cell wall formation, was highly correlated with that of the primary wall–forming *CESA* genes (Persson et al. 2005; Roudier et al. 2005). By contrast, the expression of *AtCOBL4*, which participated in secondary cell wall formation, was highly correlated with that of the secondary wall–forming *CESA* genes (Brown et al. 2005a, b; Persson et al. 2005). By searching the data from the CREP database (http://crep.ncpgr.cn/crep-cgi/home.pl; Wang et al. 2010), *OsBC1* showed high expression correlation with some secondary wall–forming *CESA* genes, including *OsCESA4*, *OsCESA7*, and *OsCESA9* (correlation coefficient >0.92). Therefore, we inferred that the high expression correlation of *OsBC1L4* with *OsCESA1*, *OsCESA8*, and *OsCESA3*, whose orthologs from *Arabidopsis*, barley, and maize had been implicated in primary wall synthesis (Burton et al. 2004a, b), further supported its roles in primary wall formation.

During cellulose synthesis, *OsBC1L4* may function by interacting with the cellulose synthase complex directly or indirectly. In addition, a putative cellulose binding domain exists in the OsBC1L4 protein (Fig. 4). The homology to a cellulose binding domain is weak and this domain has not been shown to be functional, but the carbohydrate binding domain of an endo-β-1,4-glucanase *SICel9C1* from tomato was shown to interact directly with cellulose, and this interaction could be observed both for the native protein and the chimeric fusion proteins (Urbanowicz et al. 2007).

Feedback mechanisms might exist in cellulose synthesis

Structural alterations of the plant cell wall impact on many aspects of plant life, and indirect physiological alterations are usually induced in cell wall-defective mutants (Seifert and Blaukopf 2010). Many cellulose-deficient mutants, such as *rsw1*, *rsw2*, and *rsw3,* were also found to have increased starch production in *Arabidopsis* (Peng et al. 2000). In the *Osbc1l4* mutants, in addition to decreased cellulose production, increased pectin and starch contents as well as increased expression level of cellulose-related genes were also displayed. These alterations in other wall components and gene express level might function as a putative feedback mechanism which attempts to compensate for the reduced cellulose production in *Osbc1l4* mutants and restore cell wall to the normal performance. Thus, a model for the plant cell wall integrity and performance control system has been proposed (Seifert and Blaukopf 2010). It indicates that differentials in cell wall structure and mechanical performance could be detected at the plasma membrane and translated into a compensatory response at the transcriptional and posttranslational levels to restore cell wall structure and performance to the "correct" parameters (Seifert and Blaukopf 2010).

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