Brittle Culm15 Encodes a Membrane-Associated Chitinase-Like Protein Required for Cellulose Biosynthesis in Rice*1[C][W][OA]

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Plant chitinases, a class of glycosyl hydrolases, participate in various aspects of normal plant growth and development, including cell wall metabolism and disease resistance. The rice (Oryza sativa) genome encodes 37 putative chitinases and chitinase-like proteins. However, none of them has been characterized at the genetic level. In this study, we report the isolation of a brittle culm mutant, bc15, and the map-based cloning of the BC15/OsCTL1 (for chitinase-like1) gene affected in the mutant. The gene encodes the rice chitinase-like protein BC15/OsCTL1. Mutation of BC15/OsCTL1 causes reduced cellulose content and mechanical strength without obvious alterations in plant growth. Bioinformatic analyses indicated that BC15/OsCTL1 is a class II chitinase-like protein that is devoid of both an amino-terminal cysteine-rich domain and the chitinase activity motif H-E-T-T but possesses an amino-terminal transmembrane domain. Biochemical assays demonstrated that BC15/OsCTL1 is a Golgi-localized type II membrane protein that lacks classical chitinase activity. Quantitative real-time polymerase chain reaction and β-glucuronidase activity analyses indicated that BC15/OsCTL1 is ubiquitously expressed. Investigation of the global expression profile of wild-type and bc15 plants, using Illumina RNA sequencing, further suggested a possible mechanism by which BC15/OsCTL1 mediates cellulose biosynthesis and cell wall remodeling. Our findings provide genetic evidence of a role for plant chitinases in cellulose biosynthesis in rice, which appears to differ from their roles as revealed by analysis of Arabidopsis (Arabidopsis thaliana).

Glycosyl hydrolases (GHs), the large family of enzymes that trim carbohydrates, are ubiquitous in plants and carry out essential roles in various metabolic and physiological processes. These include the metabolism of cell wall polysaccharides and glycolipids and the regulation of plant defense and secondary metabolism (Minic, 2008). The Arabidopsis (Arabidopsis thaliana) genome contains 379 genes believed to encode GHs, and they are clustered into 29 families (Henriassat et al., 2001), and the relative information has been continuously updated in the CAZy database (Cantarel et al., 2009). The rice (Oryza sativa) genome possesses a significantly different number of GHs than that of Arabidopsis, consistent with the very different carbohydrate composition of cell walls of the two species (Yokoyama and Nishitani, 2004). The large number of GHs suggests that they represent a broad range of substrate specificities. The targeting substrates of GHs provide critical clues regarding their enzyme activities and functions (Lopez-Casado et al., 2008; Minic, 2008). For example, the observation that KORRIGAN1 (KOR1), an endo-1,4-β-glucanase that belongs to GH9, degrades β-glucan with an unbranched β-(1→4)-linked backbone, which is the basic structure of cellulose (Nicol et al., 1998; Mølhøj et al., 2001), strongly suggesting that KOR1 is involved in cellulose biosynthesis. However, there is very little biochemical information for the vast majority of plant GHs, which makes it extremely challenging to understand the physiological and developmental functions of GHs.

Besides their substrates, the subcellular localization of GHs also provides important clues regarding their likely functions. Consistent with the multiple roles of GHs, their cellular localization patterns range from the cytoplasm to the extracellular matrix, vacuole, endoplasmic reticulum, and peroxisome (Roitsch and González, 2004; Lipka et al., 2005; Mega, 2005; Minic and Jouanin, 2006). Approximately 52% of Arabidopsis GHs are
predicted to be localized in the cell wall, where they likely participate in cell wall remodeling (Showalter, 1993; Minic, 2008). This is consistent with proposals that most plant GHs participate in the metabolism of cell wall polysaccharides (Cosgrove, 2005; Minic and Jouanin, 2006). β-D-Galactosidase degrades the galactan side chain prior to cell elongation (Martin et al., 2005). Xyloglucan endotransglycosylase/hydrolase (XET) is involved in the remodeling of xyloglucan and is required for wall loosening during plant cell expansion (Fry et al., 1992; Rose et al., 2002; Fry, 2004). Arabidopsis Apoplastic Xylosidase1 (XYL1/AXY3) releases xylosyl residues from xyloglucan and regulates cell wall structure (Günl and Pauly, 2011). Proteomic analysis of extracellular proteins suggests a complicated regulatory network mediated by cell wall GHs (Chivasa et al., 2002; Jamet et al., 2006; Zhu et al., 2006; Minic et al., 2007). However, the number of identified GHs is too small to describe the complicated cell wall remodeling network in any detail.

Several key questions surround plant chitinases, a group of relatively well-studied enzymes that are proposed to catalyze the hydrolysis of N-acetylglucosamine (GlcNAc) 1,4-linkages in chitin. The first question concerns the authentic in vivo substrates of plant chitinases. Because plants are devoid of chitin, the substrates of plant chitinases may differ from those of fungal chitinase (De Jong et al., 1995). A few studies have suggested that the likely substrates of plant chitinases may be arabinogalactan protein, chitooligosaccharides, N-acetylchitooligosaccharides, and other GlcNAc-containing glycoproteins or glycolipids (Wojtaszek and Bolwell, 1995; van Hengel et al., 2001; Zhong et al., 2002). Here, we report the isolation of a rice brittle culm mutant (bc15) and the characterization of its corresponding gene, BC15/OsCTL1. The findings demonstrate that BC15/OsCTL1 is a Golgi-localized membrane protein without classical chitinase activity and is required for cellulose biosynthesis in rice.

