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Molecular characterization, expression pattern, and function analysis of the OsBC1L family in rice

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Abstract COBRA-like proteins play important roles in cell expansion and cell wall biosynthesis in *Arabidopsis*. In rice, a *COBRA*-like gene, *BRITTLE CULM1* (*BC1*), has been identified as a regulator controlling the culm mechanical strength. Analysis of the rice genome indicated that *BC1* belongs to an 11-member multigene family, termed the *OsBC1L* family in this study. Based on sequence comparisons and phylogenetic analysis, the *OsBC1L* family comprises two main subgroups. Expression patterns examined by microarray and reverse transcription polymerase chain reaction revealed that *OsBC1L* genes exhibit universal or specific expression patterns. Through T-DNA or *Tos17* insertion mutant lines, the functions of six *OsBC1L* family members have been examined by investigating the phenotype variations of knockout mutants under normal growth conditions. Results suggest that the *OsBC1L* genes perform a range of functions and participate in various developmental processes in rice.

Keywords Rice · OsBC1L family · Segmental duplication · Expression profile · Insertion mutant

Abbreviations

BC1L	BRITTLE CULM1-like
GPI	Glycosylphosphatidylinositol
CCVS domain	Cysteine-rich domain
RT-PCR	Reverse transcription polymerase chain reaction

Introduction

In the angiosperms, the cell wall can be generally categorized into two types (Carpita and McCann 2000). The type I cell wall owned by dicots and some noncommelinoid monocots contains cellulose and hemicellulosic xyloglucans in nearly equal amounts as well as abundant pectin. The type II cell wall formed in commelinoid monocotyledonous plants like rice contains 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 common to two types of cell walls.

The plant cell wall has many functions, such as providing fundamental mechanical support for the plant body and sufficient plasticity for cell expansion (Roberts 1990; Albersheim et al. 1994; McCann and Roberts 1994). The primary wall is created during cytokinesis and further modified during cell expansion while the secondary wall is laid down after cessation of cell expansion in some specialized cells. By a loosening of the cell wall rather than an increase in turgor pressure, the plant cell expands and achieves the shape suited to its biological function (Cosgrove 1993). Cell expansion can occur via diffuse growth, in which new cell wall materials are distributed across the cell surface, or via tip-directed growth, in which cell wall

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expansion is highly localized at a single site at the tip of the cell, such as the growth of root hair and elongation of pollen tube (Smith and Oppenheimer 2005). Despite some advanced research on cell wall components and structure, the mechanisms that regulate cell wall synthesis and expansion remain to be elucidated.

It was previously reported that the *COBRA* gene was an essential player in controlling the orientation of cell expansion by affecting the content and direction of cellulose in *Arabidopsis* (Benfey et al. 1993; Hauser et al. 1995; Schindelman et al. 2001; Roudier et al. 2005). The *COBRA* gene encodes a glycosylphosphatidylinositol (GPI)-anchored protein that has an N-terminal signal sequence for secretion, a hydrophilic middle, a highly hydrophobic C terminus, a CCVS (Cys-rich) 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 multigene superfamily (*COBRA*-like family), a large protein family only existing in plants (Roudier et al. 2002). There are 12 members in *Arabidopsis* (*COBRA*, *AtCOBL1–11*), 11 in rice (*BC1*, *OsBC1Lp1*, *OsBC1L1–9*), and 9 in maize (*ZmBK2*, *ZmBK2L1*, *ZmBK2L3–9*; Roudier et al. 2002; Li et al. 2003; Brady et al. 2007). Members of *COBRA*-like family are involved in diverse biological processes related to cell wall biosynthesis and cell expansion. In addition to *COBRA*, five other members of this superfamily have been identified and characterized in plants. *AtCOBL4* is required for cellulose biosynthesis in the secondary cell wall in *Arabidopsis* (Brown et al. 2005; Persson et al. 2005). *AtCOBL9* is required for tip-directed growth in root hair development in *Arabidopsis* (Parker et al. 2000; Jones et al. 2006). The rice *brittle culm1* (*bc1*) mutant and the maize *brittle stalk 2* (*bk2*) mutant were found to have mutations in the putative orthologs of *AtCOBL4* that affected the mechanical strength of plant tissues and secondary cell wall biosynthesis (Li et al. 2003; Ching et al. 2006; Sindhu et al. 2007). Moreover, recent research has indicated that *ZmBk2L1* is required for the root hair development in maize (Hochholdinger et al. 2008).

As a major cereal crop in the world, rice (*Oryza sativa*) has become a model monocot plant in genomic research because of its relatively small genome size, efficient transformation system, powerful genomics and molecular analysis tools, and abundant genetic and molecular resources. Completion of the rice genome sequence has enabled gene identification and functional analysis on a large scale. In order to elucidate the gene function in rice genome, a global effort to generate insertional mutants, mainly by T-DNA and transposable elements insertion, has been carried out and a large number of mutants has been collected in the last few years (Hirochika et al. 2004; Jeon et al. 2000; Martienssen 1998; Osborne and Baker 1995; Wu et al. 2003). Further, additional research tools of functional genomics in rice, such as full-length cDNA collection and whole genome expression profiling, have been established and have enhanced the pace for elucidating gene function throughout the genome (Zhang et al. 2008). The combination of expression profiling and insertional mutagenesis strategies has been successful in assigning functions to several *COBRA*-like genes, such as *AtCOBL4* and *AtCOBL9* (Brown et al. 2005; Persson et al. 2005; Parker et al. 2000; Jones et al. 2006). As for *COBRA*-like genes in rice, only *BC1* has been characterized and the functions of the other *OsBC1Ls* remain to be elucidated.

In order to elucidate the functions of *OsBC1L* genes systematically, we analyzed the sequence information, protein characterization, chromosome localization, and phylogenetic relationships of *OsBC1L* family members in this study. Temporally and spatially regulated expression profiles of *OsBC1Ls* were examined by microarray and reverse transcription polymerase chain reaction (RT-PCR). T-DNA or *Tos17* insertional mutants for *OsBC1L* genes were collected, and their morphologic variations were examined under normal growth conditions. Morphological assays revealed that the *osbc1l4* mutant exhibited a dwarf phenotype with a reduced number of tillers. Assays also demonstrated that the *osbc1l5* line had no homozygous segregants which might result from impaired male gametophyte transmission. Our results suggest that the *OsBC1L* family members exhibit diverse functions during the development of rice.

Materials and methods

Plant materials and growth conditions

Rice (*O. sativa*) mutant lines with insertions of T-DNA or endogenous retrotransposon *Tos17* in *OsBC1L* family members were collected from different institutes (Table 2). All the lines were planted under normal growth conditions in the experimental field of Huazhong Agriculture University in Wuhan, China, in the summer of 2007. The conspicuous morphological changes were examined among more than 20 individuals per T1 family.

