

# **BRITTLE CULM1, Which Encodes a COBRA-Like Protein, Affects the Mechanical Properties of Rice Plants**

**Yunhai Li,<sup>a,1</sup> Qian Qian,<sup>b,1</sup> Yihua Zhou,<sup>a,1</sup> Meixian Yan,<sup>b</sup> Lei Sun,<sup>a</sup> Mu Zhang,<sup>a</sup> Zhiming Fu,<sup>a</sup> Yonghong Wang,<sup>a</sup> Bin Han,<sup>c</sup> Xiaoming Pang,<sup>a</sup> Mingsheng Chen,<sup>a</sup> and Jiayang Li<sup>a,2</sup>**

<sup>a</sup> Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

<sup>b</sup> China National Rice Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou 310006, China

<sup>c</sup> National Center for Gene Research, Chinese Academy of Sciences, Shanghai 200233, China

**Plant mechanical strength is an important agronomic trait. To understand the molecular mechanism that controls the plant mechanical strength of crops, we characterized the classic rice mutant *brittle culm1* (*bc1*) and isolated *BC1* using a map-based cloning approach. *BC1*, which encodes a COBRA-like protein, is expressed mainly in developing sclerenchyma cells and in vascular bundles of rice. In these types of cells, mutations in *BC1* cause not only a reduction in cell wall thickness and cellulose content but also an increase in lignin level, suggesting that *BC1*, a gene that controls the mechanical strength of monocots, plays an important role in the biosynthesis of the cell walls of mechanical tissues.**

## **INTRODUCTION**

The plant cell wall, a strong fibrillar network that provides mechanical support to cells, tissues, and the entire plant body, is a highly organized composite that may contain many different polysaccharides, aromatic substances, and proteins. The structure and composition of plant cell walls are ideally suited to the functions they perform. For example, parenchyma cells, which consist of primary walls, provide the main structural support in growing regions of the plant body. Sclerenchyma cells, which have both primary walls and thick secondary walls, provide the major mechanical support in nonelongating regions of the plant body (Carpita and McCann, 2000). Cellulose usually constitutes ~20 to 30% of the dry weight of the primary walls and ~40 to 90% of the secondary walls, depending on the cell type (Taylor et al., 1999). In some cells, lignin may be incorporated into the cell wall, enhancing its mechanical strength. Despite extensive descriptions of the chemical and physical structures of cell walls, the mechanisms that regulate the deposition of cell wall materials and that determine cell wall strength remain to be elucidated.

To understand the mechanisms that regulate the mechanical strength of the plant body and the biosynthesis of plant cell walls, mutants defective in stem strength have been isolated and characterized. For example, the barley *brittle culm* (*bc*) mutants were first described based on the physical properties of the culms, which have an ~80% reduction in the amount of cellulose and a twofold decrease in breaking strength compared with those of wild-type plants (Kokubo et al., 1989, 1991), indicating that cellulose content is related to the me-

chanical strength of the plant body. In Arabidopsis, mutants with reduced stem strength have been identified and some corresponding genes have been cloned and characterized. The *irregular xylem* mutants (*irx1* to *irx3*) show a cellulose defect in secondary walls, and the stiffness of mature stems is decreased (Turner and Somerville, 1997). The *IRX1* and *IRX3* genes encode the catalytic subunits of the cellulose synthase isoforms CesA7 and CesA8 (Taylor et al., 1999, 2000), which are essential for the production of cellulose in the cell. The *irx4* mutant is defective in a cinnamoyl-CoA reductase, resulting in a reduction in the lignin content of secondary walls and a failure to retain an upright growth habit (Jones et al., 2001). Moreover, *INTERFASCICULAR FIBERLESS1*, a gene that regulates interfascicular fiber differentiation and stem strength in Arabidopsis, was identified as a member of plant homeodomain/Leu zipper family (Zhong and Ye, 1999; Ratcliffe et al., 2000). *FRAGILE FIBER1* (*FRA1*), a kinesin-like protein, is essential for the oriented deposition of cellulose microfibrils and cell wall strength (Zhong et al., 2002), and *FRA2* encodes a katanin-like protein that regulates fiber cell length and wall thickness (Burk et al., 2001; Burk and Ye, 2002). These studies indicate that the genes involved in the biosynthesis and/or modifications of cell walls, especially secondary walls, are essential for plant mechanical strength and that the molecular mechanisms that determine plant mechanical strength are complex.

Recent studies suggest that covalent lipid modification is a major means of regulating the activity as well as the cellular localization of a protein. Glycosylphosphatidylinositol (GPI), for example, has been posited as a common means of anchoring membrane proteins in mammalian, yeast, and parasitic cells (Udenfriend and Kodukula, 1995). The addition of the GPI moiety is performed in the endoplasmic reticulum and implies the cleavage of a hydrophobic C-terminal peptide and the subsequent linkage of a preassembled GPI anchor via an amide bond onto the last amino acid residue after the cleavage, called the GPI attachment or  $\omega$ -site. GPI-anchored proteins are required

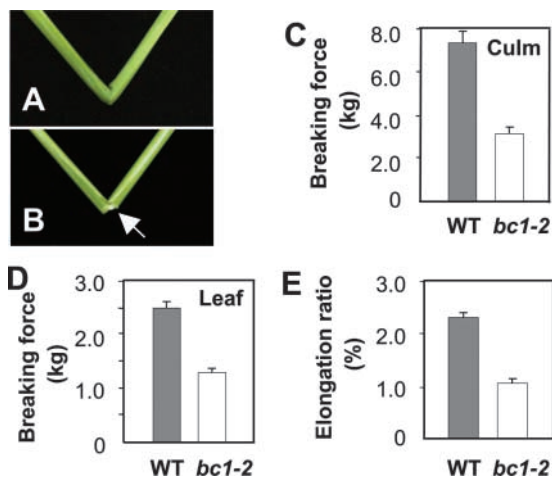
<sup>1</sup> These authors contributed equally to this work.

<sup>2</sup> To whom correspondence should be addressed. E-mail jyli@genetics.ac.cn; fax 86-10-64873428.

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for many important physiological functions, including signal transduction (Peles et al., 1997), parasitic evasion of host immune responses (Ferguson et al., 1994), and cell-to-cell recognition and nutrient uptake (Rothberg et al., 1990). In plants, several GPI-anchored proteins have been documented (Schultz et al., 1998; Sherrier et al., 1999; Schindelman et al., 2001; Sedbrook et al., 2002). However, only *COBRA*, *SKU5*, and *SOS5*, which encode the GPI-anchored proteins in Arabidopsis, have been functionally studied recently (Schindelman et al., 2001; Sedbrook et al., 2002; Shi et al., 2003). The mutation in *COBRA* affects the orientation of cell expansion and the cellulose content of the cell walls in the root elongation zone, implying a role for *COBRA* in cell wall maintenance and/or biosynthesis. *COBRA* belongs to a multigene family consisting of 12 members in Arabidopsis, all of which are predicted to encode putative GPI-anchored proteins; they are designated *COBRA*-like (*COBL*) proteins (Roudier et al., 2002). However, the functions of these *COBL* proteins in plants are poorly understood.

