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RESEARCH PAPER

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

Abbreviations: At, *Arabidopsis thaliana*; BAC, bacterial artificial chromosome; bc, brittle culm; CesA, cellulose synthase catalytic subunit; CSC, cellulose synthase complex; Csl, cellulose synthase-like protein; *fra*, *fragile fiber*; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; *irx*, *irregular xylem*; *ixr*, *isoxaben-resistant*; Os, *Oryza sativa*; pBC6:*GUS*, Promoter$_{BC6}$:*β-glucuronidase*; T65, Taichung 65.

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 *cev1* 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 *BC1* 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 α-amylase (100 U, Type VII-A from porcine pancreas, Sigma-Aldrich, St Louis MO, USA) in 50 mM 3-morpholinopropanesulphonic 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 0.04% $NaBH_4$ (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 lyophilized. 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) H_2SO_4 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 $BaCO_3$, the content of each monosaccharide was determined by high-performance anion-exchange chromatography (HPAEC) using a Dionex DX-500 liquid chromatograph equipped with a CarboPac PA-1 column and a pulsed amperometric detector (PAD) (Dionex, Sunnyvale, CA, USA) as described previously (Ishikawa *et al.*, 2000).

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 xylene/ethanol series (0–50% 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) phloroglucinol 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) OsO_4 dissolved 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-IIB ultramicrotome (Thermo Electron Corporation, Asheville, NC, USA) and 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 F_2 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://btn.genomics.org.cn:8080/rice/) were used (Table 1). Genomic DNA was extracted from leaves of F_2 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, 20 s annealing at 60 °C, and 30 s extension at 72 °C, 35 cycles.

The genomic fragment of *Bc6* was amplified by PCR using a set of specific primers, gBc6-F1 (5′-GAAGCTTTCTAGAAGTCCC-GCCAAACC-3′) and gBc6-R1 (5′-GCGGACGCATCTCACA-CAACTGAGGCC-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′-GGCTCTAGAGCGATCGATCGCCCTTCC-3′) and Bc6cDNA-R (5′-ACCTCTAGAGCTGCAATCTGAATATA-TTCC-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 digested with *Hin*dIII and subcloned into the binary vector pBIGRZ (Akiyama *et al.*, 1997) to yield the mutant construct, g*Bc6*/pBIGRZ. The wild-type *BC6* gene construct, *gBC6*/pBIGRZ, was generated by changing the mutated nucleotide at position 7112 from G to wild-type A by PCR mutagenesis with primers, PM-F1 (5′-GCAGTTCCCGCAGAGGTTCGACGGC-3′) and PM-R1 (5′-ATCGACGTCCACGACCGATACGCC-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 T_2 generation of transgenic plant was used for cell wall analysis.

Promoter:GUS assay

Promoter$_{BC6}$: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 pBC6-R1 (5′-GAGGATC-CATGGCCGCGCAACAACGGCCGG-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 ferrocyanide, 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′-CGCATGAACT-ACACCCAGTG-3′) and BC1-RTP-R1 (5′-TCCATGAGCA-GGTCGTTGTA-3′); for *BC3* (Os02g0738900), BC3-RTP-F1 (5′-GGCCGAAACGATGAGATTTA-3′) and BC3-RTP-R1 (5′-AACATCAGCAGCTTGCATTG-3′); for *BC6* (Os09g0422500), BC6-RTP-F1 (5′-TTAGCACGTTTGCGAGTTTG-3′) and BC6-RTP-R1 (5′-GAACTCGTCGTCCTCGTCTC-3′); for *OsCesA4* (Os01g0750300), OsCesA4-RTP-F1 (5′-CTAATGCGACGAA-GACGATG-3′) and OsCesA4-RTP-R1 (5′-GATT-TAACGGTGCCCTCTCA-3′); for *OsCesA7* (Os10g0467800), OsCesA7-RTP-F1 (5′-TCCATCTTCTCCCTCGTCTG-3′) and OsCesA7-RTP-R1 (5′-GAATCATCCATCCGGTCATC-3′); and for *ACTIN1* (Os03g0718100), ACT1-RTP-F1 (5′-TTCCTAC-ATCGCCCTGGACT-3′) and ACT1-RTP-R1 (5′-AGCCTTGG-CAATCCACATCT-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

