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Rice Brittle culm 6 encodes a dominant-negative form of CesA protein that perturbs cellulose synthesis in secondary cell walls

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Abstract

The brittle culm (bc) mutants of Gramineae plants having brittle skeletal structures are valuable materials for studying secondary cell walls. In contrast to other recessive bc mutants, rice Bc6 is a semi-dominant bc mutant with easily breakable plant bodies. In this study, the Bc6 gene was cloned by positional cloning. Bc6 encodes a cellulose synthase catalytic subunit, OsCesA9, and has a missense mutation in its highly conserved region. In culms of the Bc6 mutant, the proportion of cellulose was reduced by 38%, while that of hemicellulose was increased by 34%. Introduction of the semi-dominant Bc6 mutant gene into wild-type rice significantly reduced the percentage of cellulose, causing brittle phenotypes. Transmission electron microscopy analysis revealed that Bc6 mutation reduced the cell wall thickness of sclerenchymal cells in culms. In rice expressing a reporter construct, BC6 promoter activity was detected in the culms, nodes, and flowers, and was localized primarily in xylem tissues. This expression pattern was highly similar to that of BC1, which encodes a COBRA-like protein involved in cellulose synthesis in secondary cell walls in rice. These results indicate that BC6 is a secondary cell wall-specific CesA that plays an important role in proper deposition of cellulose in the secondary cell walls.

Key words: Brittle culm, cellulose synthesis, CesA protein, dominant-negative form, hemicellulose, rice, secondary cell wall, transgenic plant.

Introduction

Secondary cell walls function as skeletal frameworks and furnish the plant body with mechanical strength. Studies on Arabidopsis thaliana mutants have identified several components involved in the formation of secondary cell walls. The secondary cell wall-specific cellulose synthase catalytic subunits (CesAs), AtCesA8, AtCesA7, and AtCesA4, were first identified from their respective mutants irregular xylem 1 (irx1), irx3, and irx5, which show collapsed morphology in the xylem (Turner and Somerville, 1997; Taylor et al., 1999, 2000, 2003). A series of studies on Arabidopsis fragile fiber (fra) mutants with reduced inflorescence stem mechanical strength revealed that in addition to CesA proteins, the formation of secondary cell walls requires katanin, kinesin-like protein, and phosphatidylinositol phosphatase (Burk et al., 2001; Zhong et al., 2002, 2003, 2004). Decreased cellulose content is common to all these mutants, suggesting
that proper synthesis and accumulation of cellulose microfibrils are central events in the formation of secondary cell walls in higher plants.

*CesA* was first cloned in cotton fibre as a plant homologue of bacterial cellulose synthase (Pear *et al.*, 1996). In general, CesA proteins have eight transmembrane domains, a large cytoplasmic region between the second and third transmembrane domains, and a relatively small N-terminal cytoplasmic domain. To date, 10 CesA genes have been identified in the genome of *Arabidopsis* and nine in that of rice (*Oryza sativa*). These genes may be categorized into two groups according to their involvement in cellulose synthesis in primary or secondary cell walls. In *Arabidopsis*, the expression patterns of secondary cell wall-specific CesA genes encoding AtCesA4, AtCesA7, and AtCesA8 are highly correlated. These CesAs are known to form a cellulose synthase complex (CSC) (Taylor *et al.*, 2003; Brown *et al.*, 2005; Atanassov *et al.*, 2009). However, the specific role of each CesA component in the overall cellulose synthesis pathway remains obscure.

Some mutations in the CesA genes confer resistance to herbicide and pathogens. The *isoxaben resistant 1* (*ixr1*) and *ixr2* mutants of *Arabidopsis* have missense mutations in the conserved C-terminal regions of AtCesA3 and AtCesA6, respectively (Scheible *et al.*, 2001; Desprez *et al.*, 2002). The Arabidopsis *cevl* mutant with a mutation in the second cytoplasmic domain of AtCesA3 shows constitutive expression of the jasmonate-responsive genes, vegetative storage protein 1, protodermal factor 1.2, thionin 2.1 pathogenesis-related protein 13, and basic chitinase B pathogenesis-related protein 3, and this confers greater resistance to powdery mildew diseases (Ellis and Turner, 2001; Ellis *et al.*, 2002). In addition, altered secondary cell wall integrity due to defects in secondary cell wall-specific CesAs results in enhanced resistance to soil bacteria through activation of the abscisic acid (ABA) pathway independent of signalling by salicylic acid, ethylene, or jasmonate (Hernández-Blanco *et al.*, 2007).