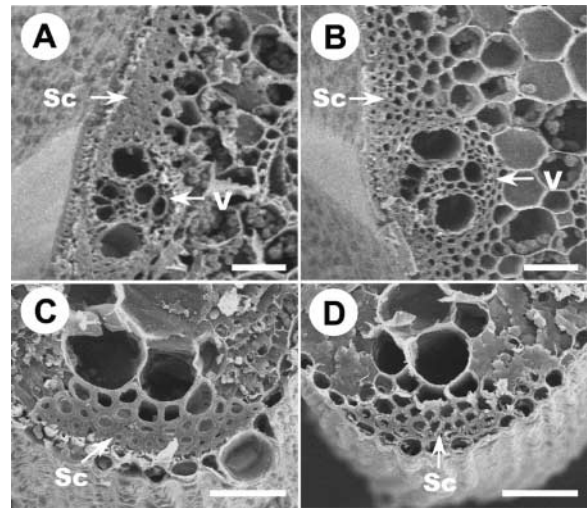
In rice, at least six *bc* mutants (*bc1* to *bc6*) have been reported, and some of them were mapped to different chromosomes using classic or molecular approaches (Kinoshita, 1995). Recently, a *bc1* allelic mutant, *bc1-2* (originally named *fp1*), was isolated from a rice *indica* variety based on its brittle culms, leaves, and leaf sheaths (Qian et al., 2001). Here, we report the in-depth characterization of the rice *bc1* mutants and the map-based cloning of the *BC1* gene as well as its spatial and temporal expression patterns. Our findings indicate that *BC1* is a *COBRA*-like protein that functions in regulating the biosynthesis of secondary cell walls to provide the main mechanical strength for rice plants.



**Figure 1.** Phenotypes and Physical Properties of Wild-Type and *bc1-2* Mutant Plants.

- (A) A wild-type culm.  
 (B) An easily broken *bc1-2* culm, as indicated by the arrow.  
 (C) The force required to break culms.  
 (D) The force required to break flag leaves.  
 (E) The elongation ratios of leaves.

Error bars were obtained from 10 measurements for (C) and from 22 measurements for (D) and (E). WT, wild type.



**Figure 2.** Scanning Electron Micrographs Showing the Differences between Sclerenchyma Cells and Vascular Bundles in Wild-Type and *bc1-2* Plants.

- (A) Cross-section of a wild-type culm.  
 (B) Cross-section of a *bc1-2* mutant culm.  
 (C) Cross-section of a wild-type leaf.  
 (D) Cross-section of a *bc1-2* leaf.

Sc, sclerenchyma cells; V, vascular bundles. Bars = 12.5  $\mu$ m.

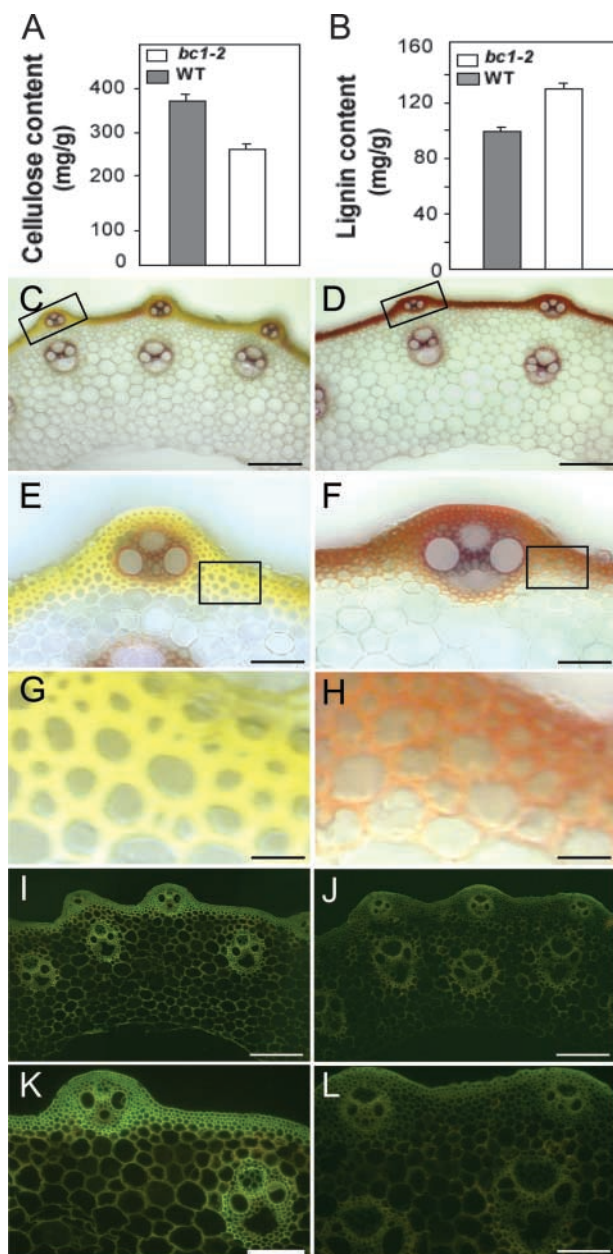
## RESULTS

### The *bc1* Mutant Has a Reduction in Mechanical Properties

To understand the mechanism that controls plant mechanical strength in cereal crops, we performed an in-depth analysis of a classic rice *bc1* mutant and its recently isolated allele, *bc1-2* (Qian et al., 2001). Although morphologically, *bc1-2* mutant plants are indistinguishable from wild-type plants (data not shown), they have brittle culms and leaves that can be broken easily by bending (Figures 1A and 1B). To accurately describe this phenotype, we quantitatively compared the breaking forces of *bc1-2* and wild-type plants, which define the forces required to break the segments of culms or leaves, and the elongation ratios of the leaves, which reflect the elasticity of plant tissues. As shown in Figures 1C and 1D, the forces required to break the mutant culms and leaves were decreased to 43 and 52% of those required for the wild type. The elongation ratio of the *bc1-2* leaves also was decreased by ~50% compared with that of the wild-type plants (Figure 1E). The striking decreases in the breaking forces and the elongation ratio of the *bc1-2* mutant indicate that the mutations in *bc1* affect both mechanical strength and the elasticity that enables plant organs or cells to maintain their proper shapes and positions.

### *bc1* Is Defective in Cell Walls of Mechanical Tissues

Reduction in the mechanical strength of culms and leaves may reflect alterations in cell wall structure, composition, or fiber length. Therefore, we examined cell wall morphology with



**Figure 3.** Measurement and Staining of Cellulose and Lignin in Wild-Type and *bc1-2* Plants.

(A) and (B) Cellulose (A) and lignin (B) contents (milligrams per gram of total cell wall residues) of the culm segments from wild-type (WT) and *bc1-2* plants. The error bars were obtained from five measurements.

(C) to (H) Wiesner's staining of the transverse culm sections of wild-type (C) and *bc1-2* (D) plants, and magnified sections (E) and (F), showing the increased level of lignin in the walls of sclerenchyma cells and vascular bundles in the mutant culm. (G) and (H) Magnified sections of (E) and (F), showing the irregular thin and defective walls of sclerenchyma cells in the mutant culm.

(I) to (L) Calcofluor staining of the transverse culm sections of wild-type (I) and *bc1-2* (J) plants, and magnified sections (K) and (L), showing the decreased level of cellulose in the cell walls of sclerenchyma cells and vascular bundles in the mutant culm.

scanning electron microscopy. In wild-type rice, several layers of mechanical cells, especially those around the peripheral vascular tissues and under the epidermal layer in culms and leaf veins, provide the mechanical support for the plants. Scanning electron microscopy observations revealed that the wild-type sclerenchyma cell walls were heavily thickened and that the cells were nearly completely filled up at the mature stages of culms and leaves (Figures 2A and 2C), in striking contrast to those of *bc1-2* mutant plants (Figures 2B and 2D). However, no differences in cell length and width were found between the *bc1-2* and wild-type plants (data not shown). These results suggest that reduction in the mechanical strength of *bc1-2* plants very likely resulted from a defect in the cell wall thickening of mechanical tissues, such as sclerenchyma cells, in the mutant plants.