Table 1. DNA markers used for positional cloning

Marker	Forward primer	Reverse primer	Length (bp)[a]	Type
5838-*Eco*RI	5′-GTCCCACATGTCAACAGAGC-3′	5′-CACACAATCCAGGAGAAGGC-3′	264	CAPS (*Eco*RI)[b]
E61522	5′-GCGTTTGAGGAAGTTACCAC-3′	5′-TTTAGTCGAGGCAGAGATCC-3′	347	CAPS (*Eco*RI)
5579-54kb	5′-TGGTATGGTCTGTGATTGGG-3′	5′-TAGAGTGAAAAGGCTGAGGC-3′	349	SNP[c]
5579-121kb	5′-CCCGGTACACACAACACACC-3′	5′-TCCCACTTTGCACTCCCTGG-3′	303	CAPS (*Eco*RI)
5420-101kb	5′-TGATCCCCTCAATCTGGCAG-3′	5′-AAGTAGCAGGTCCATCGAAC-3′	348	Indel[d]
5568-19kb	5′-TGAGCTAGCGATGTGGCTGG-3′	5′-CTAGCTACACTACACACGGC-3′	379	CAPS (*Eco*RI)

[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).

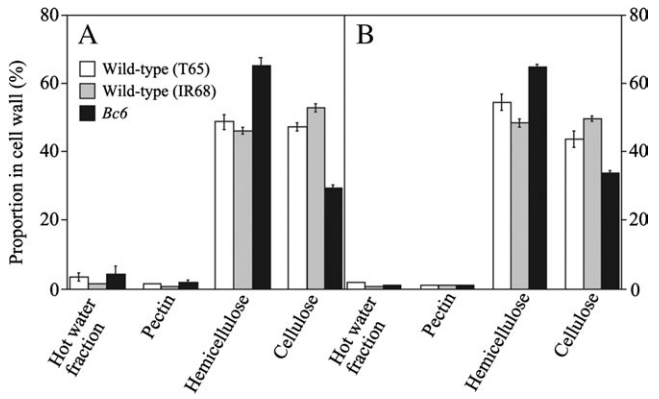

Fig. 1. Proportions of cell wall fractions. The amounts of cell wall fractions extracted from culms (A) and leaves (B) were measured in plants sampled 2 weeks after heading. Open, shaded, and filled bars indicate data for wild-type T65, IR68, and the *Bc6* mutant, respectively. Values shown are averages of three plants, and the bars represent standard errors.

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

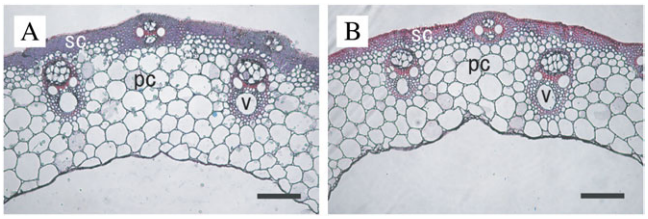

Fig. 2. Comparison of tissue morphology in culms. Transverse sections of the uppermost culms of T65 (A) and the *Bc6* mutant (B) were double-stained with Safranin O and Fast Green FCF. Scale bars indicate 100 μm. pc, parenchymal cell; sc, sclerenchymal cell; v, vascular bundle.

Cloning of the Bc6 *gene*

Positional cloning of the *Bc6* causative gene was performed using the F_2 population generated by crossing *Bc6* with the *japonica* cultivar, Toride 1. To determine the genotype of F_2 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-*Eco*RI 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 F_2 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).