The *brittle culm* (*bc*) mutants of Gramineae plants exhibit reduced mechanical strength of the plant body, especially in culms (Kokubo *et al.*, 1989, 1991; Ching *et al.*, 2006; Sindhu *et al.*, 2007). In rice, nine *bc* mutants (*bc1, bc2, bc3, bc4, bc5, bc6, bc7, bc10, and bc11*) have been found to date, and some of them were used as classic genome markers. These *bc* mutants are valuable materials for understanding the mechanism of secondary cell wall formation. Rice *BCI* and maize (*Zea mays*) *Brittle stalk 2* encode COBRA-like glycosylphosphatidylinositol-anchored proteins (Li *et al.*, 2003; Ching *et al.*, 2006). *OsDRP2B* encoding a plant classical dynamin has been identified as the causative gene for the rice *bc3* mutant (Hirano *et al.*, 2010). Rice *bc7* and *bc11* have mutations in *OsCesA4*, a secondary cell wall-specific CesA protein whose sequence is highly similar to AtCesA8/IRX1 (Yan *et al.*, 2007). Furthermore, on the basis of sequence similarity, OsCesA4, OsCesA7, and OsCesA9 have been proposed to correspond to AtCesA8, AtCesA4, and AtCesA7, respectively (Tanaka *et al.*, 2003).

With respect to its genetic semi-dominance, rice *Bc6* is unique among *bc* mutants of Gramineae plants. Here, it is reported that rice *Bc6* has a missense mutation in the *OsCesA9* gene. On the basis of cell wall properties of the *Bc6* mutant and the expression pattern of the *Bc6* gene, it is proposed that *OsCesA9* is a secondary cell wall-specific CesA of rice and that the mechanism for cellulose synthesis in secondary cell walls is highly conserved between rice and *Arabidopsis*.

### Materials and methods

#### Plant materials

Seeds of the *Bc6* mutant (RGS number 420) and *IR68* were provided by Dr Khush of the International Rice Research Institute (IRRI, Laguna, Philippines). Seeds of Taichung 65 (T65) was distributed from National Genetic Institute (Mishima, Japan). *A. japonica* cultivar, T65, was mainly used to represent wild-type control. Rice plants were grown under field conditions at the National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan.

#### Analysis of cell wall polysaccharides

Fractionation and quantification of cell wall polysaccharides were performed as described (Aohara *et al.*, 2009). Plant tissues were homogenized to a fine powder using a mortar and pestle in liquid nitrogen. The homogenates were washed twice with water, heated in 80% (v/v) ethanol at 100 °C for 15 min to inactivate endogenous cell wall enzymes, and then treated with a-amylase (100 U, Type VII-A from porcine pancreas, Sigma-Aldrich, St Louis MO, USA) in 50 mM 3-morpholinopropanesulfonic acid-NaOH buffer (pH 6.5) at 37 °C for 4 h. After removal of solubilized starch by centrifugation at 1500 g, the cell wall materials were sequentially extracted at 100 °C for 10 min with water, 50 mM EDTA (pH 6.8) (pectin fraction), and 17.5% (w/v) NaOH containing 0.4% NaBH₄ (hemicellulose fraction). The residual precipitate was washed with water, ethanol, and diethyl ether, and collected as the cellulose fraction. Hemicellulose was neutralized with acetic acid, dialysed against water at 4 °C for 1 d, and lyophillized. The sugar content in each fraction was measured by the phenol-sulphuric acid method (Dubois *et al.*, 1956) using glucose as the standard.

Klason lignin content was determined using cell walls prepared from culms of *Bc6* mutants and T65 according to the method reported by Kirk and Obst (1998).

#### Determination of sugar composition

Hemicellulose was hydrolysed in 72% (v/v) H₂SO₄ at 4 °C for 1 h, followed by addition of 8 vols of water and hydrolysis at 100 °C for 4 h. After neutralization with solid BaCO₃, the content of each fraction. Hemicellulose was neutralized with acetic acid, dialysed against water at 4 °C for 1 d, and lyophillized. The sugar content in each fraction was measured by the phenol-sulphuric acid method (Dubois *et al.*, 1956) using glucose as the standard.