### The *bc1* Plant Has an Altered Cell Wall Composition

To determine whether the cellular phenotype and the reduced mechanical strength in *bc1-2* mutant plants result from altered cellulose biosynthesis, we compared the crystalline cellulose contents of mutant and wild-type plants, because cellulose is the major component of plant cell walls. As shown in Figure 3A, the amount of cellulose in *bc1-2* culms was reduced to ~70% of that in the wild type, suggesting that *BC1* may directly or indirectly play an important role in cellulose biosynthesis. In some cells, cell walls also may contain a proportion of lignin that contributes to mechanical strength. Therefore, the lignin content of the *bc1-2* mutant plants also was assayed. As shown in Figure 3B, the Klason lignin of the *bc1-2* culms increased by ~30% compared with that of the wild-type culms, indicating that plant cells may have a sophisticated mechanism to balance the contents between cellulose and lignin.

To determine whether the alterations of cellulose and lignin are localized in particular cells, transverse sections of the culms of wild-type and mutant plants were histochemically stained with Wiesner and calcofluor solutions. Wiesner stain is known to react with cinnamaldehyde residues in lignin, and the color intensity approximately reflects the total lignin content. The color differences in mechanical tissues, especially in the sclerenchyma cells below the epidermis, between wild-type (Figures 3C and 3E) and mutant (Figures 3D and 3F) plants were clear, indicating an apparent increase in lignin quantity in mutant plants. On the other hand, calcofluor stains cellulose, callose, and other  $\beta$ -glucans. As shown in Figures 3I to 3L, much stronger fluorescent signals were observed in the sclerenchyma cells and vascular bundles in the wild type (Figures 3I and 3K) than in the *bc1-2* mutant (Figures 3J and 3L), demonstrating a significantly high level of ordered cellulose in the mechanical tissues and cells in wild-type plants. In addition, compared with the smooth and thickened walls of sclerenchyma cells of the wild type (Figure 3G), those of mutant plants were irregularly thin and uneven (Figure 3H). This finding is consistent with the scanning electron microscopy observations

Bars = 160  $\mu$ m in (C), (D), (I), and (J), 40  $\mu$ m in (E) and (F), 10  $\mu$ m in (G) and (H), and 80  $\mu$ m in (K) and (L).

**Table 1.** Comparison of the Contents of Cell Wall Sugars between *bc1-2* and Wild-Type Culms

Sugar	<i>bc1-2</i> Mutant <sup>a</sup>	Wild Type <sup>a</sup>	Difference	P Value <sup>b</sup>
Glucose	433 ± 11.1	612 ± 15.9	-179	<0.001
Xylose	149 ± 5.64	107 ± 3.94	42	<0.001
Arabinose	21.3 ± 0.94	18.0 ± 0.83	3.3	0.022
Galactose	11.9 ± 0.60	11.5 ± 0.69	0.4	0.693
Mannose	1.74 ± 0.07	1.78 ± 0.08	-0.04	0.696
Rhamnose	3.66 ± 0.21	3.58 ± 0.21	0.08	0.786

<sup>a</sup>The sugar contents of cell walls of *bc1-2* mutant and wild-type culms are given as means ± SE of eight independent assays. Each wall component was calculated as milligrams per gram of total cell wall residues.

<sup>b</sup>Determined using Student's two-sample *t* test.

(Figure 2) and indicates that the *bc1* mutant is deficient mainly in the secondary cell walls.

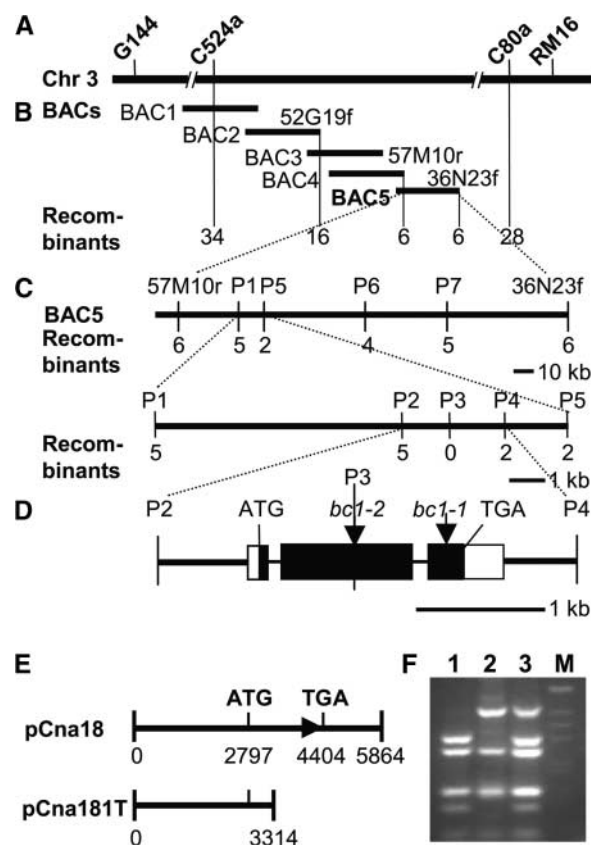
Therefore, we further compared the cell wall monosaccharide composition between *bc1-2* mutant and wild-type culms by gas chromatography. As shown in Table 1, glucose content was reduced significantly in the *bc1-2* mutant, consistent with the results obtained from cellulose assays. By contrast, the contents of xylose and arabinose were increased in the *bc1-2* mutant, whereas other analyzed sugars were not altered significantly. Because glucose and xylose are the two major sugars that constitute cellulose and hemicellulose, respectively, in the secondary cell walls, it is very likely that the *bc1* mutation mainly affects the biosynthesis of secondary cell walls, which explains the histochemical staining observation (Figure 3) and the difference in mechanical strength between wild-type and *bc1-2* plants (Figure 1).

### Positional Cloning of *BC1*

Our previous studies placed the *BC1* gene in an interval between the RM16 and C524a markers in the centromeric region of chromosome 3 (Qian et al., 2001). To fine-map the *BC1* locus, we generated a large F2 mapping population derived from a cross between *bc1-2* and C-Bao, a polymorphic rice *japonica* variety. Of ~30,000 F2 plants, 7068 segregants showing the *bc1-2* mutant phenotype were used for fine-mapping and the *BC1* gene was located between the two cleaved amplified polymorphic sequence (CAPS) markers C524a and C80a (Figure 4A). Within this region, the molecular markers and contigs of BAC or YAC clones had not been reported; therefore, we screened a rice BAC library using C524a and C80a as probes, developed new molecular markers from the ends of the BAC contig identified with the C524a probe (Table 2), and fine-mapped the *BC1* gene onto a single BAC, OSJNBa0036N23 (Figure 4B). To further narrow the *BC1* locus, we sequenced OSJNBa0036N23 using a shotgun strategy, developed seven additional markers, P1 to P7 (Table 2), and located *BC1* in an interval of a 3.3-kb DNA fragment between the P2 and P4 markers (Figure 4C). Within this region, only a single opening reading frame (ORF) was predicted, which appears to encode a protein that shows a strong similarity to Arabidopsis

COBRA, a protein required for oriented cell expansion and the proper deposition of cellulose in the root elongation zone (Schindelman et al., 2001).