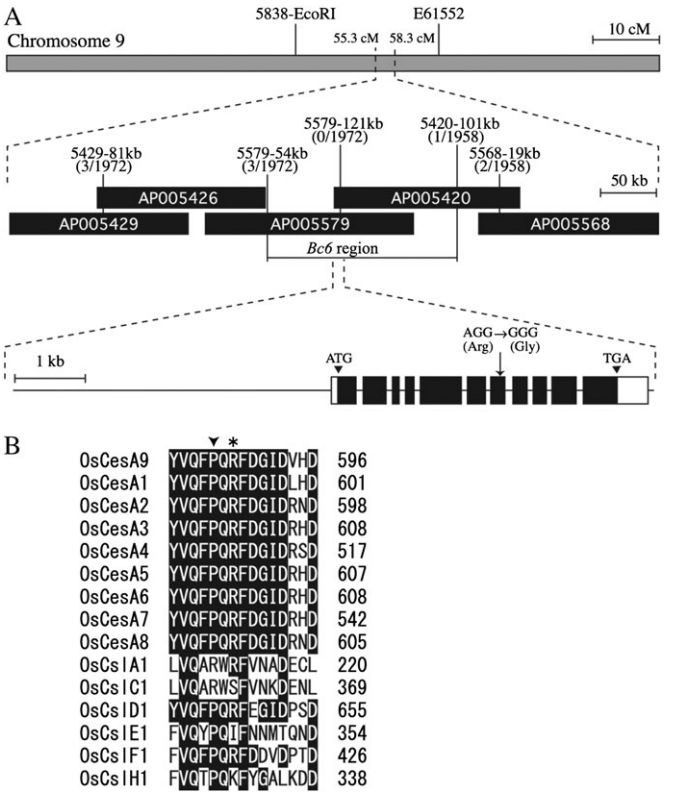

Fig. 3. Cloning of *BC6*. (A) Map-based cloning using an F_2 population of ~1000 plants. The *BC6* locus was narrowed to a 170 kb region of chromosome 9, flanked by markers 5579-54kb and 5420-101kb. BAC clones AP005579 and AP005420 encompass the *OsCesA9* gene. The *Bc6* mutant had the missense mutation, R588G, in the *OsCesA9* gene. Black and white boxes indicate coding and untranslated regions, respectively.
(B) Alignment of amino acid sequences of OsCesAs and OsCsls. The sequence of the highly conserved region of the second cytoplasmic domain of OsCesA9 was aligned with the corresponding regions of other OsCesAs and OsCsls by using the ClustalW program. Residues conserved among all OsCesA members are shown in black. The asterisk indicates the residue mutated in *Bc6*. An arrowhead indicates the residue corresponding to P557 of AtCesA7, which is substituted with threonine by semi-dominant *fra5* mutation. Accession numbers for the sequences of OsCesAs and OsCsls are as follows: OsCesA1, Os05g0176100; OsCesA2, Os03g0808100; OsCesA3, Os07g0424400; OsCesA5, Os03g0837100; OsCesA6, Os07g0252400; OsCesA8, Os07g0208500; OsCslA1, Os02g0192500; OsCslC1, Os01g0766900; OsCslD1, Os10g0578200; OsCslE1, Os09g0478100; OsCslF1, Os07g0553000; OsCslH1, Os10g0341700.