Sequence and phylogenetic analyses

The protein sequences of COBRA and OsBC1 were used to search against the *japonica* rice genome annotation database (http://rice.plantbiology.msu.edu/) and the KOME full-length cDNA database (http://cdna01.dna.affrc.go.jp/cDNA/). The resulting homologous protein sequences were manually checked for existence of the feature motifs of COBRA-like proteins. A marker-based physical map of the

japonica rice chromosome was downloaded from the International Rice Genome Sequencing Project (http://rgp.dna.avrc.go.jp/IRGSP/). BACs or PACs containing *OsBC1L* genes were searched. Information about the position of the gene on the chromosome was collected from GBROWSE (http://gbrowse.ncpgr.cn/cgi-bin/gbrowse/japonica/).

Multiple alignment analyses were performed using CLUSTALX version 1.83 (Thompson et al. 1997). 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). The phylogenetic tree was constructed with MEGA3.1 (Kumar et al. 2004).

Expression profile analysis of OsBC1L family in rice

Expression profile data of the *OsBC1L* gene family in 25 tissues for Minghui 63 (*O. sativa* L. ssp. *indica*) with 2 duplications was extracted from the CREP database (http://crep.ncpgr.cn/crep-cgi/home.pl/; Wang et al., unpublished), a database of the rice transcriptome project based on Affymetrix GeneChip microarray for the three genotypes (Minghui 63, Zhenshan 97 and Shanyou 63). The normalization method used in microarray analysis is MAS5.0 and MAS5.0 calls are used to decide whether a gene is expressed (Hubbell et al. 2002). For RT-PCR, the Trizol reagent (Invitrogen) was used according the manufacturer's instructions to extract total RNA of various tissues from Zhonghua 11 (*O. sativa* L. ssp. *japonica*). Then, 3 μg of total RNA was treated with RNase-free DNase I (Invitrogen) for 15 min to eliminate contaminating DNA. First-strand cDNA was reverse transcribed from total RNA with an oligo(dT)15 primer using an M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. About 1/20 of the resulting cDNA was used as a template to amplify the transcripts with a profile. PCR amplification started at 94°C for 3 min; then, 28–36 cycles (depending on the expression levels of different genes) 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 in *OsBC1L* gene expression analysis are listed in Supplementary Table 2. Each PCR amplification was repeated twice, and the rice *GAPDH* gene was used as an internal control.

Genotyping T-DNA or *Tos17* insertion mutants

We designed gene-specific PCR primers that flanked the T-DNA or *Tos17* insertion site for genotyping analysis. By performing two sets of PCR, one using the gene-specific primer pair and the other using a gene-specific primer and a T-DNA or *Tos17* border primer, we are able to determine whether the individual is homozygous, heterozygous, or wild type for T-DNA insertion. If T-DNA or *Tos17* is inserted, the genomic primer and vector primer should have amplified a band. If the insertion is homozygous, the length between two primers is too large to be amplified using genomic primers. The PCR was performed in an ABI 9700 thermocycler (Applied Biosystem) 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 are listed in Supplementary Table 1.

Scanning microscopy

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

In vitro pollen germination

Pollen germination in vitro was performed as described previously (Han et al. 2006), with some modifications. In brief, pollen grains from dehisced anthers (wild type and mutant) were germinated on a glass slide at 35°C for 3 h in a pollen germination medium. The humidity was maintained above 90%. The grains were observed with a microscope (DM4000B, Leica, Wetzlar, Germany), using bright-field illumination.

Results

Identification and sequence analysis of the rice OsBC1L family

In order to study the molecular characterization of the rice *COBRA*-like genes and to update the work of Li et al. (2003), we used the COBRA and BC1 protein sequences to search against the rice genome annotation database release 5.0 (http://rice.plantbiology.msu.edu/) and the full-length cDNA database (http://cdna01.dna.affrc.go.jp/cDNA) and retrieved 11 putative *OsBC1L*s, including nine *OsBC1*-like (*OsBC1L*) genes; one pseudogene, *OsBC1Lp1*; and the functionally characterized *OsBC1* gene (Li et al. 2003). Although we did not find a novel *OsBC1L* gene, some information of the *OsBC1L* genes was updated according to gene prediction of the Rice Genome Annotation database

Table 1 Members of the *OsBC1L* gene family and their predicted protein structure

Gene	Gene code	Accession no. of full-length cDNA	Amino acids	N-terminal position/ probability[a]
OsBC1	Os03g30250	AK069631	468	22–23/0.993
OsBC1L1	Os03g18910	AK119578	671	23–24/1
OsBC1L2	Os03g30260	AK103650	458	29–30/0.999
OsBC1L3	Os03g54750	AK102170	457	36–37/1
OsBC1L4	Os05g32110	AK070472	457	36–37/0.999
OsBC1L5	Os06g47110		683	27–28/0.999
OsBC1L6	Os07g41310	AK121884	446	28–29/0.998
OsBC1L7	Os07g41320		473	21–22/1
OsBC1L8	Os07g49080	AK100144	675	30–31/1
OsBC1L9	Os10g35460	AK071225	425	27–28/1
OsBC1Lp1	Os04g45700		372	22–23/0.993

[a] N-terminal signal peptide was predicted using SignalP 3.0

and the collections of full-length cDNAs (Table 1). Almost all the *OsBC1L* gene transcripts are supported by their full-length cDNA, except *OsBC1Lp1*, *OsBC1L5*, and *OsBC1L7*. Several members are noted to have a slight variation of the protein sequences by comparing the full-length cDNAs with previously predicted coding sequences (Li et al. 2003). It was previously reported that OsBC1L2, OsBC1L3, OsBC1L8, and OsBC1L9 were composed of 477, 456, 672, and 683 amino acids, respectively (Li et al. 2003). However, based on the sequence of full-length cDNAs, *OsBC1L2* should be composed of six exons encoding a 458-amino acid protein, and OsBC1L3, OsBC1L8, and OsBC1L9 proteins should contain 457, 675, and 425 amino acids, respectively. In addition, the annotation for OsBC1L7 was tentatively improved according to the gene model of Rice Genome Annotation database, whereas the previously predicted protein was shortened by four amino acids (Table 1).