To define the molecular lesions of *bc1* mutants, the genomic DNA sequences corresponding to the putative *BC1* gene from

**Figure 4.** Cloning and Confirmation of the *BC1* Gene.

**(A)** The *BC1* locus was mapped in the chromosome 3 (Chr 3) centromeric region between markers C524a and RM16.

**(B)** A BAC contig covering the *BC1* locus. The numerals indicate the number of recombinants identified from 7068 *bc1-2* F2 plants. BAC1, OSJNBa0006H20; BAC2, OSJNBa0052G19; BAC3, OSJNBa0007B10; BAC4, OSJNBa0057M10; BAC5, OSJNBa0036N23.

**(C)** Fine mapping of the *BC1* locus with the markers (P1 to P7) developed based on the OSJNBa0036N23 sequence. The *BC1* locus was narrowed to a 3.3-kb genomic DNA region between CAPS markers P2 and P4 and cosegregated with marker P3.

**(D)** *BC1* gene structure, showing the mutated sites of the two *bc1* alleles. The start codon (ATG) and the stop codon (TGA) are indicated. Closed boxes indicate the coding sequence, open boxes indicate the 5' and 3' untranslated regions, and lines between boxes indicate introns. Mutation sites in *bc1-1* and *bc1-2* also are shown.

**(E)** Complementation constructs. The construct pCna18 contains the entire *BC1* gene, including a 2795-bp upstream sequence and a 1459-bp downstream sequence. The plasmid pCna181T contains a partial *BC1* gene that encodes the first 173 amino acid residues.

**(F)** Identification of transgenic plants. The deletion of 4 bp in *bc1-2* destroys a BstNI site that is used for the CAPS marker P3. Lane 1, wild type; lane 2, *bc1-2*; lane 3, the pCna18-transformed rice line 1; lane M, 1-kb marker.

**Table 2.** List of the PCR-Based Molecular Markers Developed in This Study

Marker	Primer Pairs <sup>a</sup>	Fragment Size <sup>b</sup>	Restriction Enzyme
36N13f	F, 5'-AATCTTCTCTTACTCCACTCGC-3'; R, 5'-ATGGGAAGTACTGACTAAACCG-3'	C = 489, b = 0	
57M10r	F, 5'-AGGAACAGATGGAATCTCAGG-3'; R, 5'-TGCTCTCTGTCCAACTAGC-3'	C = 340, b = 0	
52G19f	F, 5'-GCACGCATTTAAGAAGCAGG-3'; R, 5'-TTATATGACCGTATGGCAGG-3'	502	MnII
P1	F, 5'-TTGCGTATGTCTGTCAACTGC-3'; R, 5'-TTCCAACATCTGAACCCTCG-3'	C = 1580, b = 0	
P2	F, 5'-GTTGCTCATCGTACCATCG-3'; R, 5'-AGGTATAGAGCGAGCGGTAGC-3'	1037	NsiI
P3	F, 5'-GGTAGTTGAAGTTTGTGATGGC-3'; R, 5'-ATGCTCTCTCGCTCGCTGCG-3'	1110	BstNI
P4	F, 5'-TACTACAACGACCTGCTTATGG-3'; R, 5'-TGGAGAGCAGTGTAGGTAGGG-3'	828	FokI
P5	F, 5'-AGCAGCAGTAGTCGACGTCG-3'; R, 5'-AACATAGCCATCTGGGGTCC-3'	1762	MnII
P6	F, 5'-TAGAAACAGGGTCAAGTAGGC-3'; R, 5'-GCTGAGTTTGGAGTGGTGG-3'	1470	MseI
P7	F, 5'-AGTGCCTCATCTATCGCTCG-3'; R, 5'-GTTCCACAACCGATCTAGCG-3'	2046	SalI

<sup>a</sup>F, forward primer; R, reverse primer.

<sup>b</sup>Numbers indicate the size (in bp) of amplified fragments. b, *bc1-2* mutant; C, C-Bao.

the *bc1-1* and *bc1-2* alleles were amplified by PCR and sequenced. Comparison of the sequences of the wild-type and mutant allelic genes revealed that the *bc1-1* allele carries a single nucleotide insertion in codon 425 (TTC → TTTC) in the third exon, which produces a larger protein that disrupts the GPI-attached site and the hydrophobic C terminus, and that the *bc1-2* allele contains a 4-bp deletion in codons 236 and 237 (AGGTGC → AC) in the second exon, which results in a frameshift that produces premature translational products (Figures 4D and 5), suggesting that the ORF represents the *BC1* gene.

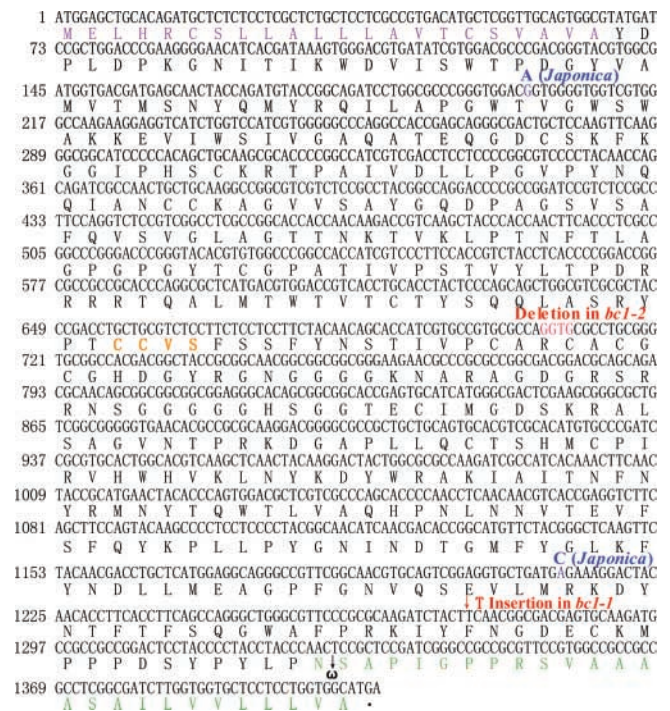
The identity of *BC1* was confirmed by genetic complementation analysis. The plasmid pCna18, containing the entire ORF, and pCna181T, containing a partial coding region of the ORF (Figure 4E), were introduced into the *bc1-2* mutant, and 80 and 62 independent transgenic lines were obtained from the two constructs, respectively. Nearly all 80 lines of pCna18 showed a complementation of the *bc1-2* phenotype, whereas all 62 lines of pCna181T failed to rescue the *bc1-2* mutant. Additionally, the deletion in the *bc1-2* allele disrupts the *Bst*NI site in the genomic DNA, which can be used as a CAPS marker to determine the *bc1-2* mutant background in the complementation test (Figure 4F). Therefore, we conclude that we have cloned the *BC1* gene in rice.

**BC1 Encodes a COBRA-Like Protein**

Sequence analysis of the products from reverse transcription (RT)-PCR and rapid amplification of cDNA ends-PCR indicated that the *BC1* cDNA is 1818 bp long, with an ORF of 1407 bp, a 90-bp 5' untranslated region, and a 321-bp 3' untranslated region (Figures 4D and 5). Sequence comparison between genomic DNA and cDNA showed that *BC1* is composed of three exons that encode a 468-amino acid protein. Interestingly, we found no difference in the *BC1* amino acid sequence between *indica* and *japonica* subspecies, although there are two synonymous base pair changes in the *BC1* coding region between the two subspecies (Figure 5).