RESULTS

The bc15 Mutant Has Reduced Mechanical Strength and Cellulose Content

The rice mutant bc15 was isolated from the embryogenic callus of the japonica rice cv Zhonghua 8. It was identified as a recessive mutant based on the segregation ratio in an F2 population generated by crossing bc15 with the indica rice cv TN1 (Supplemental Table S1). Internodes and leaves of the mutant plants are easily broken (Fig. 1, A and B). Quantification of the break force showed that the strength required to break internodes of bc15 was reduced by approximately 20% of that needed to break internodes of wild-type plants and that the strength required to break leaf blades was decreased by approximately 55% in bc15 relative to wild-type plants (Fig. 1, C and D). In addition to the reduced mechanical strength, the mutant plants have a slightly smaller stature than wild-type plants, although the mutation does not substantially reduce plant growth (Fig. 2C).

We next performed microscopy analysis to investigate the underlying cause of the observed brittleness. Analysis of cross-sections through comparable internode samples revealed that the wall thickness of sclerenchyma cells is thinner in internodes of bc15 plants than in those
of wild-type plants, although the anatomic structure of the mutant internodes is comparable to that of wild-type plants (Supplemental Fig. S1). Transmission electron microscopy further confirmed that sclerenchyma cells in the mutant plants had thinner cell walls than those of wild-type plants, although there were no discernible differences in cell wall structure evident using microscopy (Fig. 1, E–H). Given that the change of wall thickness may reflect differences in cell wall composition, we next examined the cell wall composition of comparable tissues harvested from the internodes of bc15 and wild-type plants. As shown in Table I, the cellulose content was substantially decreased by approximately 23% in bc15 relative to wild-type plants, whereas Ara and Xyl contents were dramatically increased by 58% and 77%, respectively. The contents of other neutral sugars and lignin differed only slightly between bc15 and wild-type samples.

We next used several monoclonal antibodies directed against cell wall polysaccharides for in situ analysis of changes in the levels of neutral sugars in different cell types in the wild-type and mutant internodes. LM10 and LM11 are two antibodies recognizing xylan, and antibody LM6 labels arabinan. All three antibodies generated stronger fluorescent signals in the mutant sclerenchyma cells than in wild-type plants (Supplemental Fig. S2, A–F). However, when labeled with either of the antibodies MLG and LM15, which target β-1,3-1,4-glucan and xyloglucan, respectively, the level of signal, which was generated primarily in parenchyma cells, could not be distinguished between bc15 and wild-type plants (Supplemental Fig. S2, G–J). Wiesner staining, which reflects the total lignin content, did not reveal any evidence of ectopic deposition of lignin or alterations in the abundance of lignin in bc15 internodes relative to internodes from wild-type plants (Supplemental Fig. S2, K and L).

Taken together, our findings indicated that the inferior mechanical strength in bc15 correlates with reduced cellulose content in the mutant.
Map-Based Cloning of the bc15 Locus

We conducted map-based cloning to identify the defective gene responsible for the bc15 phenotype. After screening with 70 simple sequence repeat markers that distribute evenly across the 12 rice chromosomes, the mutant locus was mapped between markers RM242 and RM160 on chromosome 9 (Fig. 2A). Fine-mapping further placed this locus in the 114-kb region between markers M2 and M3, which includes 17 predicted ORFs. After sequencing all of the candidate genes, a missense mutation was found in an ORF, which was annotated as LOC_Os09g32080 by the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and as Os09g0494200 in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). This mutation changes a highly conserved Ala residue at position 213 in the predicted protein to Leu (Fig. 2A; Supplemental Fig. S3A).

In an attempt to complement the bc15 mutation, we introduced a 3,874 bp genomic DNA fragment containing the putative promoter, the coding region, and the 3’ untranslated region (pBC15F; Fig. 2B) into the bc15 mutant background. We evaluated the phenotypes of the obtained transgenic plants. Complete rescue of the mechanical strength and cell wall composition phenotypes in the transgenic bc15 mutants provides compelling evidence that LOC_Os09g32080 is the BC15 gene responsible for the mutant phenotypes described above (Fig. 2C; Table I). In addition, the mutant backgrounds of transgenic plants expressing pBC15F were confirmed by using a BstUI-cleaved amplified polymorphic sequence marker (Fig. 2D).

BC15 Encodes a Chitinase-Like Protein

The coding sequence of BC15 (981 nucleotides) encodes a protein comprising 326 amino acids. Pfam analysis showed that BC15 belongs to the GH19 family (EC 3.2.1.14). Plaza 2.5 software further identified 27 orthologs (ORTHO004207) and 306 homologs (HOM000272) of BC15 in different plant species. Because all of them are plant chitinases,

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**Table I. Cell wall composition analysis of internodes of wild-type and bc15 plants**

Alcohol insoluble residues were prepared from the second internodes of bc15, wild-type, and complementary transgenic plants as described in Supplemental Materials and Methods S1. The alditol derivatives were analyzed by gas chromatography-mass spectrometry for glycosyl residue composition. The results are given as means ± s.e. of three independent assays. Cellulose and lignin contents were measured as described in Supplemental Materials and Methods S1. Asterisks indicate significant differences (t test at P < 0.01) when compared with the wild type (n = 4).

| Sample    | Rha    | Fuc    | Ara    | Xyl    | Man    | Gal    | Glc    | Cellulose | Lignin |
|-----------|--------|--------|--------|--------|--------|--------|--------|-----------|--------|
| Wild type | 1.1 ± 0.0 | 0.6 ± 0.0 | 17.2 ± 0.5* | 143.4 ± 5.9* | 0.9 ± 0.0 | 6.2 ± 0.2 | 33.8 ± 1.4 | 347.6 ± 6.8* | 160.2 ± 4.3 |
| bc15      | 1.3 ± 0.0 | 0.6 ± 0.0 | 27.1 ± 0.4* | 253.7 ± 9.0* | 1.0 ± 0.0 | 8.4 ± 0.2 | 33.0 ± 1.5 | 265.2 ± 3.6* | 173.5 ± 1.8 |
| pBC15F    | 1.2 ± 0.0 | 0.6 ± 0.0 | 21.1 ± 0.3* | 163.3 ± 2.9* | 1.0 ± 0.0 | 8.1 ± 0.2 | 47.8 ± 1.7 | 366.6 ± 5.9* | 141.6 ± 3.3 |