We analyzed the GPI-anchored characteristic of OsBC1Ls with the updated protein sequences. As shown in Table 1, all the OsBC1L homologs have an N-terminal signal peptide for secretion with a length of 20–40 amino acids. Except OsBC1L2 and OsBC1L9, all the other members of this family have a potential ω-cleavage site for GPI modification as reported by Li et al. (2003). By analyzing the hydropathy plot of this family (Kyte and Doolittle 1982), we found that most of the amino acid sequences displayed a similar profile with a central hydrophilic portion located between two hydrophobic regions. OsBC1L9, however, lacks a hydrophobic C terminal required for the cleavage during GPI linkage (Fig. 1a). This is consistent with the null ω-site, which suggests that OsBC1L9 might not be a GPI-anchored protein.

Sequence alignment of the updated OsBC1L proteins showed that most of the OsBC1L proteins, except OsBC1Lp1, contained a CCVS (Cys-rich) domain similar to COBRA (Fig. 1b). In addition, OsBC1L1, OsBC1L5, and OsBC1L8 were characterized by an additional N-terminal region of 170 amino acids (Fig. 1b), which was also found in the *Arabidopsis* AtCOBL7 subgroup and maize ZmBk2L1 subgroup (Roudier et al. 2002; Brady et al. 2007).

The duplication of *OsBC1L* genes in rice chromosomes

By analyzing the chromosomal distribution of the 11 *OsBC1L* homologs, we found that *OsBC1Lp1*, *OsBC1L3*, *OsBC1L4*, *OsBC1L5*, and *OsBC1L9* were distributed on chromosomes 4, 3, 5, 6, and 10, respectively (Fig. 2a). The position of *OsBC1L3* is different from the previous study, which has located this gene on chromosome 4 (Li et al. 2003). In addition, a group of genes (*OsBC1L1*, *OsBC1*, and *OsBC1L2*) is located on the upper arm of chromosome 3, and another group of genes (*OsBC1L6*, *OsBC1L7*, and *OsBC1L8*) is present in the lower arm of chromosome 7. Sequence identity between *OsBC1L1* and *OsBC1L8*, *OsBC1* and *OsBC1L7*, and *OsBC1L2* and *OsBC1L6* is 73, 81, and 81% at the DNA level, respectively. The rice genome annotation database (http://rice.plantbiology.msu.edu/segmental_dup/index.shtml) was used to analyze the superimposition of segmental duplication on the dataset of chromosomal location of *OsBC1L* genes. It is predicted that the formation of the three gene pairs, *OsBC1L1* and *OsBC1L8*, *OsBC1* and *OsBC1L7*, and *OsBC1L2* and *OsBC1L6* (Fig. 2a), may be due to the segmental duplication of the rice genome.

Phylogenetic analysis of the OsBC1L family

To study the evolutionary relationships of rice OsBC1Ls with all the COBRA-like proteins functionally characterized so far from other species, an unrooted tree was constructed by MEGA3.1 (Kumar et al. 2004). The results revealed that all OsBC1L proteins fell into two main subgroups which are the same as reported by Li et al. (2003)

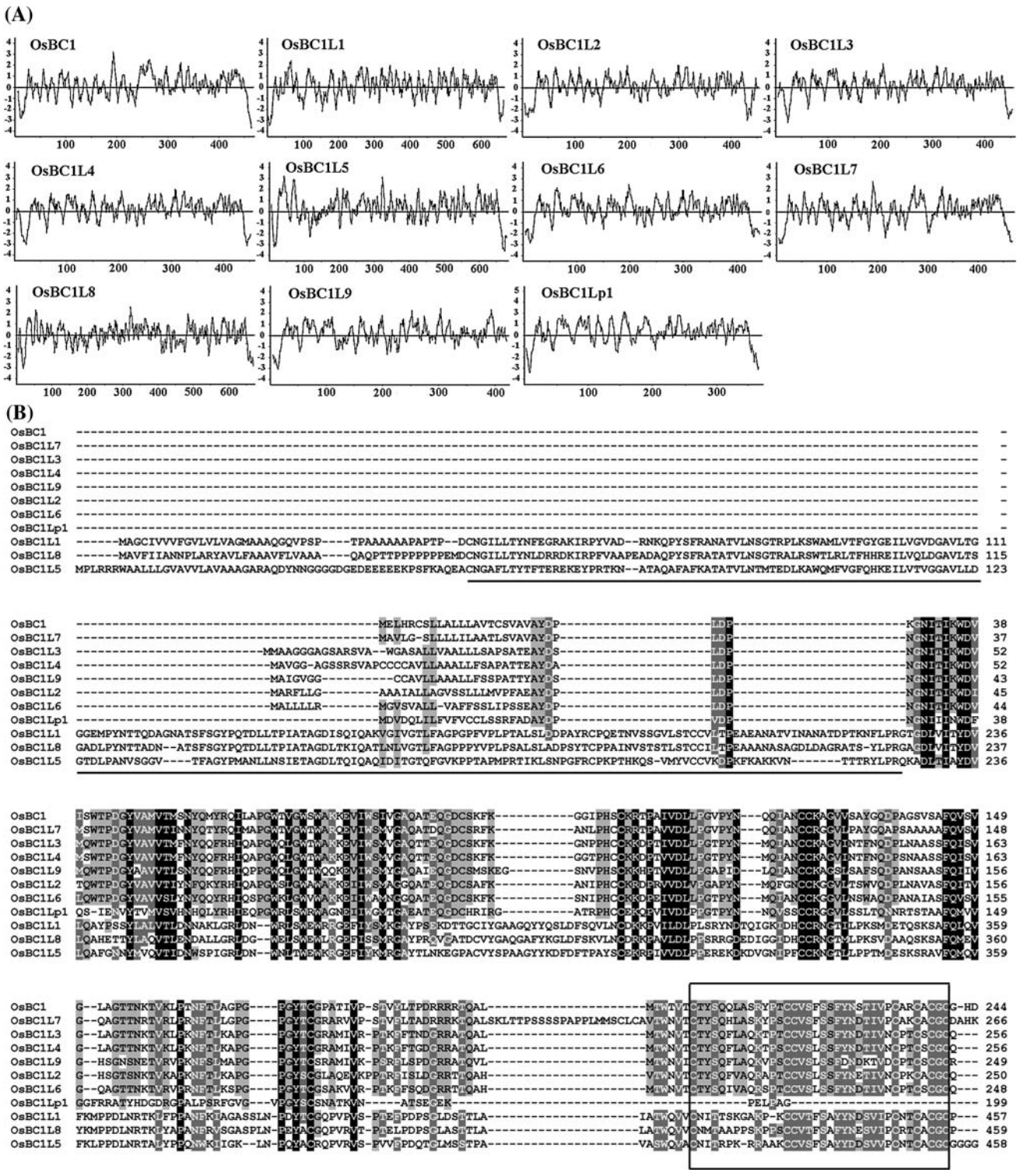

Fig. 1 Features of the rice OsBC1L proteins. **a** Hydropathy plot for OsBC1L proteins by the Kyte–Doolittle method. The *vertical axis* represents the degree of hydrophilicity (positive values) or hydrophobicity (negative values). The *horizontal axis* represents the length of the protein in amino acids. **b** Sequence alignment of the OsBC1L proteins by the CLUSTALX program. The *underlined* sequence represents the N-terminal domain specific to the subgroup II (signal peptide was excluded). The CCVS (*Cys-rich*) motif is indicated in the *box*