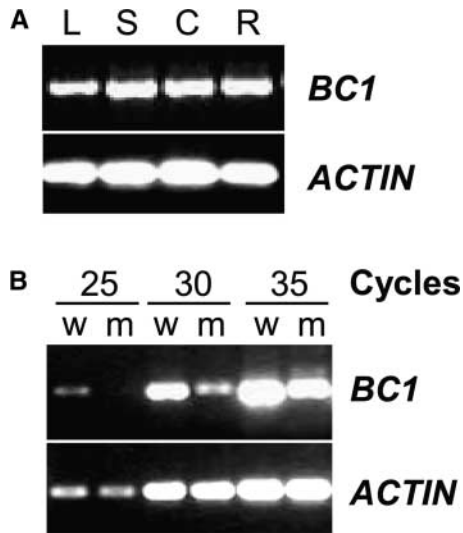
Basic Local Alignment Search Tool (BLAST) analysis revealed that the rice *BC1* protein shares the highest identity (>72% over the entire length) with COBL4, a predicted member of the COBRA family proteins in Arabidopsis (Roudier et al.,

2002). COBRA recently was identified as a putative GPI-anchored protein required for the orientation of cell expansion and the cellulose deposition of cell walls in the Arabidopsis root elongation zone (Schindelman et al., 2001). Predicted by its sequence characteristics, *BC1* appears to be a member of the COBRA family, because it contains all of the conserved features of the COBRA family, including a CCVS motif, an N-termi-



**Figure 5.** *BC1* cDNA and Predicted Amino Acid Sequences.

Numbers at left refer to the positions of nucleotides. Red letters indicate the 4-bp deletion in *bc1-2* and the 1-bp insertion in *bc1-1*; blue letters indicate different nucleotides between *indica* and *japonica* subspecies; purple letters indicate the N-terminal signal; orange letters indicate the conserved CCVS motif; and green letters indicate the C terminus, including the predicted ω-site and the hydrophobic tail.



**Figure 6.** *BC1* Expression Analysis.

(A) RT-PCR analysis of *BC1*. Total RNA was isolated from leaves (L), leaf sheaths (S), culms (C), and roots (R) of wild-type plants. Amplification of actin cDNA was used to ensure that approximately equal amounts of cDNA were loaded.

(B) Comparison of *BC1* transcripts between *bc1* mutant (m) and wild-type (w) plants with RT-PCR.

nal signal peptide sequence for secretion, a highly hydrophobic C terminus, and specific features around the  $\omega$ -site required for processing (Figure 5) (Udenfriend and Kodukula, 1995).

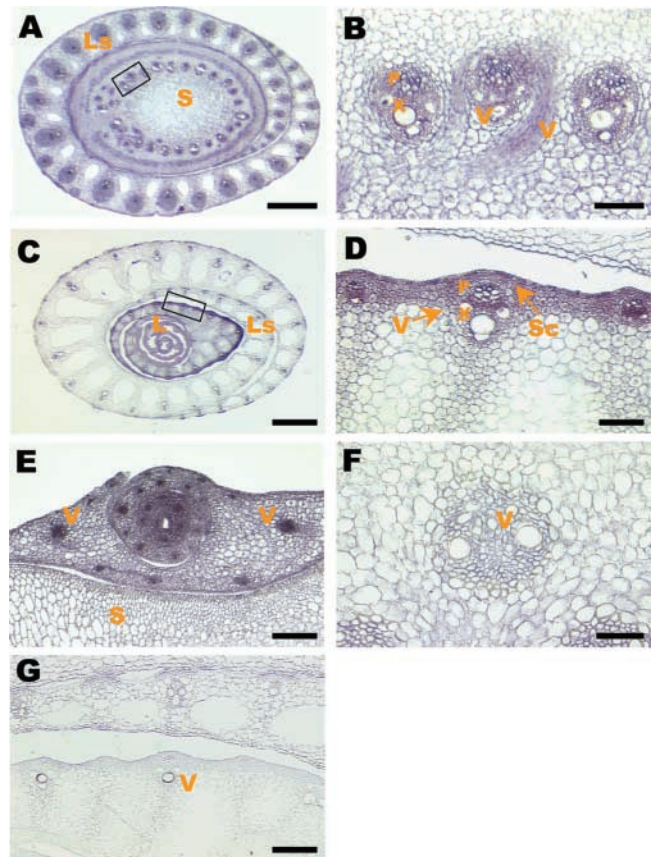
### ***BC1* Is a Spatially and Temporally Expressed Gene**

Although the steady state levels of *BC1* mRNA were undetectable by RNA gel blot analysis when whole plants were sampled, its expression could be examined by RT-PCR. As shown in Figure 6A, *BC1* was expressed universally throughout the wild-type plant organs, including leaves, stems, and roots, consistent with the brittleness phenotype of *bc1* mutant plants. It should be noted that RT-PCR analysis demonstrated a dramatic reduction in the abundance of transcripts in the homozygous *bc1-2* seedlings (Figure 6B), indicating that the mutation in *bc1-2* plants may affect not only the *BC1* protein function but also the steady state level of the transcript.

The precise expression patterns of *BC1* were explored further by RNA in situ hybridization. As shown in Figures 7A to 7D, *BC1* was expressed mainly in the cells of mechanical tissues, including the vascular bundles, and in the subepidermal regions where sclerenchyma cells differentiate, explaining the phenotypes observed with scanning electron microscopy (Figure 2) and histochemical staining (Figures 3C to 3L). The much stronger expression of *BC1* in tiller buds (Figure 7E) than in the developing culms (Figure 7F) and in young leaf sheaths (Figure 7D) than in old ones (Figure 7C) indicate that *BC1* expression also was regulated developmentally, suggesting that the biosynthesis of cell walls of mechanical tissues is temporally dynamic.

### ***BC1* Belongs to a Multimember Gene Family in Rice**

A genome-wide search of DNA and protein databases identified nine *BC1*-like (*BC1L*) genes and one pseudogene, *BC1L-p1*, in the rice genome (Table 3); their overall structures are illustrated in Figure 8A. All of the *BC1* family proteins except *BC1L2* and *BC1L9* contain an N-terminal signal peptide sequence for secretion, a highly hydrophobic C terminus, the conserved C CVS domain, and a potential  $\omega$ -cleavage site (Ta-



**Figure 7.** Expression Patterns of the *BC1* Gene Revealed by in Situ Hybridization in Transverse Sections of Wild-Type Rice Plants.

(A) Young stem and leaf sheath.

(B) A magnified section from (A), showing the strong expression of *BC1* in the developing vascular bundles.

(C) Leaves and leaf sheath, showing the intense expression of *BC1* in young leaves and sheaths.

(D) A magnified section from (C), showing strong signals in the developing vascular and other mechanical tissues.

(E) Tiller bud, showing strong signals in vascular bundles.

(F) Mature vascular bundles, showing the sharply decreased expression level of *BC1* compared with that shown in (E).

(G) Background control, in situ hybridization of a young leaf sheath with a sense probe.

L, leaf; Ls, leaf sheath; P, phloem; S, stem; Sc, sclerenchyma cells; V, vascular bundles; X, xylem. Bars = 70  $\mu$ m in (E) and (G), 450  $\mu$ m in (A) and (C), and 35  $\mu$ m in (B), (D), and (F).