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**Figure 3.** BC15/OsCTL1 is an N-glycosylated membrane protein. A, Prediction of the domain structure of BC15/OsCTL1. TM, Transmembrane domain. B, Protein gel blotting of BC15-GFP in the fractionated total proteins with anti-GFP antibody. The marker protein antibodies that recognize CESA9, HSP, and XET5 were used for monitoring the success of fractionation. S, Supernatant; TM, total membrane; CW1, cell wall fraction extracting with CaCl2; CW2, cell wall fraction extracting with LiCl. C, Extraction of the total membrane fraction of transgenic plants expressing BC15-GFP with different chemicals. Each treatment was separated into supernatant (S) and microsomes (M) and probed with anti-GFP and OsCESA9 antibodies. D, Protein gel blotting BC15-GFP in the absence (−) and presence (+) of PNGase F.
BC15 thus encodes a plant chitinase. To check whether BC15 preserves the catalytic and substrate-binding domains of chitinases, we aligned the sequences of BC15 and its orthologs. As shown in Supplemental Figure S3A, BC15 possesses the conserved signatures of GH19. One common signature of GH19 proteins is devoid of the N-terminal Cys-rich region that is considered for chitinase binding. Moreover, the H-E-T-T motif required for catalytic activity is replaced by the S-K-T-S motif. Construction of a phylogenetic tree that includes all of the BC15 orthologs and its homologs from rice and Arabidopsis (Supplemental Fig. S3B) indicated that the closest ortholog of BC15 is AtCTL1. Therefore, BC15 was designated as Chitinase-Like Protein1 (OsCTL1) and will henceforth be referred to as BC15/OsCTL1 in this paper.

BC15/OsCTL1 Is an N-Glycosylated Membrane Protein

Plant chitinases often target the extracellular matrix or various organelles. To investigate the locations wherein BC15/OsCTL1 exists, we used the TMHMM 2.0 and SignalP 4.0 software to predict whether BC15/OsCTL1 has any localization motifs. These analyses indicated that BC15/OsCTL1 is a classical type II membrane protein and possesses an N-terminal transmembrane domain (Fig. 3A; Supplemental Fig. S4A). To verify this prediction in vivo, we generated transgenic bc15 mutant plants that express GFP-fused BC15/OsCTL1 (pBC15GFP; Fig. 2B). The resulting construct fully rescued the mutant phenotypes (Supplemental Fig. S4, C and D), indicating that the tagging with GFP does not affect BC15/OsCTL1 function. We extracted the total proteins from pBC15GFP transgenic plants, fractionated them into soluble, total membrane, and cell wall fractions, and probed them using anti-GFP antibody; BC15/OsCTL1 was mainly detected in the membrane fraction (Fig. 3B), suggesting that BC15/OsCTL1 is membrane associated. This result prompted us to treat the membrane fraction with several chemicals. As shown in Figure 3C, BC15/OsCTL1 was partially solubilized following Triton X-100 treatment. BC15/OsCTL1 is thus an integral membrane protein. In addition, the Prositescan algorithm predicts that BC15/OsCTL1 has three N-glycosylation sites. Changes in the extent of migration of BC15/CTL1-GFP on a SDS-PAGE gel after treatment with PNGase F, an amidase widely used to remove N-linked glycans from glycoproteins, suggested that BC15/OsCTL1 is N-glycosylated (Fig. 3D).

BC15/OsCTL1 Is Localized in the Golgi Apparatus with Its C-Terminus Facing the Golgi Lumen

To determine whether BC15/OsCTL1 resides on the plasma membrane or in intracellular compartments,
we further separated the total membrane fraction isolated from pBC15GFP transgenic plants into the plasma membrane and endomembrane fractions. Protein gel blotting showed that BC15/OsCTL1 is predominantly present in the cellular endomembrane systems (Fig. 4A). We then investigated whether BC15/OsCTL1 targets a particular subcellular organelle. Signal peptide (SP) sequences provide valuable clues regarding the subcellular localization of chitinases. In contrast to AtCTL1, which has a secretion SP at the N terminus (D score = 0.865 in the SP prediction by SignalP 4.0), BC15/OsCTL1 lacks this motif (D score = 0.277; Supplemental Fig. S4B), suggesting that BC15/OsCTL1 and AtCTL1 have different subcellular localization patterns. We viewed the root epidermal and coleoptile cells of plants expressing the pBC15GFP transgene, and the dot-like fluorescent signals were observed in those living cells (Fig. 4B; Supplemental Fig. S4, E and F). The dot-like signals were almost identical to those labeled by a Golgi marker, while we coexpressed CTL1-GFP and Man49-mCherry in rice protoplast cells (Fig. 4C). Therefore, OsCTL1 is localized in the Golgi apparatus. As a type II membrane protein, the C terminus of BC15/OsCTL1 that contains a catalytic domain is proposed to face the Golgi lumen. To verify this topological structure, we treated the membrane extracts from plants expressing BC15/OsCTL1-GFP with proteinase K in the presence or absence of the detergent Triton X-100. Immunoblotting assays revealed that GFP fused to the C terminus of BC15/OsCTL1 was sensitive to proteinase K treatment in the presence of Triton X-100 (Fig. 4D), indicating that the C terminus of BC15/OsCTL1 faces the Golgi lumen.