and Brady et al. (2007) (Fig. 2b). Subgroup I contains the most members (BC1, OsBC1L2, OsBC1L3, OsBC1L4, OsBC1L6, OsBC1L7, and OsBC1L9) showing structure similar to that of BC1. In subgroup I, BC1 and its two orthologs, AtCOBL4 and ZmBk2, are all identified as being involved in forming the secondary cell wall in rice,

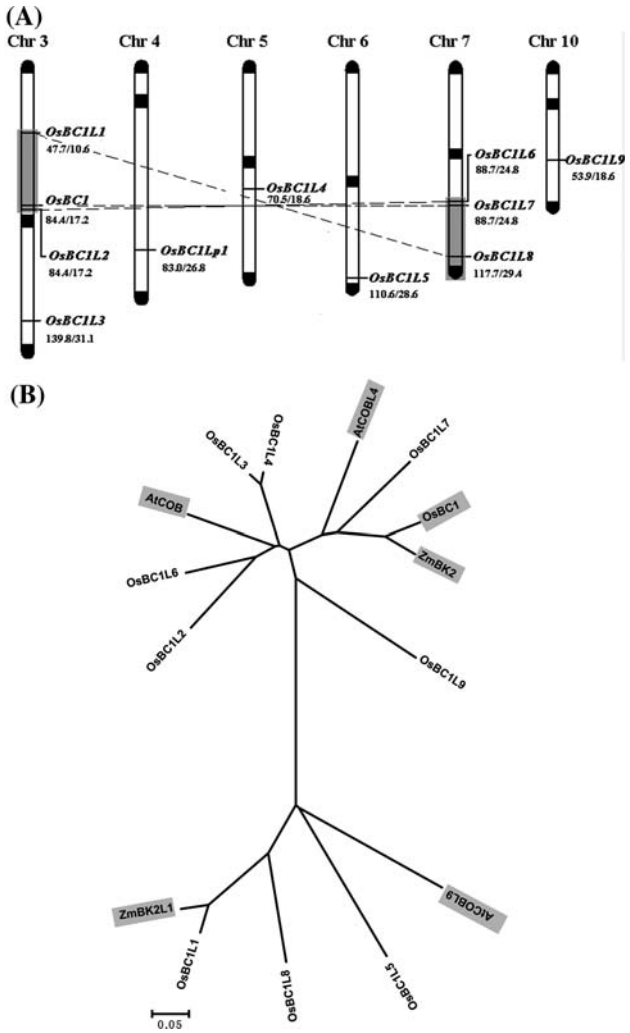

Fig. 2 Chromosomal location and phylogenetic analysis of the rice *OsBC1L* family members. **a** The distribution of *OsBC1L* genes on rice chromosomes. The telomeric and centromeric regions are represented in black. The genetic and physical distance of every gene is given in centimorgans (*left number*) and megabases (*right number*), respectively. The *gray box* indicates the possible duplicated segment on the chromosome. Duplication events are represented by *dashed lines*. **b** Phylogenetic relatedness of OsBC1L members and functionally characterized COBRA-like proteins. The unrooted tree is generated using MEGA3.1 program by the neighbor-joining (*NJ*) method. The COBRA-like proteins that have been functionally characterized are indicated in *gray boxes*

Arabidopsis, and maize, respectively, suggesting the function of COBRA-like proteins in this subclade is highly conserved among different species. Subgroup II (OsBC1L1, OsBC1L8, and OsBC1L5) is characterized by the N-terminal stretch of 170 amino acids. In subgroup II, two characterized proteins, AtCOBL9 and ZmBk2L1, are all involved in root hair development, a type of tip-directed expansion (Parker et al. 2000; Jones et al. 2006; Hochholdinger et al. 2008). However, AtCOBL9 and ZmBk2L1 are not orthologous based on their different mutant phenotype in root hair of *Arabidopsis* and maize (Jones et al. 2006; Hochholdinger et al. 2008).

Expression patterns of *OsBC1L* family members

To understand the expression patterns of *OsBC1L* genes, we firstly extracted the expression data from the CREP database (http://crep.ncpgr.cn/crep-cgi/home.pl; Wang et al., unpublished); the data had been well confirmed to reveal the expression profiles of various genes (Nayidu et al. 2008; Nuruzzaman et al. 2008; Gupta et al. 2008). The results from the database for 25 tissues of Minghui 63 (*O. sativa* L. ssp. *indica*) with two replications indicate that *OsBC1L* genes are expressed at different levels and exhibit a variety of expression patterns (Fig. 3). In general, the expression patterns of the 10 *OsBC1L* genes can be roughly classified into two categories. Category I, including *OsBC1*, *OsBC1L1*, *OsBC1L3*, *OsBC1L4*, *OsBC1L8*, and *OsBC1L 9*, has higher expression in several tissues. *OsBC1* is detected in both the vegetative tissues and the reproductive organs and has highest expression in the culms at heading stage (Fig. 3a). *OsBC1L1* is expressed in most of the tissues with the highest expression level in the flag leaves at 14 days after heading stage (Fig. 3b). *OsBC1L3* and *OsBC1L4* have similar expression patterns, with highest levels in embryos and radicles after germination and culms 5 days before heading stage, respectively (Fig. 3d, e). *OsBC1L3* also shows high expression in the sheath (Supplementary Fig. 1). *OsBC1L8* is observed to have highest expression in flag leaves at 14 days after heading stage (Fig. 3i). *OsBC1L9* is mainly expressed in vegetative tissues and shows highest level in sheath at young panicles of secondary branch primordium differentiation stage (Fig. 3j). Four genes (*OsBC1L2*, *OsBC1L5*, *OsBC1L6*, and *OsBC1L7*) belong to category II, characterized by tissue- and organ-specific expression patterns. *OsBC1L2* shows high expression in the young panicles but very low expression level in the panicles of heading stage (Fig. 3c). *OsBC1L5* shows a very high expression level only in the stamen before flowering (Fig. 3f). *OsBC1L6* is mainly expressed in endosperms of various stages (Fig. 3g). *OsBC1L7* has the highest expression in the culms at heading stage but almost no expression in the culms 5 days before heading (Fig. 3h).