#### Histology

The uppermost internode was fixed in an FAA solution (water/ethanol/acetic acid/formaldehyde=45:45:5:5, v/v), dehydrated through a graded ethanol/t-butyl alcohol series (0–100% t-butyl alcohol), embedded in paraffin, and sectioned with a microtome (Leica, RM2125RT, Leica Microsystems, Wetzlar, Germany) at a thickness of 10 μm. The sections were washed in a xylen/ethanol series (0–100% ethanol, v/v), dehydrated with a grade ethanol/water series (90–50% ethanol, v/v), stained with 1% (w/v) Safranin O (Waldeck, Münster, Germany) for 24 h, washed with an ethanol/water series (50–95% ethanol, v/v), stained with 0.5% (w/v) Fast Green FCF (Wako, Tokyo, Japan) for 45 s, and washed with 95%
and 100% ethanol (v/v). To detect lignin in cell walls, hand-cut sections of culm were stained with 2% (w/v) osmium tetroxide and then treated with 18% (w/v) HCl. The sections were observed under a light microscope (Eclipse E400, Nikon, Tokyo, Japan).

Transmission electron microscopy

Tissues were fixed in 50 mM phosphate buffer (pH 7.0) containing 2% (v/v) glutaraldehyde. After washing with the phosphate buffer, tissues were post-fixed with 2% (w/v) osmium tetroxide in the phosphate buffer. Tissues were dehydrated through a graded series of acetone and gradually infiltrated with Spurr’s resin, which was polymerized by incubation at 70 °C overnight. Ultra-thin, 90–100 nm sections were cut with a diamond knife on a Sorvall MT-IIIB ultramicrotome (Thermo Electron Corporation, Asheville, NC, USA) and then stained with 2% (w/v) uranyl acetate for 15 min followed by lead citrate for 5 min. The sections were observed with a Hitachi H-7500 electron microscope (Hitachi Science Systems, Ibaraki, Japan) at an acceleration voltage of 100 kV.

Positional cloning

For positional cloning of the BC6 gene, the BC6 mutant was crossed with a japonica cultivar, Toride 1, and the resulting F2 population was analysed for their brittle phenotype and genotype. Together with DNA markers reported previously (Yamamoto and Sasaki, 1997), the co-dominant DNA markers designed on the basis of genomes of a japonica cultivar, Nipponbare (http://rgp.dna.affrc.go.jp/), and an indica cultivar, 93-11 (http://brad.genomics.org.cn:8080/rice/) were used (Table 1). Genomic DNA was extracted from leaves of F2 plants as described (Aohara et al., 2009). PCR was performed with Phusion DNA polymerase (Finnzymes, Espoo, Finland) under the following conditions: 10 s denaturation at 98 °C, 35 cycles.

The genomic fragment of BC6 was amplified by PCR using a set of specific primers, gBc6-F1 (5'-GAAGCTTCTTACACAGGCCAAGGACACGTCGTCCT-3') and gBc6-R1 (5'-GGCGGACGCATCTCACAAG-3') of specific primers, gBc6-F1 (5'-GAAGCTTCTTACACAGGCCAAGGACACGTCGTCCT-3') and gBc6-R1 (5'-GGCGGACGCATCTCACAAG-3'). The nucleotide sequence was determined with an ABI genetic analyzer (PRISM 3100, ABI, Foster City, CA, USA), and compared with that of 93-11. BC6 cDNA was amplified by reverse transcription-PCR (RT-PCR) using a set of primers, Bc6cDNA-F (5'-GGCTCTAGAGCTGCAATCTGAATATAGA-3') and Bc6cDNA-R (5'-ACCTCTAGAGCTCAATCATGAATAATTCC-3'), and sequenced.

Introduction of the BC6 gene

A genomic fragment containing the mutant BC6 gene (9374 bp), including 4.8 kbp of upstream sequence and 492 bp downstream, was generated by changing the mutated nucleotide at position 7112 from G to wild-type A by PCR mutagenesis with primers, PM-F1 (5'-GCAGCTCTCCGCGAGGTTCGCGG-3') and PM-R1 (5'-ATCGAGTCCACAGGCAGATGCC-3'). These constructs were introduced into the wild-type plant, T65, by an Agrobacterium (Rhizobium radiobacter)-mediated method using strain EHA101 (Hood et al., 1986; Toki, 1997). The T2 generation of transgenic plant was used for cell wall analysis.