**Table 3.** Rice *BC1* Family Genes

Locus	Accession No.	Gene Position	EST Accession No.	Amino Acid <sup>a</sup>	$\omega$ -Site
<i>BC1</i>	AAAA01003837	16545 to 18148	D47139	468	N (443)
<i>BC1L1</i>	AAAA01008372	1321 to 3336	AU030042	671	N (644)
<i>BC1L2</i>	AAAA01003837	12631 to 15956		477	None
<i>BC1L3</i>	AAAA01000310	31175 to 33981	AU225729; C74834	456	N (429)
<i>BC1L4</i>	AAAA01006032	1439 to 4300	C91883; AU096812	457	N (430)
<i>BC1L5</i>	AAAA01005547	9426 to 11477		683	A (658)
<i>BC1L6</i>	AAAA01000918	6284 to 9445	C97636	446	N (419)
<i>BC1L7</i>	AAAA01000918	10062 to 12498	AU173066	477	A (459)
<i>BC1L8</i>	AAAA01019714	756 to 2774	AU1738855; AU1738866	672	S (644)
<i>BC1L9</i>	AAAA01005473	10047 to 12814		683	None
<i>BC1L-p1</i>	AL606587	73953 to 75957			

<sup>a</sup> Amino acid residue number in each predicted BC1L protein.

ble 3). However, *BC1L2* and *BC1L9* lack the predicted  $\omega$ -cleavage sites in the deduced protein sequences (Table 3).

Although ESTs of *BC1L2*, *BC1L5*, and *BC1L9* are not found in any known EST database, all of the *BC1* family genes identified in the rice genome are expressed, as revealed by RT-PCR analysis with total RNA isolated from aerial organs of rice seedlings (Figure 8B). This finding indicated that all *BC1* family genes are transcriptionally active and may have different functions.

### Phylogenetic Analysis of *BC1* Family Genes

To determine the evolutionary relationships of rice *BC1* family and Arabidopsis *COBRA* family members, an unrooted tree was built using the neighbor-joining method (Figure 8D). Phylogenetic analysis indicated that the rice *BC1* family and the Arabidopsis *COBRA* family belong to a single gene superfamily, the *COBRA* family. As determined by phylogenetic analysis, the *COBRA* family is divided into two subfamilies, which is in agreement with the exon/intron organization of each subfamily member. Members in one subfamily have multiple introns/exons, whereas those in the other subfamily have a single exon (Figures 8A and 8D). *BC1* and *COBRA* belong to two divergent clades, implying that they have related but distinct functions. This distinction was reflected by the different phenotypes of *bc1* and *cobra* mutants. *BC1* and *BC1L7* in rice and *COBL4* in Arabidopsis form a monophyletic clade with 99% bootstrap support. *BC1* and *BC1L7* are located on chromosomes 3 and 7 in rice, respectively (Figure 8C). They resulted from a gene duplication event that took place after the divergence of monocot and dicot phyla, suggesting that they might have partially redundant functions.

### DISCUSSION

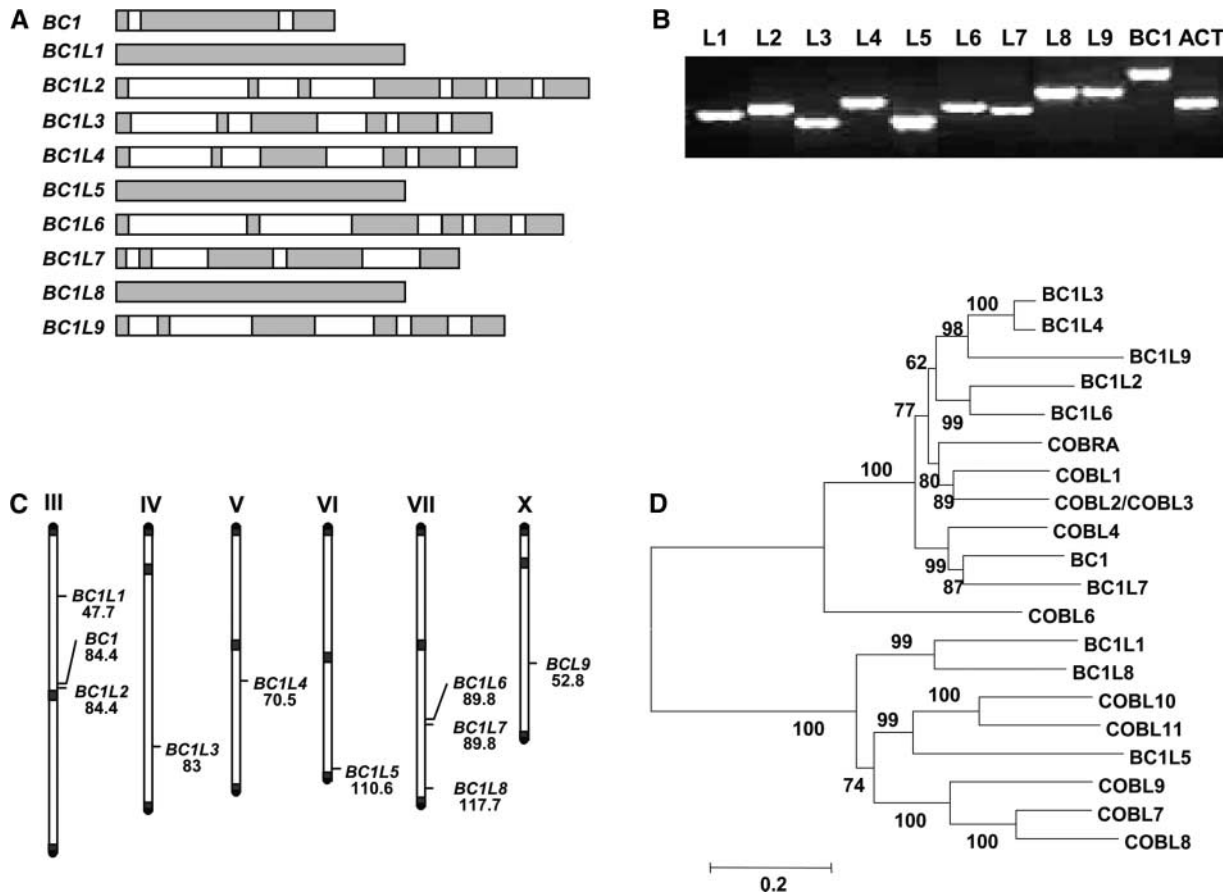
We have described the molecular genetic characterization of the classic rice mutant *bc1*, its positional cloning, and its temporally and spatially regulated expression in the cells of mechanical tissues. *BC1* encodes a protein that is homologous with Arabidopsis *COBRA* family members and represents a 10-member family in rice. Deficiency in *BC1* causes the altered

biosynthesis of cellulose, hemicellulose, and lignin, leading to a reduction in the secondary cell wall thickness and the mechanical strength of rice plants.

### Mutation in *BC1* Alters the Formation of Secondary Cell Walls

Our results clearly demonstrate that mutations in *BC1* affect the biosynthesis of secondary cell walls and result in alterations in the contents of cellulose and lignin. The prominent differences between the wild type and the *bc1-2* mutant are localized mainly in the mechanical tissues, such as sclerenchyma cells and vascular bundles (Figures 2 and 3). It has known that deficiency in cellulose biosynthesis in the primary cell walls often leads to a globally altered morphology, sterility, or even lethality (Arioli et al., 1998). By contrast, plants with mutations that affect the formation of secondary cell walls usually grow relatively normally (Taylor et al., 1999, 2000). The similarities of the plant morphology and the sclerenchyma and parenchyma cell sizes between wild-type and *bc1-2* plants suggest that *BC1* very likely plays a role in regulating the biosynthesis of secondary cell walls. This notion was substantiated further by comparing the cell wall sugar contents of mutant and wild-type culms. Compared with wild-type plants, the *bc1-2* mutant showed a dramatic alteration in glucose and xylose, the two major sugars that constitute cellulose and hemicellulose in the secondary cell walls, respectively.