**BC15/OsCTL1 Is Ubiquitously Expressed in Many Organs**

The expression pattern of BC15/OsCTL1 was analyzed by quantitative real-time (qRT)-PCR using RNAs isolated from various organs from wild-type plants. As shown in Figure 5A, BC15/OsCTL1 is expressed in several organs, with relatively high levels in the internodes and nodes. This expression pattern fits with the brittle culm phenotype of bc15. To further examine its expression at the tissue level, the promoter of BC15/OsCTL1 was fused to the GUS reporter gene and the construct was expressed in the wild-type plants. We detected GUS activity in the coleoptile, roots, shoots, and lamina joints of transgenic seedlings (Fig. 5B–E). Careful observation revealed stronger GUS-derived signal in the elongation zone of the roots than in the root tips (Fig. 5C) and strong GUS-derived signal in the vascular bundles of the root mature zone (Fig. 5D). GUS activity was also observed in the panicle branches and glumes of mature plants (Fig. 5F). Fresh hand-cut sectioning of the stained internodes clearly

**Figure 5.** Expression pattern of BC15/OsCTL1. A, qRT-PCR analysis of BC15 expression in various rice organs, using the Ubiquitin5 (UBQ5) gene as an internal control. B to G, GUS activity staining assay in various organs of BC15pro::GUS transgenic plants, showing GUS signals in a rice seedling (B), including the elongation root regions (C), the vascular bundles of root mature regions (D), and the leaf lamina joint (E). GUS activity was also observed in mature plants, such as in flower branches and glumes (F) and vascular bundles of mature internodes (G). Arrows show the GUS activity signal. Bars = 1.0 cm (B, E, and F) and 100 μm (C, D, and G).
showed strong GUS activity in the vascular bundles (Fig. 5G). Therefore, BC15/OsCTL1 is ubiquitously expressed, especially in the tissues that confer mechanical strength in fully developed rice plants.

BC15/OsCTL1 Does Not Function as a Classical Chitinase

Characterization of the enzyme activities of plant chitinases is critical for elucidation of their biological functions. We first chose a yeast assay system to investigate the possible substrates of BC15/OsCTL1. The cts1 mutant of *Saccharomyces cerevisiae*, a deletion strain mutated in the yeast chitinase CTS1, is defective in cell separation (Kuranda and Robbins, 1991). Expression of BC15/OsCTL1 in this strain could not rescue the cell separation defect (Fig. 6). Next, we purified recombinant BC15/OsCTL1 in *Escherichia coli* and assayed its enzyme activity. As shown in Table II, no obvious reaction products were detected when we incubated the recombinant BC15/OsCTL1 protein with any of the three 4-methylumbelliferone-labeled variants of GlcNAc, chitobiose, or chitotriose. Furthermore, we isolated microsomes from wild-type, mutant, and transgenic plants overexpressing pBC15GFP for chitinase activity assays. Although the enzyme activities were detected by using the above three artificial substrates, the activities were indistinguishable between microsomes isolated from the bc15 mutant and those from wild-type or transgenic plants (Table II). Therefore, BC15/OsCTL1 does not possess classical chitinase activity.

Comparison of Genome-Wide Expression Profiles of bc15 and Wild-Type Plants

To elucidate the molecular mechanism(s) by which BC15/OsCTL1 regulates the mechanical strength of rice plants, we used Illumina RNA sequencing to investigate the global expression profiles of wild-type and bc15 internodes. Mapping the sequencing reads to

| Sample          | Recombinant Proteins in E. coli | Microsomes Isolated from Different Plants |
|-----------------|--------------------------------|------------------------------------------|
|                 | Control                        | CTL1                                    | Wild Type       | bc15        | BC15-GFP     |
| 4MU-GlcNAc      | 5,845.4                        | 0                                       | 26,064.6        | 19,998.5    | 28,195.8     |
| 4MU-chitobiose  | 2,948.8                        | 0                                       | 1,030.6         | 966.4       | 1,021.1      |
| 4MU-chitotriose | 1,645.4                        | 0                                       | 1,356.8         | 1,437.9     | 1,435.7      |
rice reference complementary DNAs (cDNAs; Michigan State University Rice Genome Annotation Project Release 6.1) generated 10.21 and 10.89 million unique reads from 11.78 and 12.46 million clean reads for wild-type and bc15 plants, respectively (Fig. 7A). The sequencing quality is reliable because the detected gene number was saturated, and the reads were distributed uniformly at random on reference genes (Supplemental Fig. S5). Of the 28,342 genes shown to be expressed in either wild-type or bc15 plants, we detected 1,064 differentially expressed genes (DEGs), including 486 genes that were up-regulated and 578 genes that were down-regulated in bc15 relative to wild-type plants (Fig. 7B; Supplemental Data Set S1).