In order to reveal the expression patterns of *OsBC1Ls* in the rice subspecies *japonica*, RT-PCR was performed to examine the transcription levels of *OsBC1L* genes in selected tissues from Zhonghua11 (*O. sativa* L. ssp. *japonica*): seedlings (three-leaf stage), roots (tillering stage), culms (5 days before heading stage), leaves (heading stage), sheaths (heading stage), and panicles (heading

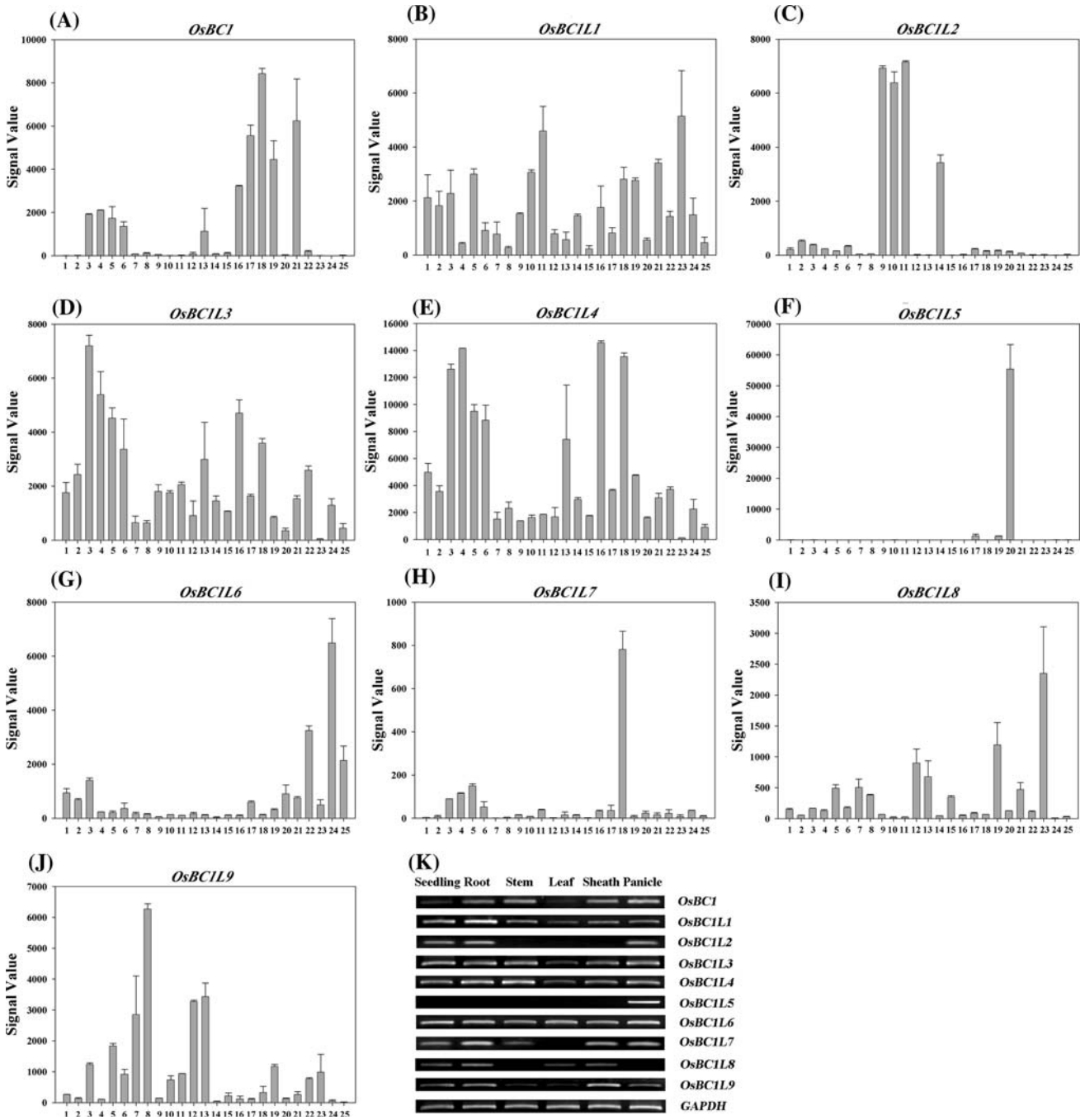

Fig. 3 The expression patterns of the *OsBC1L* family members in 25 different tissues of Minghui 63 (*O. sativa* L. ssp. *indica*) (**a**–**j**) and reverse transcription polymerase chain analysis (RT-PCR) analysis (**k**). Tissues: (*1*) seed at 72 h after imbibition; (*2*) calli at 15 days after subculture; (*3*) embryo and radicle after germination; (*4*) leaf and root at three-leaf stage; (*5*) root at seedling with two tillers; (*6*) shoot at seedling with two tillers; (*7*) leaf at young panicle of secondary branch primordium differentiation stage; (*8*) sheath at young panicle of secondary branch primordium differentiation stage; (*9*) young panicle of secondary branch primordium differentiation stage; (*10*) young panicle at pistil/stamen primordium differentiation stage; (*11*) young panicle at pollen-mother cell formation stage; (*12*) leaf at 4–5 cm young panicle stage; (*13*) sheath at 4–5 cm young panicle stage; (*14*) panicle at 4–5 cm young panicle stage; (*15*) flag leaf at 5 days before heading; (*16*) culm at 5 days before heading stage; (*17*) panicle at heading stage; (*18*) culm at heading stage; (*19*) hull at 1 day before flowering stage; (*20*) stamen at 1 day before flowering stage; (*21*) spikelet at 3 days after pollination stage; (*22*) endosperm at 7 days after pollination stage; (*23*) flag leaf at 14 days after heading stage; (*24*) endosperm at 14 days after pollination stage; (*25*) endosperm at 21 days after pollination stage. Signal value represents expression level. The *error bars* are obtained from two replications. In RT-PCR (**k**), total RNAs were from seedling (three-leaf stage), root (tillering stage), culm (5 days before heading stage), flag leaf (heading stage), sheath (heading stage), and panicle (heading stage) of Zhonghua 11 (*O. sativa* L. ssp. *japonica*). The expression level of a *GAPDH* gene was used as an internal control

stage). The result demonstrated that no clear expression specificity could be seen in *OsBC1L* genes except *OsBC1L5* which shows exclusive expression in panicles at heading stage (Fig. 3k). *OsBC1Lp1* shows no expression in any of the examined tissues by RT-PCR and microarray (data not shown), indicating it may be a putative pseudogene in rice. The expression patterns of *japonica* detected by RT-PCR are a little different from those of *indica* revealed by microarray. For example, *BC1* shows lower expression in seedlings at three-leaf stage compared with in roots at tillering stage by RT-PCR, but the similar signal values in two tissues was detected by microarray assay (Fig. 3a, k). Previous studies have verified that the microarray data in CREP database match well with the expression patterns of many genes which are tested by RT-PCR (Nayidu et al. 2008; Nuruzzaman et al. 2008; Gupta et al. 2008). Because the tissues used in microarray and RT-PCR were taken from different rice subspecies and also the tissues chosen for RT-PCR are not exactly same at developmental stages and growth conditions with those chosen for the microarray analysis, we think that it is apprehensible that there are some expression differences between microarray and RT-PCR results.