The mechanism that regulates the biosynthesis of cell walls is complicated and requires the coordination of a number of metabolic pathways. Recent studies with Arabidopsis mutants defective in cellulose production have demonstrated that cellulose biosynthesis is affected if a mutation occurs either in enzymes involved in cellulose biosynthesis or in other proteins, such as 1,4- $\beta$ -endoglucanase, mannose-1-phosphate guanylyltransferase, a katanin-like protein, or membrane proteins (Arioli et al., 1998; Nicol et al., 1998; Taylor et al., 1999, 2000; Fagard et al., 2000; Burk et al., 2001; Lukowitz et al., 2001; Schindelman et al., 2001; Pagant et al., 2002). In all cases, these defects cause abnormal plant growth and development, suggesting the functional importance of highly dynamic cell wall biosynthesis. The finding that mutations in *BC1* cause ab-



**Figure 8.** The *BC1* Gene Family in Rice.

(A) Intron/exon structure of rice *BC1* family genes. Exons are indicated with gray boxes, and introns are indicated with white boxes.

(B) Expression of rice *BC1* family genes in aerial parts of rice seedlings revealed by RT-PCR. Amplification of actin (*ACT*) transcript was used as an internal control. L1 to L9, *BC1L1* to *BC1L9*.

(C) Chromosomal positions of *BC1* family genes. The chromosomes are labeled with roman numerals. The number below the gene symbol represents the genetic position of each putative gene (centimorgans).

(D) Neighbor-joining tree of rice *BC1L* proteins and Arabidopsis *AtCOBRA* family members. The numbers at each node represent the bootstrap support (percentage). The scale bar is an indicator of genetic distance based on branch length.

normalities of the cellulose and lignin contents and the defective formation of secondary cell walls of mechanical tissues suggests that *BC1* may act as an auxiliary protein to regulate secondary cell wall biosynthesis in rice. The *in situ* hybridization study showed that the *BC1* gene is expressed mainly at the young, elongating regions of organs (Figure 7). However, at this developmental stage, sclerenchyma cells below the epidermis and bundle sheath fiber cells are not yet undergoing secondary wall thickening.

Because the expression of *BC1* is decreased dramatically in nonelongating organs, in which sclerenchyma cells undergo secondary wall formation (Figure 7), it appears that *BC1* is not involved directly in secondary wall formation. Arabidopsis *COBRA* family members are likely to be important players at the plasma membrane–cell wall interface (Roudier et al., 2002). It is very possible that *BC1*, as a putative GPI-anchored protein, is located at the cell wall–plasma membrane interface, where

cellulose deposition takes place. This hypothesis is strengthened by our findings that *BC1* is expressed mainly and functions in the cells of developing mechanical tissues. Clearly, additional studies, particularly of the biochemical nature of *BC1* and its targets, will be essential to fully understand its function during cell wall biosynthesis.

### ***BC1* Is a New Member of the *COBRA* Family**

*BC1* encodes a putative GPI-anchored *COBRA*-like protein. Based on hydrophobicity analysis using a method described by Kyte and Doolittle (1982), *BC1* has a hydrophobic N-terminal signal peptide and a highly hydrophobic peptide at its C terminus and is predicted to be GPI anchored based on the big-PI Predictor (Eisenhaber et al., 2000) and PSORT (Nakai and Horton, 1999) programs. Normally, at the  $\omega$ -site, only amino acid residues such as Ser, Asn, Ala, Gly, Asp, or Cys may be



present, whereas at the  $\omega+2$ -site, only Ala, Gly, Thr, or Ser are commonly found, and the  $\omega+2$ -residue usually is followed by a spacer of five to seven amino acid residues rich in charged and/or Pro residues preceding a stretch of 10 to  $\sim 30$  hydrophobic residues (Udenfriend and Kodukula, 1995). The predicted BC1 protein meets all of these requirements. The *bc1-1* allele was frameshifted by a single nucleotide insertion in codon 425, which results in a translational product without the GPI attachment site and the hydrophobic tail. The mutation in *bc1-2* contains a 4-bp deletion in codons 236 and 237, which results in a frameshift mutation and produces a truncated protein product that lacks the C terminus. Both *bc1-1* and *bc1-2* are likely to be loss-of-function mutations, because the GPI attachment site and the hydrophobic C-terminal peptide required for post-translational processing are destroyed in their aberrant protein products; therefore, BC1 processing is likely to be disrupted.

Genomic analysis identified nine additional *BC1L* genes in the rice genome. Phylogenetic analysis indicates that *BC1L*, *COBL*, and homologs from other plants form a gene superfamily, the COBRA gene family. The COBRA gene family is an ancient family that arose before the divergence of monocot and dicot phyla. Determining the functions of all members of this family is a major challenge. Based on phylogenetic analysis and intron/exon structure, the COBRA family can be divided into two subfamilies. BC1 and COBRA belong to the same subfamily. However, BC1 and COBRA are located on two different clades (Figure 8), suggesting that BC1 and COBRA might have different functions in the cell.

The function of the GPI-anchored proteins is not understood fully. Recent studies on *COBRA* (Schindelman et al., 2001), *SKU5* (Sedbrook et al., 2002), and *SOS5* (Shi et al., 2003) have shown the importance of GPI-anchored proteins in cell expansion, proper cell wall structure, and cell surface adhesion. The fact that the rice BC1 protein shares the highest identity with Arabidopsis *COBL4* suggests that members of the COBRA family may have different functions in plants and that BC1 represents a subgroup that is essential for secondary cell wall biosynthesis and plant mechanical strength.

Brittleness is one of the most important agronomic traits that affect not only grain production but also the usefulness of cereal straws as animal forage. As an important player regulating rice brittleness, *BC1* (and its orthologs in other cereals) could make a significant contribution to the future improvement of these crops.

## METHODS

### Plant Materials and Growth Conditions

The rice (*Oryza sativa*) brittle culm mutant *bc1-2* was isolated from the *indica* cultivar Shuang Ke Zao irradiated with  $\gamma$ -rays (Qian et al., 2001), and the *bc1-1* mutant was renamed for the previously reported *bc1* mutant derived from a *japonica* cultivar (Kinoshita, 1995). *bc1-2* and C-Bao, a polymorphic *japonica* variety, were crossed to generate a large F2 mapping population. Rice plants were cultivated in the experimental field at the China National Rice Research Institute in the natural growing season.

### Measurements of Physical Properties

The breaking force and elongation ratio of rice culms or leaves were measured with a universal force/length testing device (model DC-KZ300; Kaiming, Sichuan, China). To avoid inaccuracies from sampling, the first internodes of culms and flag leaves were used for an immediate measurement. The elongation ratio (%) was defined by the formula  $100 \times (L_1 - L_2)/L_2$ , where  $L_1$  represents the length of the leaf segments at breaking and  $L_2$  stands for the original length of the leaf segments.

### Scanning Electron Microscopy

Samples were prepared as described previously (Mou et al., 2000) with some modifications. Briefly, rice tissues were excised with a razor and immediately placed in 70% ethanol, 5% acetic acid, and 3.7% formaldehyde for 18 h. Samples were critical point dried, sputter-coated with gold in an E-100 ion sputter (Mito City, Japan), and observed with a scanning electron microscope (S570; Hitachi, Tokyo, Japan).

### Carbohydrate and Lignin Measurement

Carbohydrate was assayed according to the methods described previously (Updegraff, 1969; Hoebler et al., 1989). Briefly, the first internodes of culms were ground into fine powder in liquid nitrogen. 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. For the measurement of cell wall sugars, the dried cell wall materials were hydrolyzed by incubation in 72% (w/w)  $H_2SO_4$  at room temperature for 1 h and then in 2 M  $H_2SO_4$  at 121°C for 1 h. The sugar alditol acetates were analyzed by gas chromatography. To measure lignin content, the first internodes of 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).