Based on the considerable effects of the bc15 mutation on mechanical strength and cellulose content, we examined the expression levels of the BC and Cellulose Synthase (CESA) genes, which contribute to both phenotypes. Levels of transcripts for both genes were slightly increased in bc15 (Table III), which may result from the feedback responses of the mutation. Therefore, the bc15 mutation probably regulates the mechanical property through a different pathway. We further employed Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to reveal the pathways affected by the bc15 mutation. Although the detected DEGs may be involved in 87 pathways, Phe metabolism (ko00360), plant-pathogen interaction pathways (ko04626), DNA replication and repair (ko03030, ko03410, ko03440, ko03430, and ko00240), and phos- phatidylinositol signaling (ko04070) seem to be affected (Table IV). In the Phe metabolism pathway, 15 DEGs annotated as peroxidase and three annotated as Phe ammonia lyase are up-regulated in bc15 (Supplemental Table S2). However, no significant alterations in expression were found for genes involved in lignin monomer biosynthesis in rice (Supplemental Table S3). This result is consistent with only slight changes in the lignin contents of bc15 mutants relative to wild-type plants (Table I). Nine putative calcium-binding proteins that exist in both plant-pathogen interaction and phosphatidylinositol signaling pathways were up-regulated in bc15 mutant plants (Supplemental Tables S4 and S5). The bc15 mutation up-regulates the expression of 10 chitinase genes (Supplemental Table S6) and also alters the expression of three genes required for ethylene

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**Figure 7.** RNAseq analysis of the genes expressed in wild-type and bc15 internodes. A, Statistical analysis of reads from the wild type (WT) and bc15, showing the high quality of RNAseq data. B, Number of genes that are up-regulated and down-regulated in bc15 compared with those in the wild type. C, Scatterplot to show the overall expression alterations in bc15. Red and green spots indicate the up-regulated and down-regulated genes in bc15, respectively.

**Table III.** Expression levels of BC and CESA genes in wild-type and bc15 RNAseq analysis

| Name | Locus | Length | RPKM Wild Type | RPKM bc15 | Log2 Ratio | p | FDR | Change |
|------|-------|--------|----------------|------------|------------|---|-----|--------|
| CTL1 | Os09g32080 | 1,850 | 209.48 | 250.93 | 0.26 | 1.49E-11 | 2.70E-10 | Up |
| BC1  | Os03g30250 | 2,565 | 72.37 | 96.49 | 0.41 | 2.94E-12 | 6.15E-11 | Up |
| BC3  | Os02g50550 | 3,397 | 128.66 | 124.68 | -0.05 | 1.34E-01 | 2.93E-01 | Up |
| BC12 | Os09g02650 | 4,300 | 60.66 | 65.05 | 0.10 | 8.38E-03 | 3.11E-02 | Up |
| BC14 | Os02g40030 | 1,799 | 10.89 | 14.09 | 0.37 | 5.30E-03 | 2.10E-02 | Up |
| BC10 | Os05g07790 | 2,035 | 12.27 | 18.50 | 0.59 | 1.89E-07 | 1.97E-06 | Up |
| CESA4| Os01g54620 | 3,479 | 448.38 | 582.01 | 0.38 | 0.00 | 0.00 | Up |
| CESA7| Os10g32980 | 3,575 | 296.64 | 434.20 | 0.55 | 1.11E-11 | 2.04E-10 | Up |
| CESA9| Os09g25490 | 3,698 | 237.10 | 350.59 | 0.56 | 0.00 | 0.00 | Up |
| CESA1| Os05g08370 | 4,069 | 377.98 | 488.12 | 0.37 | 3.66E-12 | 7.48E-11 | Up |
| CESA2| Os03g59340 | 4,488 | 34.16 | 29.14 | -0.23 | 1.37E-05 | 1.03E-04 | Down |
| CESA4| Os07g24190 | 4,622 | 370.88 | 397.55 | 0.10 | 0.00 | 0.00 | Up |
| CESA5| Os03g62090 | 4,365 | 129.70 | 160.90 | 0.31 | 1.10E-11 | 2.02E-10 | Up |
| CESA6| Os07g14850 | 4,109 | 162.27 | 179.78 | 0.15 | 4.87E-10 | 7.32E-09 | Up |
| CESA8| Os07g10770 | 4,025 | 412.12 | 480.18 | 0.22 | 0.00 | 0.00 | Up |
biosynthesis and two genes involved in ethylene signaling (Supplemental Data Set S1). These findings suggested that mutation of BC15/OsCTL1 may trigger defense responses and activate signaling systems in rice plants.

**DISCUSSION**

The presence of chitinases in a wide range of organisms, including fungi, animals, and plants, suggests that the biochemical and physiological processes in which these enzymes participate are fairly important (Graham and Sticklen, 1994; Hossain et al., 2010). The rice and Arabidopsis genomes encode 50 (Supplemental Table S6) and 24 putative chitinase-encoding genes, respectively (Xu et al., 2007). In contrast to the several Arabidopsis chitinase mutants reported to date (Zhong et al., 2002; Hermans et al., 2010; Hossain et al., 2010), no mutations in rice chitinase genes have been characterized. Here, through isolation of the rice bc15 mutant, we identified a rice chitinase gene. It was designated as OsCTL1 because its sequence shows highest similarity to that of AtCTL1. Its different localization features and cellular behavior than AtCTL1 highlight a hitherto undescribed action of chitinase in rice.

Plant chitinases are regarded as a super gene family. Two common characteristics are often shared by super gene families: (1) multiple expression patterns or subcellular localization features and (2) enzyme activity. Chitinases from various plant species were reported to have diverse expression profiles. Some of them are constitutively expressed in different organs or at different developmental stages (van Hengel et al., 1998; Takakura et al., 2000; Zhong et al., 2002; Hermans et al., 2010), whereas others are regulated in a tissue-specific or pathogen/stress-inducible manner (Ancillo et al., 1999; Takakura et al., 2000; Hossain et al., 2010). The results of qRT-PCR and GUS activity assays indicated that BC15/OsCTL1, like its Arabidopsis homolog AtCTL1, is ubiquitously expressed in many organs, suggesting that the two gene products may play similar biological roles. Apart from the expression pattern, the subcellular localization of chitinases also influences their function. Plant chitinases have been proposed to be targeted to the endoplasmic reticulum, chloroplast, mitochondrion, vacuole, and extracellular matrix according to whether they possess SPs, glycosylphosphatidylinositol anchors, and/or transmembrane domains (Xu et al., 2007). Many chitinases, such as AtCTL1 (Zhong et al., 2002), are considered to enter the secretory pathway due to the absence or presence of SP that is not targeted to the vacuole or other organelles. In contrast, BC15/OsCTL1 has a transmembrane domain instead of an N-terminal SP. Consistent with expectations on the basis of these features, we further demonstrated it to be an integral membrane protein that is targeted to the Golgi apparatus. Therefore, BC15/OsCTL1 appears to participate in a cellular pathway, which, to our knowledge, has not been described in plant chitinases.