Promoter:GUS assay

Promoter gBc6:β-glucuronidase (pBC6:GUS) activity was assayed according to the method of Kosugi et al. (1991). The 4.8 kbp promoter region of BC6 was amplified by PCR using specific primers, gBc6-F1 and gBc6-R1 (5'-GAGGATCCCATGGCCGCGCAACACGGCGG-3') and fused with the GUS gene in the pBIGRZ vector, yielding pBC6:GUS. The nucleotide sequence of the construct was confirmed before transformation into the wild-type plant, T65, as described above. Culms, leaves, nodes, and seedlings of the transgenic plants harbouring the pBC6:GUS gene were cut into pieces, fixed in 5% (w/v) agar, and hand-sectioned. Sections from the transgenic plants were stained with a solution containing 0.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferricyanide, and 50 mM phosphate buffer (pH 7.4) at 37 °C for 24 h, and observed under a microscope (Eclipse E400).

Quantitative analysis of BC1 and BC6 mRNAs

Relative amounts of BC1, BC3, BC6, OsCesA4, and OsCesA7 mRNA were estimated by quantitative RT-PCR. Single-stranded cDNA was synthesized from total RNA of the tissues or organs using oligo(dT)12-18 primer. The following specific primers were designed using the Primer3 program (http://frodo.wi.mit.edu/): for BC1 (Os03g0416200), BC1-RTP-F1 (5'-CGCATGAACATCACCCATGT-3') and BC1-RTP-R1 (5'-TCCATTGAGCAGTTCGTTGTA-3'); for BC3 (Os02g0738900), BC3-RTP-F1 (5'-GGCCGAAACATGAGATTATTA-3') and BC3-RTP-R1 (5'-AACATACGAAGCTGATGTTG-3'); for BC6 (Os09g0422500), BC6-RTP-F1 (5'-TTAGCACGTTTCGAGGTG-3') and BC6-RTP-R1 (5'-GAACATGTCGTCCCTGCTC-3'); for OsCesA4 (Os01g0750300), OsCesA4-RTP-F1 (5'-CTAATGCGACGAGACGATG-3') and OsCesA4-RTP-R1 (5'-GATT-TAACCGTGCCCTTCTCA-3'); for OsCesA7 (Os10g0467800), OsCesA7-RTP-F1 (5'-TCCATTCTTCTCCCTCTCGTCT-3') and OsCesA7-RTP-R1 (5'-GAATCATCCCATCGCCTAC-3'); and for ACTIN1 (Os03g0718000), ACTIN1-RTP-F1 (5'-TTCCATACATGCGCTGACT-3') and ACTIN1-RTP-R1 (5'-AGCGTCTGG-CATTCCACAT-3'). The PCR was performed with a SYBR Premix Ex Taq kit (Takara Bio Inc, Otsu, Japan) under the following conditions: 10 s denaturing at 95 °C, 30 s annealing at 60 °C, and 30 s extension at 72 °C, 35 cycles.

Table 1. DNA markers used for positional cloning

| Marker     | Forward primer                      | Reverse primer                      | Length (bp)a | Type   |
|------------|-------------------------------------|-------------------------------------|--------------|--------|
| 5838-EcoF1 | 5'-GTCGCCACATGTCACACACAC-3'         | 5'-CACACACATCCAGGAGAAGCG-3'         | 264          | CAPS (EcoRI) |
| E61522     | 5'-GGCTTTGAGGAGAATCTCAC-3'          | 5'-TTAGTGGAGGAGAAGATCC-3'           | 347          | CAPS (EcoRI) |
| 5579-54kb  | 5'-TGATATGCGCTGCTGAGG-3'            | 5'-TAGATGGAAGGAGGTGAGG-3'           | 349          | SNF   |
| 5579-121kb | 5'-CCCGGCTACACACACAC-3'            | 5'-TCCACCTTGGCTACCTCCTG-3'          | 303          | CAPS (EcoRI) |
| 5420-101kb | 5'-TACTCCCTTCAATCCTGACG-3'         | 5'-AGCTGCACTCATGCTGAC-3'           | 348          | Indel |
| 5568-19kb  | 5'-TGAGTGGCGAGTCTGCTGCTG-3'         | 5'-CTAGCTACATACACACACG-3'           | 379          |        |

a The length of the fragment amplified by PCR from genomic DNA of Toride 1 is shown.

b Cleaved amplified polymorphic sequence. The restriction enzyme used is shown in parentheses.

c Single nucleotide polymorphism.

d Marker with a difference amplified fragment length due to an insertion or deletion event.
60 °C, and 20 s amplification at 72 C, 40 cycles. The PCR products were detected with Opticon 2 (Bio-Rad, Hercules, CA, USA), and the mRNA amounts relative to ACTIN1 mRNA were calculated.