### Histochemical Staining

For histochemical localization of lignin, a Wiesner reaction was performed according to a standard protocol (Strivastava, 1966). Fresh hand-cut sections ( $\sim 20 \mu\text{m}$  thick) from rice culms were incubated for 2 min in phloroglucin solution (2% in ethanol:water [95:5, v/v]; Sigma), mounted in 50% HCl, and photographed using a 3CCD (charge-coupled device) color video camera (DXC-390P; Sony, Tokyo, Japan). For cellulose staining, paraffin-embedded sections (10  $\mu\text{m}$  thick) were stained with a 0.005% aqueous solution of calcofluor (fluorescent brightener 28; Sigma) for 2 min and visualized with a fluorescent microscope (Leica, Wetzlar, Germany).

### DNA Isolation and DNA Gel Blot Analysis

The rice genomic DNA preparation and DNA gel blot analysis were performed as described (Mou et al., 2000; Qian et al., 2001). Briefly, rice genomic DNA (20  $\mu\text{g}$ ) was digested completely with restriction enzymes and then separated by 0.8% agarose gel electrophoresis. The DNA then was transferred onto a Hybond N<sup>+</sup> membrane (Amersham) and hybridized with a  $^{32}\text{P}$ -labeled probe under high-stringency hybridization conditions.

### Genetic Analysis and Marker Development

The genetic linkage between the *BC1* locus and molecular markers was determined using Mapmaker (Lander et al., 1987). To fine-map the *BC1*

locus, new molecular markers, especially the cleaved amplified polymorphic sequence markers, were developed (Table 2).

### Construction of the *BC1* BAC Contig

To construct a BAC contig covering the *BC1* locus, we screened a BAC library with two flanking markers, C80a and C524a, identified the overlapped BAC clones based on their fingerprints (<http://www.genome.clemson.edu>), and determined the linkage with the new markers developed from the BAC ends. By repeating this process, the *BC1* locus eventually was located in a single BAC, OSJNBa0036N23.

### DNA Sequencing

The BAC clone OSJNBa0036N23 was sequenced using a shotgun approach. To sequence the *bc1-1* and *bc1-2* alleles, the entire genomic regions were amplified from the mutants (*bc1-1* and *bc1-2*) and their corresponding wild-type plants by PCR with LA-Taq (TaKaRa, Dalian, China). The PCR program included 3 min at 94°C, followed by 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were sequenced directly, and the mutations in *bc1-1* and *bc1-2* were identified and verified further by sequencing two additional independent PCR products. The mutation in *bc1-2* also can be detected by restriction analysis with BstNI.

### Complementation Test

A 5.86-kb genomic DNA fragment containing the entire *BC1* coding region, the 2796-bp upstream sequence, and the 1459-bp downstream sequence was inserted into the binary vector pCAMBIA1300 to generate the transformation plasmid pCna18 for the complementation test. A control plasmid, pCna181T, containing the 3' truncated *BC1* gene that encodes the first 173 amino acid residues, also was constructed (Figure 3E) according to the strategy described previously (Li et al., 2003). The two binary plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation, and the rice *bc1-2* mutant was transformed according to a published method (Hiei et al., 1994).

### Reverse Transcription– or Rapid Amplification of cDNA Ends–PCR Analysis

Total RNA was extracted from leaves, leaf sheaths, culms, roots, and entire plants according to the method described by Wadsworth et al. (1988). For reverse transcription (RT)–PCR, first-strand cDNA was transcribed reversibly from total RNA with oligo(dT) as the primer and used as the template to amplify the transcripts with a profile (35 cycles) of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. Primers for *BC1* RT-PCR were BC1F and BC1R (5'-GGTAGTTGAAGTTTGATGGC-3' and 5'-GCTCTCTCCTCGCTCGGCTCC-3'), and those for actin were ActF and ActR (5'-TCCATCTTGGCATCTCTCAG-3' and 5'-TCCATCTTGGCATCTCTCAG-3'). The 5' and 3' ends of the *BC1* cDNA were amplified from wild-type Shuang Ke Zao total RNA using the rapid amplification of cDNA ends (RACE) kit (TaKaRa) according to the kit manual. Primers for 5' RACE were RACE1F (5'-CCCACGATGGACCAGATGACC-3'), the first nested primers N1F and N1R (5'-GTCACGCGAGGAGCCGAGC-3' and 5'-ACATCACGTTAAAGTGGGACG-3'), and the second nested primers N2F and N2R (5'-GAGCCGAGCGAGGAGAGAGC-3' and 5'-CCAGATGTACCGGCAGATCC-3'). Primers for 3' RACE were BC4R (5'-GGAGAGCAGTGTAGGTAGGG-3') and the nested primer BC11R (5'-CAGTCCCCAGTTAGGCCAGC-3'). The RACE-PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced.

### RNA in Situ Hybridization

RNA in situ hybridization was performed as described (Kao et al., 2002). The 3' end of *BC1* was subcloned into pGEM-T vector (Promega) and used as a template to generate RNA probes. Transverse sections (10 μm thick) were probed with digoxigenin-labeled antisense probes (DIG Northern Starter Kit; Roche, Indianapolis, IN). The slides were observed with a microscope (Leica) and photographed using a 3CCD color video camera (DXC-390P; SONY).

### Sequence and Phylogenetic Analyses

*BC1L* genes were identified by searching the whole genome draft sequence database of rice subspecies *indica* (Yu et al., 2002) using the DNA or protein sequences of the rice *BC1* gene and Arabidopsis COBRA family genes as queries. The *japonica* ortholog of each *BCL1* gene was determined by searching the nonredundant and high-throughput genomic sequence databases using Basic Local Alignment Search Tool (BLASTN). The retrieved *japonica* BAC or PAC sequences were used to posit each *BC1L* gene to a particular chromosomal location based on the rice physical map (Chen et al., 2002).

Gene prediction was performed using Fgenesh (Salamov and Solovyev, 2000), and intron/exon structures were verified by alignment of the EST sequences of rice and other grass species with the genomic DNA. The signal peptide was predicted with SignalP version 2.0 (Nielsen and Krogh, 1998), and the hydrophobic profile was determined with the program provided by the Weizmann Institute of Sciences (<http://bioinformatics.weizmann.ac.il>). Glycosylphosphatidylinositol modification was predicted using big-PI ([http://mendel.imp.univie.ac.at/gpi/index\\_content.html](http://mendel.imp.univie.ac.at/gpi/index_content.html)) and PSORT (<http://psort.nibb.ac.jp>).

Phylogenetic analysis included all rice *BC1L* proteins and Arabidopsis COBRA family members except AtCOBL5, because of the incompleteness of its sequence. Multiple sequence alignments were conducted using CLUSTAL X version 8.0 (Thompson et al., 1997) with the PAM matrix (Dayhoff, 1979). A neighbor-joining tree (Saitou and Nei, 1987) was built using MEGA version 2.1 (Kumar et al., 2001) adopting Poisson correction distance, and the tree was presented using TreeView (Page, 1996). Support for the tree obtained was assessed using the bootstrap method (Felsenstein, 1985). The number of bootstrap replicates was 1000. Similar topology was obtained by using the protpas program in the Phylip package (Felsenstein, 1993) to estimate maximum parsimony and the proml program in the Phylip package to estimate maximum likelihood. Alignments with the full-length sequences or only the conserved regions of each family member produced trees of similar topology.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact J. Li, [jyli@genetics.ac.cn](mailto:jyli@genetics.ac.cn).

### Accession Numbers

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