The plant Golgi apparatus is the site for the synthesis of various noncellulosic cell wall polymers and glycoproteins. As a Golgi-localized GH, the enzyme activity of BC15/OsCTL1 is critical for understanding its function. It is presumed that, being in a super gene family, they may share similar enzyme activity. Members of CESAs that catalyze cellulose elongation all use UDP-Glc as substrates. This is because 36 CESA members are required to form one complex (Somerville, 2006). Given that plant chitinases appear unlikely to contribute to heterogeneous molecular complexes, it is reasonable to suspect that they may have different substrate specificities and catalyze different reactions (Levorson and Chlan, 1997; Sasaki et al., 2006). Many attempts have been made to identify the likely substrates of plant chitinases, and the sequences that contribute to the catalytic and substrate-binding domains often provide clues regarding enzyme activities. Chitin may be the substrate of those chitinases that contain the Cys-rich chitin-binding domain and the H-E-T-T catalytic site (Meins et al., 1994; Bishop et al., 2000). Genes encoding such enzymes are often induced by pathogens and contribute to the degradation of fungal cell walls in order to limit pathogen invasion (Eckardt, 2008). Plants indeed possess many chitinase-like proteins that do not have either a typical chitin-binding domain or a typical chitinase activity domain. These chitinases are members of the GH19 family and clustered into class II. Their substrates are poorly defined. Nonetheless, some evidence suggests that arabinogalactan proteins and other GlcNAc-containing glycoproteins or chitoooligosaccharides may be the substrates of class II chitinases (De Jong et al., 1993;
Domon et al., 2000; Passarinho et al., 2001; Zhong et al., 2002; Hermans et al., 2010). The BC15/OsCTL1 protein is classified as a class II chitinase because it lacks both an N-terminal Cys-rich domain and a chitinase activity H-E-T-T motif. Recombinant BC15/OsCTL1 produced in either S. cerevisiae or E. coli can neither rescue a yeast mutant deficient in CTS1 nor exhibit classical chitinase activity in vitro. In vivo, microsomes isolated from bc15, wild-type, and transgenic plants also have not shown different enzyme activities. All these data lead us to conclude that BC15/OsCTL1 does not possess classical chitinase activity. Further studies are required to reveal the reaction likely to be catalyzed by BC15/OsCTL1 in rice plants.

Although the biochemical process in which BC15/OsCTL1 participates remains unclear, the reduced cellulose content in bc15 clearly indicates that BC15/OsCTL1 functions in cell wall synthesis or remodeling. The first evidence of chitinases regulating wall properties was from the experiment of treating cowpea (Vigna unguiculata) hypocotyls with a chitinase (Okamoto and Okamoto, 1995). Arabidopsis ctl1 mutants are also characterized by reduced cellulose content and cell wall defects (Zhong et al., 2002; Mouille et al., 2003; Hématy et al., 2007). AtCTL1 was proposed to enter the secretory pathway (Zhong et al., 2002), whereas BC15/OsCTL1 is a Golgi-targeted integral membrane protein. Therefore, the role of BC15/OsCTL1 in cellulose synthesis may differ from that mediated by AtCTL1. Like AtCTL1, which is coregulated with CESA proteins or some other BC-encoding genes, the rice BC15/OsCTL1 is coexpressed with OsCESA1, OsCESA3, OsCESA8, and BC1L4 (Supplemental Fig. S6). Genetic evidence suggests that rice CESAs, including CESA4, CESA7, CESA9, and BC1, are required for cellulose biosynthesis in the secondary cell wall. Mutations in those genes result in brittleness and abnormal cell wall content (Li et al., 2003; Tanaka et al., 2003; Zhang et al., 2009). Our RNAseq data indicated that the expression levels of various CESA- and other BC-encoding genes are not significantly altered in bc15 mutant plants. The deficiency in cellulose synthesis may be a secondary effect of the bc15 mutation. However, we could not eliminate the possibility that BC15/OsCTL1 may function in the modification of CESA proteins or some polymers during cellulose production, which could alter the localization and/or moving velocity of CESA proteins on the plasma membrane and consequently affect cellulose synthesis. Elucidation of the primary action of BC15/OsCTL1 in cell wall synthesis or remodeling will likely remain enigmatic until elucidation of its enzyme activity.

In conclusion, our findings provide genetic evidence for the roles of plant chitinases in rice, which appears different from its ortholog in Arabidopsis.

**MATERIALS AND METHODS**

**Phenotyping of the Mutant Plants and Microscopy**

The details for growth conditions of plant materials, quantification of mechanical strength and cell wall composition, and microscopy of bc15 and wild-type cell walls are described in Supplemental Materials and Methods S1.

**Map-Based Cloning**

The bc15 locus was mapped using 375 mutant F2 plants selected from the progeny of a cross between bc15 and indica cv TN1 of rice (Oryza sativa). Molecular markers for mapping (Supplemental Table S7) were developed to narrow the mutant locus to a 114-kb region on chromosome 9. The DNA fragments that correspond to the 17 ORFs in this region were amplified from mutants and wild-type plants using LA-Taq (TaKaRa; http://www.takara-bio.com/) and sequenced using an Applied Biosystems 3730 sequencer (ABI; http://www.appliedbiosystems.com). The complementation assays were performed as described in Supplemental Materials and Methods S1.