In addition, we obtained an *OsBC1L3* promoter trap line harboring GUS as a reporter, which was inserted in the third exon with the same transcriptional orientation (Fig. 4a). The GUS expression pattern mimicks the target gene in the promoter trap lines (Jeon et al. 2000; Jeong et al. 2002), and the GUS expression pattern in the *OsBC1L3* promoter trap line is similar with the results from RT-PCR and microarray analysis (mainly in culms, roots, and sheaths; Supplementary Fig. 1).

Identification and characterization of *OsBC1L* insertion mutants

To understand the biological functions of *OsBC1L* family members, we collected T-DNA or *Tos17* insertion mutant lines for *OsBC1L* genes. Individual *OsBC1L* gene sequences, including 1 kb upstream of the translation start codon ATG, the coding region, and 100 bp downstream of the translation stop codon, were used in a BLAST search against the RiceGE database (http://signal.salk.edu/cgi-bin/RiceGE/). Altogether, we obtained seven insertion mutant lines for *OsBC1L2*, *OsBC1L3*, *OsBC1L4*, *OsBC1L5*, *OsBC1L6*, and *OsBC1L9* (Table 2). We then confirmed the inserted site of these lines via PCR, using a respective pair of primers located in the gene region and the border of T-DNA or *Tos17* (Fig. 4b, c), which are listed in Supplementary Table 1. The insertion sites of most of the insertion lines, except one line (NE8527), were confirmed (Fig. 4a). In brief, the *osbc1l2* has a *Tos17* insertion in

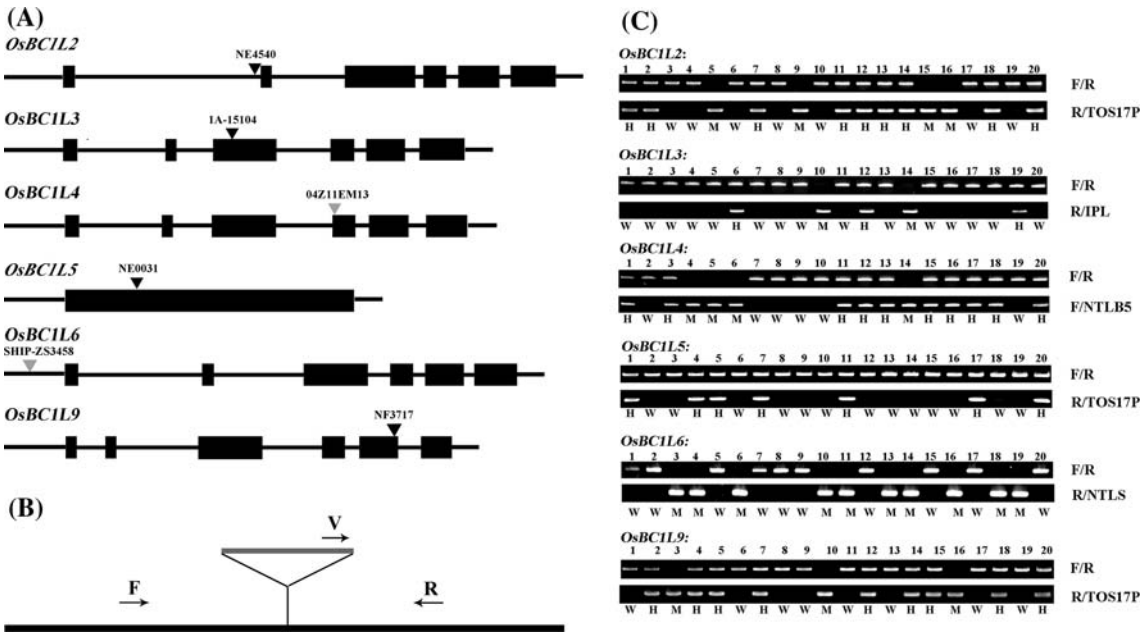

Fig. 4 Genotyping analysis of *OsBC1L* insertion mutants. **a** Locations of T-DNA or *Tos17* insertions in six *OsBC1L* genes. *Boxes* represent exons. *Gray* and *black triangles* represent the T-DNA or *Tos17* insertions, respectively. **b** Schematic diagram for genotyping T-DNA or *Tos17* inserted plants. Genomic primers (F and R), should amplify the genomic DNA size <1,500 bp; *V* vector primer (TOS17P for NE4540, NE0031, NE8527, and NF3717; IPL for 1A-15104; NTLB5 for 04Z11EM13; NTLS for SHIP-ZS3458). **c** Genotyping of *OsBC1L* insertional mutant progeny. If T-DNA/*Tos17* is inserted, the length between two genomic primers is too large to be amplified while the genomic primer and vector primer should have amplified a band. *M* homozygous for the insertion, *W* wild type, *H* heterozygous

Table 2 Collection of T-DNA or *Tos17* insertional mutants of *OsBC1L* genes in rice

Gene	Mutant line	Insertion position	Mutagen	Sources
OsBC1L2	NE4540	Intron	*Tos17*	NIAS, rice *Tos17* insertion mutant database (http://tos.nias.affrc.go.jp/)
OsBC1L3	1A-15104	Exon	T-DNA	POSTECH, rice T-DNA insertion sequence database (http://www.postech.ac.kr/life/pfg/risd/)
OsBC1L4	04Z11EM13	Exon	T-DNA	RMD, rice mutant database (http://rmd.ncpgr.cn/)
OsBC1L5	NE0031	Exon	*Tos17*	NIAS rice *Tos17* insertion mutant database
OsBC1L6	SHIP-ZS3458	Promoter	T-DNA	SIPPE, T-DNA insertion population (http://ship.plantsignal.cn/links.do)
OsBC1L6	NE8527	Not confirmed	*Tos17*	NIAS, rice *Tos17* insertion mutant database
OsBC1L9	NF3717	Exon	*Tos17*	NIAS, rice *Tos17* insertion mutant database

intron I, *osbc1l3* has a T-DNA insertion in the coding sequence of exon III, *osbc1l4* has a T-DNA insertion in exon IV, *osbc1l5* has a *Tos17* insertion in exon I, *osbc1l6* has a T-DNA insertion in the promoter region, and *osbc1l9* has a *Tos17* insertion in the coding sequence of exon V.