Results

Reduced cellulose content in the Bc6 mutant

The rice Bc6 mutant was generated from an indica cultivar, IR68, by treatment with ethyl methanesulphonate (Singh et al., 1994). As with rice bc1 and bc7 mutants (Li et al., 2003; Yan et al., 2007), culms and leaves of Bc6 mutants were easily broken when bent. The brittle phenotype was also observed in Bc6/BC6 heterozygotes, suggesting that Bc6 is a dominant mutation as reported previously (Sanchez and Khush, 2000; Singh et al., 1994). No pleiotropic phenotypes in the appearance of Bc6 were observed, such as dwarfism or withering that are observed for rice bc3 and bc11 mutants (Iwata and Omura, 1989; Zhang et al., 2009; Hirano et al., 2010). Because the genetic background of IR68 and Bc6 could not be confirmed to date, homozygous Bc6 mutant plants were compared with the wild-type japonica cultivar, T65.

To characterize cell wall defects in the Bc6 mutant, cell wall polysaccharides were extracted, and the amount in different fractions (hot water, pectin, hemicellulose, and cellulose) were compared with those in wild-type plants. As shown in Fig. 1A, culms of Bc6 exhibited a 38% decrease in the proportion of cellulose in cell wall polysaccharides compared with that of T65 (Fig. 1). The value corresponds to a 31% decrease in the cellulose content based on weight compared with that of the wild-type plant, T65 (here, the content means the cellulose amount per fresh weight). The decrease in the cellulose content was comparable with that of bc1 (30% of the content based on weight) (Li et al., 2003). Conversely, the proportion of hemicellulose in the cell wall polysaccharides was increased by 34% compared with that of T65, which corresponds to a 48% increase in hemicellulose content based on weight. The increased hemicellulose content is probably a compensation reaction of rice with reduced cellulose content. Indeed, a similar increase in the hemicellulose content has also been observed for the bc1 mutant (Li et al., 2003). A decrease in the cellulose proportion and an increase in the hemicellulose proportion were also observed in the matured leaves of Bc6 mutants in samples taken at the same time as the culms, but the alterations in the components were rather mild compared with those of the culms (Fig. 1B). Essentially the same results were also obtained in a comparison of Bc6 mutants with the parental line, IR68 (Fig. 1).

Sugar composition analysis by HPAEC-PAD was used to examine whether the proportion of particular polysaccharides was increased by the Bc6 mutation. In both Bc6 and wild-type plants, the hemicellulose mainly consisted of xylose, glucose, and L-arabinose (Table 2). These results indicate that Bc6 mutation did not affect the sugar composition of hemicellulose in culms; therefore Bc6 mutation does not seem to cause the accumulation of particular hemicellulosic polysaccharides in the cell walls.

Tissue organization of Bc6

To address the effects of Bc6 mutation on tissue morphology, transverse sections of Bc6 culms stained with Safranin O and Fast Green FCF were observed microscopically. Bc6 mutants exhibited neither the altered xylem tissue organization nor the incomplete cell wall found in Arabidopsis irx mutants (Turner and Sommerville, 1997) (Fig. 2A, B).

To examine lignin accumulation in the tissue, transverse sections were treated with phloroglucinol to stain lignin-rich cell walls selectively. Although phloroglucinol staining appeared slightly stronger in Bc6 mutants than in T65 (see Supplementary Fig. S1 available at JXB online), quantitative analysis by the Klason method (Kirk and Obst, 1988) did not demonstrate a significant increase in lignin content in culms of Bc6 (Bc6, 2.23±0.65 mg g⁻¹ fresh weight; T65, 2.42±0.20 mg g⁻¹ fresh weight). Other cell wall components such as cellulose and hemicellulose may influence the staining.