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*

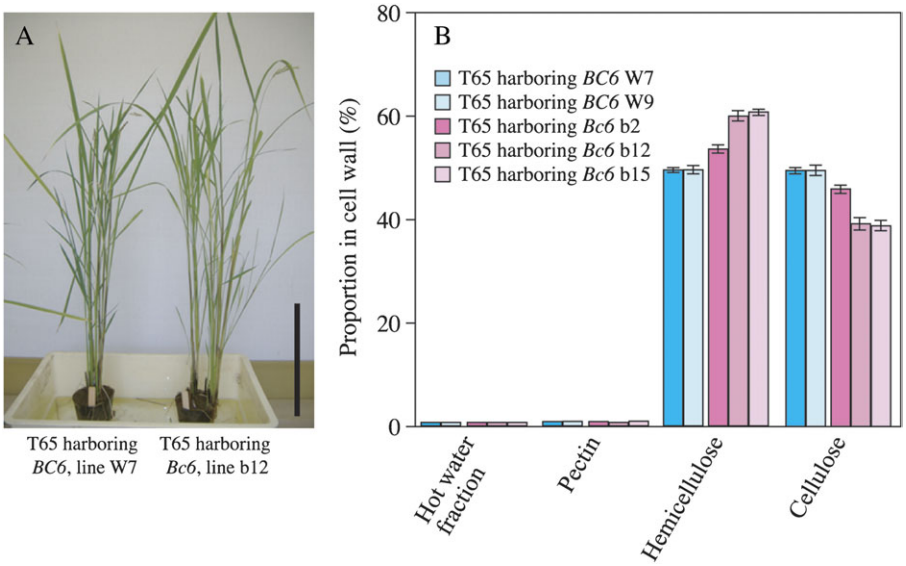

Fig. 4. Introduction of the semi-dominant *Bc6* gene into wild-type plants. Introduction of the genes, including the 4.8 kbp upstream region, into T65 yielded 15 lines of transgenic plants harbouring the mutant *Bc6* gene and seven lines harbouring the wild-type *BC6* gene. (A) Appearance of representative lines of wild-type *BC6* (W7, left) and mutant *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 *BC6* (lines W7 and W9, blue bars) and the mutant *Bc6* (lines b2, b12, and b15, pink bars). Values shown are averages of three plants, and the bars represent standard errors.

(line W7, Fig. 4B). These facts confirm that *Bc6* mutation is due to the R588G substitution. Introduction of the mutant *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 *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 *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.

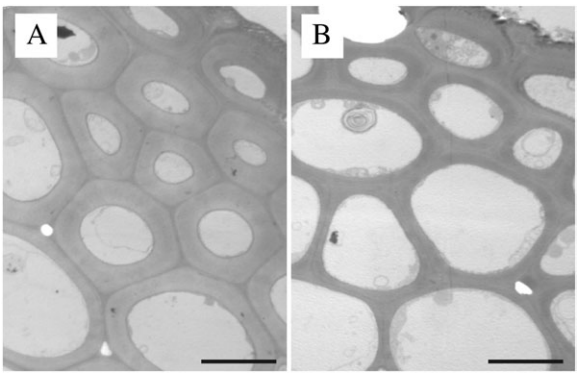

Fig. 5. Cell wall structure in transgenic plants. Transmission electron microscopic images of sclerenchymal cells in culms of transgenic plants harbouring the wild-type *BC6* (line W7) (A) or mutant *Bc6* gene (line b12) (B). Scale bars indicate 5 μm.

Expression pattern of BC6

The pattern of expression of *BC6* was assessed by analysing T65 plants transformed with pBC6:*GUS*, a binary vector in which the 4.8 kbp region upstream of the *BC6* gene was fused with the *GUS* reporter gene. *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 *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 *BC6* gene product, OsCesA9 protein. These patterns of *BC6* promoter-driven gene expression were similar to the expression pattern of *BC1* demonstrated by *in situ* hybridization (Li *et al.*, 2003).

In *Arabidopsis*, the *COBL4* gene is co-expressed with the secondary cell wall-specific *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 *et al.*, 2001; Roudier *et al.*, 2005). To examine the relationship between the secondary cell wall-specific CesA and COBRA-like proteins in rice, *BC6* and *BC1* mRNAs were quantitated in several tissues. Consistent with the results of the pBC6:*GUS* analysis, relatively high levels of *BC6* mRNA were detected in culms and nodes (Fig. 7A). The level of *Bc6* mRNA in roots was relatively low. Indeed, the brittle phenotype in