**Bioinformatics Analysis**

Domain prediction for BC15/OsCTL1 was performed using the Pfam database (http://pfam.sanger.ac.uk/). A search for BC15/OsCTL1 orthologs and homologs in plants was performed using the PlaZa 2.5 database (http://bioinformatics.psb.ugent.be/plaza/). Multiple alignments were conducted with the ClustalX and Jalview programs (Waterhouse et al., 2009). A phylogenetic tree of BC15/OsCTL1 orthologs and BC15/OsCTL1 homologs in rice and Arabidopsis (Arabidopsis thaliana) was generated using MEGA4 with 1,000 bootstrap replicates (Tamura et al., 2007). Bootstrap values more than 50% were shown. The TMHMM 2.0 and SignalP 4.0 servers (http://www.cbs.dtu.dk/services/) were used to identify the putative transmembrane domain and SIs in BC15/OsCTL1.

**Membrane Fractionation and Protein Gel Blotting**

To analyze the biochemical features of BC15/OsCTL1, the coding region of BC15/OsCTL1 without the stop codon was amplified and inserted into the pC5-GFP vector between the Xhol and Ksfl sites for fusion to the N terminus of GFP. The resulting construct, pBC15GFP, was introduced into the bc15 mutant using Agrobacterium tumefaciens-mediated transformation. The background and phenotypes of the transgenic plants were confirmed in both T0 and T1 plants.

Extraction of the soluble and microsomal fractions was performed as described in Supplemental Materials and Methods S1. Comparable amounts of proteins from each fraction were subjected to protein gel blotting involving anti-GFP, anti-CESA9, anti-HSP, and anti-XET5 primary antibodies and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Sigma). Anti-GFP was used to label BC15/OsCTL1 protein. The three other primary antibodies were used to monitor the microsomal, soluble, and cell wall fractions, respectively. To test the solubility of BC15/OsCTL1, the microsomal fraction of the transgenic plants expressing pBC15GFP was resuspended in 150 μL of extract buffer, high-salt buffer (1 M NaCl, 100 mM HEPES-KOH, pH 7.5, 0.3 mM Suc, 5 mM EDTA, and 5 mM EDA), alkaline buffer (0.1 M Na2CO3, pH 11, 0.3 mM Suc, 5 mM EGTA, and 5 mM EDTA), or Trition X-100 buffer (1% [v/v] Triton X-100, 100 mM HEPES-KOH, pH 7.5, 0.3 mM Suc, 5 mM EGTA, and 5 mM EDTA). After incubation for 1 h, each treated solution was ultracentrifuged at 100,000g for 1 h at 4°C to obtain both supernatant and pellet fractions. Each fraction was subjected to immunoblot analysis using the anti-GFP and anti-CESA9 antibodies with 1:1,000 dilutions. The microsomal proteins were further separated in the fractioning buffer (5 mM sodium phosphate, pH 7.2, 0.25 mM Suc, 1 mM diithiothreitol [DTT], 12.4% polyethylene glycol [PEG] 3350, and 12.4% Dextran [DEX] T500) by centrifuging at 8,000g for 10 min at 4°C to generate the plasma membrane (PEG) and endomembrane (DEX) fractions. Proteins in the PEG and DEX fractions were collected separately and concentrated by centrifugation at 100,000g for 1 h. After dissolving them in suspension buffer (2 mM Tris, pH 6.5, 1 mM DTT, and 0.25 μM...
Suc), comparable amounts of proteins from the two fractions were subjected to immunoblot analysis using 1:1,000 dilutions of the anti-GFP, anti-Arf, and anti-PIP1 antibodies. The latter two antibodies preferably detect endomembrane and plasma membrane proteins, respectively.

To determine whether BC15/OsCTL1 is N-glycosylated, microsomes from BC15/OsCTL1-GFP transgenic plants were treated with PNGase F (New England Biolabs) according to the manufacturer’s instructions. Approximately 30 μg of microsomal protein prepared in glycerol containing detergent (0.5% SDS and 40 mM DTT) at 100°C for 10 min. After the addition of Nonidet P-40, G7 reaction buffer (50 mM sodium phosphate, pH 7.5), and 2-fold dilutions of PNGase F, the reaction mix was incubated at 37°C for 2 h. Finally, the samples were separated using SDS-PAGE and subjected to immunoblot analysis using anti-GFP (1:1,000 dilution) and anti-PIP2 (1:1,000 dilution) antibodies as probes. To determine the membrane topology of BC15/OsCTL1, the microsomal fraction of plants expressing BC15/OsCTL1-GFP was resuspended in the extract buffer in the presence or absence of 1% (v/v) Triton X-100 and then incubated with 2 ng μL⁻¹ proteinase K (Invitrogen) for 20 min on ice. The reactions were terminated by heating at 70°C to denature the enzyme and then subjected to immunoblot analysis with antibodies against GFP.

The primary commercial antibodies anti-XET5, anti-Arf, anti-Sec21p, and anti-PIP1 used in these analyses were purchased from Agrisera anti-GFP was from Sigma, and anti-HSP was purchased from Beijing Protein Innovation. Anti-CESA9 polyclonal antibodies were produced in rabbit against the polypeptide from the 65th to 170th amino acids of rice CESA9 and then purified using an antigen-conjugated agarose column.