Table 2. Sugar composition of hemicellulose

| Sugar       | Composition (mol %) |
|-------------|---------------------|
|             | T65     | IR68    | Bc6     |
| L-Arabinose | 8.5     | 13.7    | 9.3     |
| L-Fucose    | 0.0     | 0.1     | 0.0     |
| Galactose   | 1.3     | 4.0     | 1.5     |
| Glucose     | 18.4    | 10.4    | 15.3    |
| Mannose     | 0.0     | 0.0     | 0.0     |
| L-Rhamnose  | 0.3     | 0.7     | 0.3     |
| Xylose      | 69.6    | 69.8    | 72.0    |
| Galacturonic acid | 1.2 | 0.5 | 1.2 |
| Glucuronic acid  | 0.7 | 0.9 | 0.4 |
Cloning of the Bc6 gene

Positional cloning of the Bc6 causative gene was performed using the F2 population generated by crossing Bc6 with the japonica cultivar, Toride 1. To determine the genotype of F2 plants, several DNA markers were designed on the basis of the genomes of the japonica cultivar, Nipponbare, and the indica cultivar, 93-11 (Table 1). The BC6 gene was mapped between 5838-EcoRI and E61552 markers on chromosome 9 (Fig. 3A), consistent with its location on chromosome 9 in the classical linkage map (Sanchez and Khush, 2000). By using ~1000 F2 plants, the BC6 locus was further narrowed to a 170 kb region covered by bacterial artificial chromosome (BAC) clones AP005579 and AP005420 (Fig. 3A). This region included the Os09g0422500 gene encoding OsCesA9, which at 79% identity shares the highest sequence similarity with AtCesA7 among 10 Arabidopsis CesAs. Sequence analysis of OsCesA9 identified a missense mutation that substitutes a highly conserved arginine residue with glycine (R588G, accession no. AB527075; Fig. 3A). The R588 residue is located in the middle region of the second cytoplasmic domain, but is not part of a QXXRW motif (791–795 in OsCesA9): the motif was shown to be required for catalytic activity of chitin synthase in yeast (Nagahashi et al., 1995; Somerville 2006). In Arabidopsis, fra5, a semi-dominant P557T mutation of AtCesA7, causes decreased cellulose content in fibre cells, perturbing cell wall thickening. Importantly, P557 of AtCesA7 corresponds to P586 of OsCesA9, a site quite near the R588 of the OsCesA9 residue mutated in Bc6 (Fig. 3B). Taken together, these results suggest that this region of CesA is essential for proper cellulose synthesis in secondary cell walls of both Arabidopsis and rice. Supporting this hypothesis, the region was found to be highly conserved among all CesAs of rice (Fig. 3B).

Introduction of the Bc6 gene into the wild-type plant

To confirm that the missense mutation in OsCesA9 is responsible for the brittle phenotype and decreased cellulose content of Bc6, the mutant Bc6 gene was introduced into the wild-type plant, T65. As a control, the missense R588G mutation was corrected by PCR, and this wild-type BC6 gene was introduced into T65. Transgenic plants harbouring mutant Bc6 (lines b12 and b15) had the brittle phenotype in culms and leaves, but did not show any morphological alterations such as dwarfism as observed for Bc6 mutants (Fig. 4A). Along with the brittle phenotype, a transgenic plant harbouring mutant Bc6 (line b12) also showed an apparently reduced proportion of cellulose in the cell walls (21% decrease in proportion), which corresponds to a 24% decrease in cellulose content based on weight compared with that harbouring the wild-type gene, BC6.
These facts confirm that \textit{Bc6} mutation is due to the R588G substitution. Introduction of the mutant \textit{Bc6} gene appeared to reduce the thickness of secondary cell walls in epidermal and sclerenchymal cells (Fig. 5). These results suggested that Bc6 protein with the R588G mutation perturbs cellulose synthesis in secondary cell walls by acting as a dominant-negative form. The cellulose deficiency in these transgenic plants was milder than that in \textit{Bc6} mutants (31\% decrease based on weight) (Figs 1A, 4B), presumably because of the presence of wild-type BC6 protein in the transgenic plants. Furthermore, the decrease in the proportion of cellulose of one transgenic line harbouring the mutant \textit{Bc6} (line b2) was milder than that of other lines (lines b12 and b15) (Fig. 4B). Hence, this mutant gene behaved as a dose-responsive semi-dominant rather than fully dominant form in this experiment.

**Expression pattern of BC6**

The pattern of expression of \textit{BC6} was assessed by analysing T65 plants transformed with pBC6:GUS, a binary vector in which the 4.8 kbp region upstream of the \textit{BC6} gene was fused with the \textit{GUS} reporter gene. \textit{BC6} promoter activity was detected in leaves, culms, and nodes, with relatively strong expression in culm vascular bundles 2 weeks after heading (Fig. 6A–C). Promoter activity was also observed in young tissues such as developing leaves (Fig. 6D). Although \textit{Bc6} mutation appeared to reduce cell wall thickness in sclerenchymal cells (Fig. 5), promoter activity was not detected in developed sclerenchymal cells (Fig. 6A, B). It is possible that the reporter gene activity did not completely mirror the expression of the \textit{BC6} gene product, OsCesA9 protein. These patterns of \textit{BC6} promoter-driven gene expression were similar to the expression pattern of \textit{BC1} demonstrated by \textit{in situ} hybridization (Li \textit{et al.}, 2003).