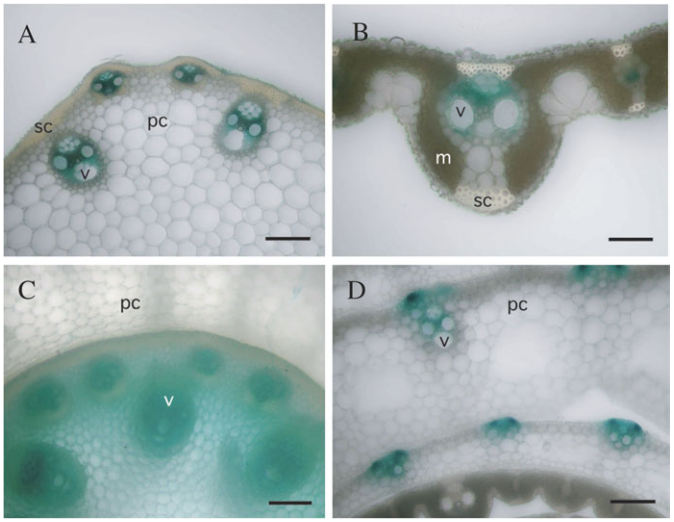

Fig. 6. Pattern of *BC6* promoter-driven GUS activity. The transverse sections of a culm (A), a mature leaf (B), a node (C), and a young leaf (D) in pBC6:*GUS* rice plants were GUS stained and observed under an optical microscope. Identical expression patterns were observed for three independent pBC6:*GUS* lines. Scale bars indicate 100 μm in A, C, and D, and 250 μm in B. m, mesophyll cell; pc, parenchymal cell; sc, sclerenchymal cell; v, vascular bundle.

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*).

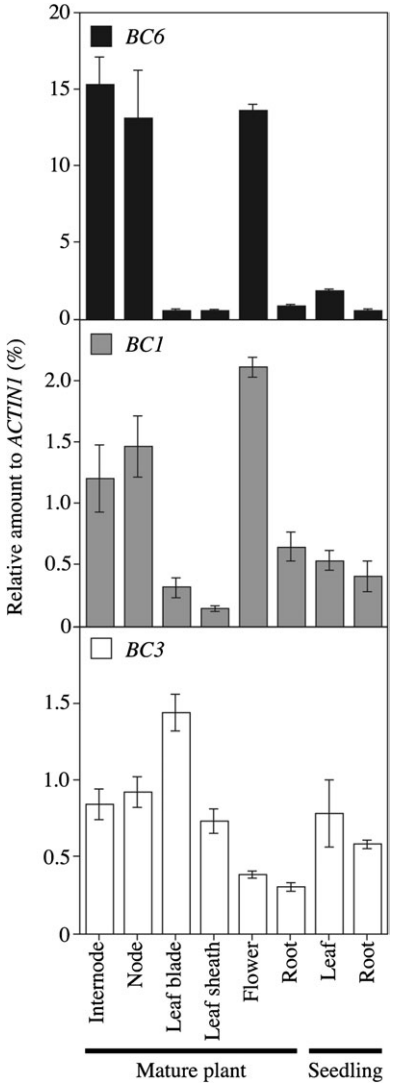

Fig. 7. Comparison between *BC6*, *BC1*, and *BC3* expression patterns. The amounts of *BC6*, *BC1*, and *BC3* mRNA relative to that of *ACTIN1* mRNA were determined by quantitative RT-PCR. The template cDNA was prepared using total RNA extracted from two wild-type plants. Values are averages of three technical replicates, and the bars represent standard errors.

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, *bc7* mutation comprising deletions in exon 10 and intron 10 does not affect growth

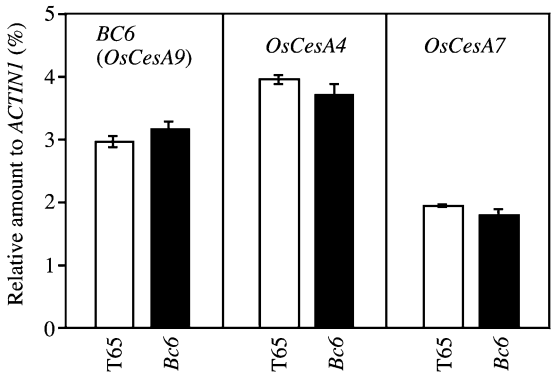

Fig. 8. Expression levels of *BC6*, *OsCesA4*, and *OsCesA9* in the *Bc6* mutant. Relative amounts of *BC6*, *OsCesA4*, and *OsCesA9* mRNA in developing leaf blades of *Bc6* mutants were compared with those in T65. Values shown are averages of three plants, and the bars represent standard errors.

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, R588G, 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 R588 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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