GUS Activity and Subcellular Localization Assays

For GUS activity analysis, the putative promoter of BC15/C.TL1 (1,890 bp) was amplified and cloned into the pCAMBIA1301 vector upstream of the GUS reporter gene. The construct was introduced into Nipponbare plants using Agrobacterium-mediated transformation. GUS activity was assayed in T1 and T2 transgenic plants. Different rice organs were picked up and stained in 1 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-Gluc) for 2 to 24 h at 37°C. After washing with PBS buffer, the samples were mounted in water and observed with a light microscope (Leica TCS SP5).

To observe the subcellular localization of BC15/OsCTL1, the roots and coleoptiles from T0 and T1 transgenic plants were observed using a confocal microscope (Leica TCS SP5). For transient subcellular analysis, the coding region of BC15/OsCTL1-GFP was resuspended in the extract buffer in the presence or absence of 1% (v/v) Triton X-100 and then incubated with 2 ng μL⁻¹ proteinase K (Invitrogen) for 20 min on ice. The reactions were terminated by heating at 70°C to denature the enzyme and then subjected to immunoblot analysis with antibodies against GFP.

Yeast Assay

The Saccharomyces cerevisiae csl1 strain Y00947 (BY4741; Mata; his3Δ1; leu2Δ10; met15ΔD; ura3Δ0; YLR286c::kanMX4) and the wild-type BY4741 strain was obtained from EUROscarf (the European Saccharomyces cerevisiae Archive for Functional Analysis). The ORF of BC15/OsCTL1 was cloned into the yeast expression vector pDR196. The resulting construct and empty vector were then transformed into the yeast csl1 mutant strain using the lithium acetate method (Sherman, 1991). Three positive clones from each transformation procedure were selected for culture on yeast peptone dextrose medium containing 1% (v/v) yeast extract, 2% Bacto peptone, and 2% Cz at 30°C for 72 h. The cultures were diluted in water and observed using a differential interference contrast microscope.

Analysis of BC15/OsCTL1 Enzyme Activity

The coding region of BC15/OsCTL1 was amplified and inserted into the expression vector pET32a between the EcuRI and BamHI sites. The resulting construct was transformed into the Origami B strain. Recombinant proteins with both thioredoxin and 6×His tags were purified using nickel-nitrilotriacetic acid agarose resin according to the manufacturer’s instructions. About 5 μg of purified recombinant protein and 10 μg of microsome proteins prepared from wild-type, bc15, and pBC15GFP transgenic seedlings (the microsome preparation procedure is described in Supplemental Materials and Methods S1) were used for each reaction according to the chitinase assay kit (Sigma). Three substrates each labeled with 4-methylumbelliferyl-N,N'-diacetyl-D-β-chitobiose, N-acetyl-D-glucosaminide, and β-O-2,3-α-N,N'-triacylchitohexitroxide were used to detect both exochitinase (chitobiosidase activity and β-N-acetylglucosaminidase activity) and endochitinase activities. In brief, the enzymes were incubated with each of the substrates at 37°C for 30 min. After the reaction was stopped and cooled, levels of released fluorescent groups were assayed at an excitation wavelength of 360 nm and an emission wavelength of 450 nm upon ionization in basic pH. A chitinase from Trichoderma viride was used as a positive control.

RNAseq Analysis

Total RNAs was extracted from developmentally matched second internodes of wild-type and bc15 plants. The oligo(dT)-enriched mRNA was fragmented into molecules, each of approximately 200 nucleotides in length, for CDNA synthesis. The library was then constructed and sequenced using an Illumina HiSeq 2000 instrument (Beijing Genomics Institute). The raw sequence data were collected, and the dirty raw reads were filtered out. Totals of 11,785,714 and 12,459,184 clean reads were obtained for the wild-type and bc15 samples, respectively. After aligning to the rice reference CDNAS (MSU version 6.3) using SOAP aligner/soap2 (Li et al., 2009), 10,719,649 and 11,393,821 reads from wild-type and bc15 plants were evenly mapped. The levels of gene expression were calculated using the RPKM method (for reads per kb per million reads). The significance of DEGs was determined as described previously (Audic and Claverie, 1997). The false discovery rate (FDR) method was used to determine the threshold of P values. The DEGs were finally identified using the criteria FDR < 0.01 and absolute value of the log2 ratio ≥ 1. To perform pathway enrichment analysis using the KEGG database (http://www.genome.jp/kegg/), all 1.064 DEGs were used to identify the significantly enriched metabolic or signal transduction pathways compared with the whole-genome background. Nine pathways were significantly enriched, with 2 ≤ 0.05. DEGs with more than 2-fold alterations are listed in Supplemental Data Set S1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phenotypes of the bc15 mutant plant.

Supplemental Figure S2. Immunodetecting the distribution of wall polysaccharides in wild-type and bc15 mutant internodes.

Supplemental Figure S3. BC15/OsCTL1 encodes a chitinase-like protein.

Supplemental Figure S4. Biochemical properties of BC15/OsCTL1.

Supplemental Figure S5. Statistical charts revealing the quality of RNAseq data of wild-type and mutant plants.

Supplemental Figure S6. BC15/OsCTL1 is coexpressed with certain genes involved in cell wall biosynthesis.

Supplemental Table S1. Segregation ratio of bc15 mutant plants in the F2 population.

Supplemental Table S2. Two-fold alterations of genes involved in Phe and peroxidase metabolism pathways in a comparison of bc15 RNAseq data with those of the wild type.

Supplemental Table S3. Two-fold alterations of genes involved in lignin monomer biosynthesis in a comparison of bc15 RNAseq data with those of the wild type.

Supplemental Table S4. Two-fold alterations of genes involved in plant-pathogen interaction in a comparison of bc15 RNAseq data with those of the wild type.

Supplemental Table S5. Two-fold alterations of genes involved in the phosphatidylinositol signaling system in a comparison of bc15 RNAseq data with those of the wild type.
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