In \textit{Arabidopsis}, the \textit{COBL4} gene is co-expressed with the secondary cell wall-specific \textit{CesA} genes and is presumed to play a role in the cellulose synthesis of secondary cell walls, although the precise molecular functions of COBRA-like proteins remain unclear (Schindelman \textit{et al.}, 2001; Roudier \textit{et al.}, 2005). To examine the relationship between the secondary cell wall-specific CesA and COBRA-like proteins in rice, \textit{BC6} and \textit{BC1} mRNAs were quantitated in several tissues. Consistent with the results of the pBC6:GUS analysis, relatively high levels of \textit{BC6} mRNA were detected in culms and nodes (Fig. 7A). The level of \textit{Bc6} mRNA in roots was relatively low. Indeed, the brittle phenotype in

![Fig. 4. Introduction of the semi-dominant \textit{Bc6} gene into wild-type plants. Introduction of the genes, including the 4.8 kbp upstream region, into T65 yielded 15 lines of transgenic plants harbouring the mutant \textit{Bc6} gene and seven lines harbouring the wild-type \textit{BC6} gene. (A) Appearance of representative lines of wild-type \textit{BC6} (W7, left) and mutant \textit{Bc6} (b12, right) transgenic plants. The scale bar indicates 30 cm. (B) Proportions of cell wall fractions in culms of transgenic plants harbouring wild-type \textit{BC6} (lines W7 and W9, blue bars) and the mutant \textit{Bc6} (lines b2, b12, and b15, pink bars). Values shown are averages of three plants, and the bars represent standard errors.

![Fig. 5. Cell wall structure in transgenic plants. Transmission electron microscopic images of sclerenchymal cells in culms of transgenic plants harbouring the wild-type \textit{BC6} (line W7) (A) or mutant \textit{Bc6} gene (line b12) (B). Scale bars indicate 5 μm.](https://example.com/fig5.png)
roots was not clear compared with those in culms and leaves (data not shown). Despite the apparent brittle phenotype in the leaves of Bc6 mutants, the level was low in both leaf blades and sheaths. Importantly, both BC6 and BC1 were highly expressed in culms, nodes, and flowers (Fig. 7A, B). These results indicated that BC6 and BC1 are co-expressed during development of secondary cell walls. On the other hand, the expression of BC6 was not related to that of BC3, suggesting that BC6 and BC3 are differently regulated.

The effect of the mutation on the expression of Bc6 was also examined in developing leaf blades. Bc6 mutants showed accumulation of BC6 mRNA comparable with T65 (Fig. 8). Furthermore, Bc6 mutation barely influenced the mRNA levels of other CesA genes, OsCesA4 and OsCesA7, which are expected to participate in cellulose synthesis in secondary cell walls, together with BC6 (OsCesA9).

Discussion

Brittle phenotypes caused by mutation of OsCesA9

The bc loci have been used for genetic analysis of rice for >70 years, but several causative genes are yet to be cloned. The present study demonstrates that the BC6 gene encodes OsCesA9, a secondary cell wall-specific CesA. Insertional disruption of OsCesA9 by the endogenous retrotransposon Tos17 was previously reported (Tanaka et al., 2003). While ND2395 and NF1011 mutations in that study were recessive, the Bc6 missense mutation, R588G, in OsCesA9 reported here is semi-dominant. The phenotypes of Bc6 were milder than those of ND2395 and NF1011, i.e. Bc6 caused a 31% decrease in cellulose content relative to T65, while ND2395 and NF1011 showed a 91% and 46% decrease, respectively, compared with the wild-type plant. Furthermore, Bc6 did not affect plant growth, whereas ND2395 and NF1011 plants exhibited dwarfism, small leaves, and thin culms. These differences suggest that the transposon insertions in OsCesA9 affect the formation of primary cell walls, while the Bc6 missense mutation, R588G, does not. Indeed, a link between secondary cell wall integrity and primary cell wall deposition and remodelling has also been reported in Arabidopsis. The AtCesA7 mutation, murus10, results in altered structure of pectins and xyloglucan, leading to dwarfism (Bosca et al., 2006). In the case of the OsCesA4 gene, be7 mutation comprising deletions in exon 10 and intron 10 does not affect growth
and development, but the bc11 missense mutation, G858R, causes severe dwarfism (Yan et al., 2007; Zhang et al., 2009). The severity of the phenotype probably depends on both mutation type and site.