We determined the genotypes of the insertion mutant lines by PCR. If a T-DNA or *Tos17* insertion site is homozygous, the length between two genomic primers is too large to be amplified. In this way, we identified the homozygous plants for most of the *OsBC1L* lines, except *osbc1l5*. Most of the plants homozygous for T-DNA or *Tos17* insertion were knockout mutants because their expression was not detected by RT-PCR but was amplified in wild type. Only the expression of the *osbc1l6* mutant was not significantly affected (Supplementary Fig. 2). We investigated the phenotypes of homozygous progenies with each of the insertion lines throughout their lifecycles under normal growth conditions. However, except for *osbc1l4* and *osbc1l5* mutant lines, the insertion lines did not show any obvious phenotypic variation under normal growth conditions.

The *osbc1l4* mutant exhibited a dwarf and less tiller phenotype

The *osbc1l4* mutant line was identified from our T-DNA insertion Rice Mutant Database (RMD; http://rmd.ncpgr.cn/; Wu et al. 2003; Zhang et al. 2007). The mutant exhibited a

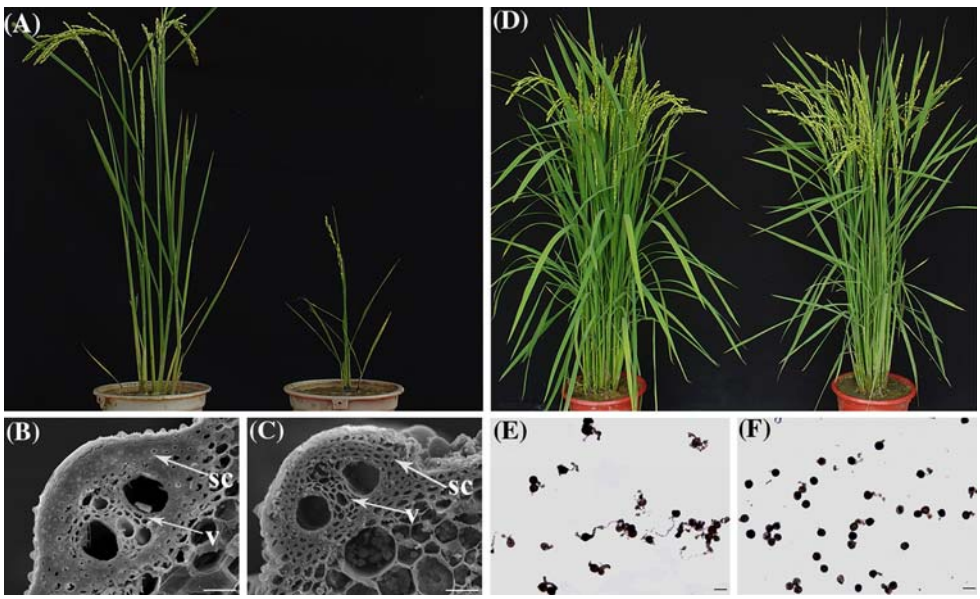

Fig. 5 Phenotype of the *OsBC1L4* and *OsBC1L5* inserted lines. **a** Wild type (*WT*; *left*) and *osbc1l4* (*right*) plants at maturity stage. Scanning electron micrographs showed the differences between sclerenchyma cells and vascular bundle in wild-type (**b**) and *osbc1l4* culms (**c**). *SC* sclerenchyma cells, *V* vascular bundles. *Bars* = 20 μm. **d** Wild type (*left*) and *OsBC1L5/osbc1l5* (*right*) heterozygous plants at maturity stage. **e**, **f** Pollen grains from wild-type (**e**) and *OsBC1L5/osbc1l5* (**f**) anthers at the mature stage were germinated for 3 h in pollen germination medium, and then observed via bright-field microscopy. *Bars* = 80 μm

dwarf phenotype with a reduced number of tillers; the plant height was about one-third that of the wild type (Fig. 5a). This line has a T-DNA insertion in the fourth exon of *OsBC1L4* (Fig. 4a). Since *OsBC1L4* belongs to the same subgroup as *OsBC1* in phylogenetic analysis (Fig. 2b), we examined secondary cell wall morphology of the mutant using scanning electron microscopy to reveal whether its secondary wall was also affected like that of *osbc1* (Li et al. 2003). The results suggested that the wild-type sclerenchyma cell walls around the peripheral vascular tissues were heavily thickened and nearly completely filled by secondary wall materials in the second internodes of mature stage rice (Fig. 5b), while the *osbc1l4* cell walls around the peripheral vascular bundles exhibited relative empty structure (Fig. 5c).

Male gametophyte transmission was specifically blocked in *osbc1l5* mutants

In the *osbc1l5* line, we could not find any homozygous plants of *Tos17* insertion when we determined the genotypes of heterozygous progeny. The heterozygous and wild-type plants segregated at a 1:1 ratio (92:100, $\chi^2 = 0.333$) in the T1 population. However, neither fertility nor seed germination was affected in *OsBC1L5/osbc1l5* heterozygous plants (Fig. 5d), which suggested that the defect was not a result of embryo death. Therefore, we infer that either the male or the female gametophyte that carries *osbc1l5* allele is defective in this *Tos17* insertion line.

To verify whether *osbc1l5* was a male gametophytic mutant or a female gametophytic mutant, *OsBC1L5/osbc1l5* heterozygous plants were reciprocally crossed with the wild type and then the genotypes of their progeny were tested. When *OsBC1L5/osbc1l5* plants were used as a female, we obtained both heterozygous and wild progeny at a 1:1 ratio (24:28, $\chi^2 = 0.308$). However, when *OsBC1L5/osbc1l5* plants were used as a male, only wild-type progeny were produced (60:0). The results suggest that the male gametophyte rather than female gametophyte that carries *osbc1l5* mutant gene has a severe defect and can not transmit the *osbcl5* allele to the progeny.