**Dominant-negative form of CesA**

The semi-dominant brittle phenotype of Bc6 was found to be caused by a missense mutation, R586G, in the second cytoplasmic domain of OsCesA9. Importantly, this mutated residue was located near to P586, and this site corresponds to P557 of AtCesA7 that is altered in the semi-dominant fra5 mutant of *Arabidopsis*. This region, which is distinct from the putative catalytic motif, QXXRW, is highly conserved among the CesA genes of higher plants (Fig. 3). Such conservation is not seen in the corresponding region in cellulose synthase-like proteins (Csls) catalysing related biosynthetic reactions of polysaccharides such as the β-1,4-mannan, β-1,3:1,4-glucan, and the β-1,4-glucan backbone of xyloglucan (Liepman et al., 2005; Burton et al., 2006; Cocuron et al., 2007). These facts suggest the possibility that the region has a function other than the catalysis of β-1,4-glucan synthesis. In the study on the *Arabidopsis* fra5 mutant, Zhong et al. (2003) suggested that the missense P557T mutation of AtCesA7 affects the interaction between CesA proteins or between CesA and other cellular components. In the present study, introduction of the semi-dominant Bc6 mutant gene into T65 caused decreased cellulose content and brittle phenotype. It is possible that the presence of the mutated CesA protein interferes with proper formation of functional CSC. Supporting this hypothesis, the phenotypes of the transgenic plants were milder than those of Bc6 homozygous mutants, possibly because of the presence of wild-type Bc6 protein. Involvement of the second cytoplasmic domain in the formation of CSC has not yet been demonstrated, whereas the interaction of CesAs through their N-terminal RING-finger-like motifs, depending on the redox state, has been demonstrated (Kurek et al., 2002). It remains unknown, however, how the CesA dimers formed through interaction of RING-finger-like motifs assemble to form the rosette structure that includes perhaps 36 CesAs. An epitope tagging analysis of the interaction between secondary cell wall-specific AtCesAs detected higher order CesA oligomerization beyond dimerization (Atanassov et al., 2009). The conserved region of the second cytoplasmic domain of OsCesA9, including R586 and P586, could be a site for a protein–protein interaction to form such higher order oligomerization.

**Cellulose synthesis of secondary cell walls in rice**

Quantitative analysis of gene expression revealed correlated expression of *Bc6* and *Bc1* in rice. Bc6 and Bc1 represent rice counterparts of AtCesA7/IRX3 and AtCOBL4/IRX6, respectively. *AtCesA7*/*IRX3* and *AtCOBL4/IRX6* are co-expressed in tissues during secondary cell wall development, and loss-of-function mutation of either of these genes results in diminished cellulose content and loss of mechanical strength of the plant body (Brown et al., 2005). In addition, disruptions of OsCesA4 and OsCesA7, the respective rice orthologues of *AtCesA8/IRX1* and *AtCesA4/IRX5*, cause the brittle phenotype and decreased cellulose content in rice (Tanaka et al., 2003; Yan et al., 2007; Zhang et al., 2009). The shared requirement for these components in secondary cell wall cellulose synthesis suggests that the mechanism of cellulose synthesis is highly conserved between *Arabidopsis* and rice.

On the other hand, Gramineae plants, including rice, generally show resistance to isoxaben, a strong inhibitor of cellulose synthesis in dicotyledonous plants, including *Arabidopsis* (Desprez et al., 2002). The differential sensitivity to isoxaben suggests that the mechanism of cellulose synthesis differs at least partially between rice and *Arabidopsis*. Future analyses of rice cell wall mutants, including *bc* mutants, should help to clarify the differences in cellulose synthesis between Gramineae and dicotyledonous plants.

**Supplementary data**

Supplementary data are available at JXB online.

**Figure S1.** Lignin staining of sclerenchymal cells. Sclerenchymal cells of T65 (A) and the Bc6 mutant (B) were stained with phloroglucinol-HCl. Scale bars indicate 50 μm. pc, parenchymal cell; sc, sclerenchymal cell; v, vascular bundle.

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