We then analyzed in vitro pollen germinability and found that most of the wild-type pollen grains germinated in a pollen germination medium that was suitable for pollen tube growth (Han et al. 2006; Fig. 5e). In contrast, only about one half of the pollen grains from *OsBC1L5/osbc1l5* anthers could germinate under the same condition (Fig. 5f). The result demonstrates that the disruption of the *OsBC1L5* gene might cause a defect in pollen germination, which prevents the *osbc1l5* allele from being transmitted to the progeny by the male gametophyte.

Discussion

The *OsBC1L* gene family in rice

The rice genome contains 11 *COBRA*-like genes. Almost all members of the rice OsBC1L protein family have the same characteristics as the COBRA family: an ω-site (except OsBC1L2 and OsBC1L9), a CCVS (Cys-rich) motif (except OsBC1Lp1), an N-terminal signal peptide sequence, highly hydrophobic N and C terminal (except OsBC1L9), and a hydrophilic middle portion. By analyzing the chromosomal distribution of the *OsBC1L* genes, we found that the two groups of genes resided within two duplicated segments present on the upper arm of chromosome 3 (*OsBC1L1*, *OsBC1*, and *OsBC1L2*) and the lower arm of chromosome 7 (*OsBC1L8*, *OsBC1L7*, and *OsBC1L6*). Recent studies have revealed that the rice genome contains 10 duplicated blocks accounting for 45% of its total size (Wang et al. 2005). Several rounds of whole genome duplication have been found in *Arabidopsis* and rice genomes as reported in previous studies (Wang et al. 2005; Lynch and Conery 2000; Simillion et al. 2002; Raes et al. 2003). Two rounds of large-scale genome duplications have been predicted in most of the rice chromosomes; one occurred around 40–50 million years ago and the other occurred before the monocot–dicot divergence at about 120–150 million years ago (Goff et al. 2002; Yu et al. 2002). Gene duplication events are important to gene family evolution, which can occur via three major mechanisms: segmental duplication, tandem duplication, and transposition events such as retroposition and replicative transposition (Kong et al. 2007). The distribution of *OsBC1L* genes on chromosomes indicates that the chromosomal segmental duplication has been an important reason for *OsBC1L* genes expansion in the rice genome evolution.

The *OsBC1L* family genes show diverse expression patterns

Our microarray data indicate that all the *OsBC1L* genes are expressed at different level and exhibit various expression patterns. Six *OsBC1L* genes are transcribed in several kinds of tissues and four *OsBC1L* genes show high expression in certain tissues (Fig. 3). In addition, it appears that in almost every tissue, at least one *OsBC1L* gene is expressed. Because the expression pattern of a gene can be indicative of its functional relevance, the wide expression patterns of some *OsBC1L* family members suggest that these genes may play regulatory roles at multiple development stages while the unique expression patterns of other members might indicate these genes participate in specific biological processes. For example, *OsBC1L5* shows high expression

in the stamen before flowering, and we have determined that it plays an important role in pollen tube growth at this stage. Although the functions of some members of the *OsBC1L* family still remain to be elucidated, we can speculate about their functions according to their universal or specific expression patterns. *OsBC1L2* is mainly transcribed in young panicles, suggesting that it might participate in the development of young panicles. *OsBC1L6* is primarily expressed in endosperm at various stages, suggesting that it might be needed in the development of endosperm. High expression level of *OsBC1L7* in the culm at heading stage might indicate that it is involved in the development of culm then.

Potential function of OsBC1L4

Of the 11 *OsBC1L* family genes in rice, only one has a known function: *BC1* was identified as controlling the mechanical strength of the rice culm (Li et al. 2003). By investigating the phenotypes of six *OsBC1L* gene insertion mutant lines, we found that most of the lines failed to show an obvious visible phenotype variation. Although it is certainly possible that phenotypic difference might be detected under stressed conditions or that there are very subtle changes in phenotype that can not be detected easily, functional redundancy among some *OsBC1L* genes is another speculation. Redundancy could occur among the most evolutionarily related *OsBC1L* genes or members whose expression pattern overlaps with each other. However, the *OsBC1L3* and *OsBC1L4* genes have high identity (93% identity at the protein level) and similar expression pattern, but the mutant in *osbc1l4* still caused an obvious dwarf phenotype, suggesting that the two genes might have obtained some functional difference after duplication. It is also possible the difference in the *osbc1l3* and *osbc1l4* phenotype is due to different positions of the insertion or the mutant phenotype of *osbc1l4* line is caused by an additional insertion.

The *osbc1l4* mutant line exhibited a dwarf phenotype with a reduced number of tillers as well as some defect in the secondary wall. So far, several members of the *COBRA* gene family have been found to be involved in the synthesis of cellulose, which is responsible for the expansion and formation of the cell wall (Schindelman et al. 2001; Li et al. 2003; Brown et al. 2005; Ching et al. 2006). In addition, *osbc1l4* shows similar mutant phenotype and expression pattern with some cellulose synthase genes (*OsCESA4*, *OsCESA7*, and *OsCESA9*; Tanaka et al. 2003). These genes also exhibited a dwarf mutant phenotype and decreased secondary wall synthesis and were expressed in seedlings, culms, panicles, and roots, but not in mature leaves (Tanaka et al. 2003). Therefore, we infer that *OsBC1L4* might be involved in the biosynthesis of cell wall and cellulose in rice.

OsBC1L5 is required for pollen tube elongation in rice

By microarray and RT-PCR analyses, we understand that *OsBC1L5* is particularly expressed at an extremely high level at the mature stamen, which indicates that it functions at that stage. In addition, we could not find any *osbc1l5/osbc1l5* homozygous plants because of defective male gametophytes. Further, we found that the pollen germination ratio of heterozygous plant was reduced almost half compared with that of wild type. As a member of clade II in the *OsBC1L* gene family, *OsBC1L5* shows higher similarity to *AtCOBL9* and *ZmBk2L1*, which are required for the growth of root hair in *Arabidopsis* and maize, respectively. It has been reported that both root hair and pollen tube elongation represent tip-directed growth, a situation characterized by locally focused deposition of cell wall material (Brady et al. 2007). Therefore, we speculate that *OsBC1L5* might also participate in the tip-directed cell wall expansion and be required for the elongation of the pollen tube in rice.

In conclusion, our study provides an updated annotation of COBRA superfamily in rice. Sequence and phylogenetic analyses benefit the function elucidation for the *OsBC1L* genes. Expression profile and phenotype analyses of six new knock-out mutants indicate that *OsBC1L* genes perform diverse roles during the development of rice. The construction of double and triple mutant plants among *OsBC1L* members and the collection of more *OsBC1L* mutants will drive future approaches to elucidate COBRA protein function in rice. More investigation is also needed to reveal the detail functions of OsBC1L4 and OsBC1L5 by examination of the confirmed mutants.

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