Rice Brittle Culm19 Encoding Cellulose Synthase Subunit CesA4 Causes Dominant Brittle Phenotype But Has No Distinct Influence on Growth and Grain Yield

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Original article

Keywords: Rice, Dominant mutation, Brittle culm, Cellulose synthase, Secondary cell wall

Posted Date: July 13th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-696804/v1>

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Version of Record: A version of this preprint was published at Rice on November 25th, 2021. See the published version at <https://doi.org/10.1186/s12284-021-00536-2>.

Abstract

Background: Mechanical strength is a crucial agronomic trait in rice (*Oryza sativa*), and brittle mutants are thought suitable materials to investigate the mechanism of cell wall formation. So far, almost all brittle mutants are recessive, and most of them are defected in multiple morphologies and/or grain yield, limiting their application in hybrid breeding and in rice straw recycling.

Results: We identified a semi-dominant brittle mutant *Brittle culm19* (*Bc19*) isolated from the *japonica* variety Nipponbare through chemical mutagenesis. The mutant showed the same apparent morphologies and grain yield to the wild type plant except for its weak mechanical strength. Its development of secondary cell wall in sclerenchyma cells was affected, along with reduced contents of cellulose, hemicellulose, lignin and sugars in culms and leaves. Positional cloning suggested that the *Bc19* gene was allelic to *OsCesA4*, encoding one of the cellulose synthase A (CesA) catalytic subunits. In this mutant, a C-to-T substitution occurred in the coding sequence of *BC19*, causing the P507S missense mutation in its encoded product, which was located in the second cytoplasmic region of the OsCesA4 protein. Furthermore, introducing mutant gene *Bc19* into the wild-type plant resulted in brittle plants, confirming that the P507S point mutation in OsCesA4 protein was responsible for the semi-dominant brittle phenotype of *Bc19* mutant. Reverse correlation was revealed between cellulose contents and expression levels of mutant gene *Bc19* among the homozygous mutant, the hybrid F₁ plant, and the *Bc19* overexpression transgenic plants, implying that gene *Bc19* might affect cellulose synthesis in a dosage-dependent manner.

Conclusions: *Bc19*, a semi-dominant brittle mutant allele of gene *OsCesA4*, was identified using map-based cloning approach. The mutated protein of *Bc19* possessing the P507S missense mutation behaved in a dosage-dependent semi-dominant manner. Unique brittle effect on phenotype and semi-dominant genetic quality of gene *Bc19* indicated its potential application in grain-straw dual-purpose hybrid rice breeding.

Background

Mechanical strength is one of the most important traits for cereal crops, and is determined by plant cell walls, which constitute the skeletal structures of the plant bodies. Ninety-percent components of plant cell walls are polysaccharides, which exist mainly as cellulose and hemicellulose in primary cell walls (PCW) and secondary cell walls (SCW), and as lignin only in SCW (Darley et al. 2001; Taylor et al. 2000). SCW generally determines the mechanical strength of cell walls, so defects in biosynthesis of cellulose, hemicellulose and/or lignin always result in inferior mechanical index and brittle plant bodies, which in turn make these brittle culm mutants valuable materials for understanding the mechanism of SCW formation.

As a matter of fact, a number of brittle mutants have been studied and some responsible genes have been identified in Arabidopsis, rice and other cereal crops, which to some extent revealed the mechanism

regulating mechanical strength of the plant body and metabolic pathway of plant cell walls. Initially, physical characteristics of crop stems were first described through three barley brittle mutants (Kokubo et al. 1989; Kokubo et al. 1991). Cellulose, lignin and noncellulose components, maximum bending stresses and mole number of cellulose molecules were determined and compared between mutants and nonbrittle strains, which indicated that weak culms of the three barley mutants were related to decrease in number of cellulose molecules (Kokubo et al. 1991). Later on, three *Arabidopsis irregular xylem* mutants, *irx1*, *irx3* and *irx5*, were identified (Turner and Somerville 1997), and the relevant corresponding genes were cloned and were confirmed to encode the three basic cellulose synthase A (CesA) catalytic subunits, AtCesA8, AtCesA7 and AtCesA4, respectively (Taylor et al. 1999; Taylor et al. 2000; Taylor et al. 2003). In addition, several genes involved in other different steps of cell wall formation were reported in *Arabidopsis*. *IRX4* encoding a cinnamoyl-CoA reductase (CCR) is essential for lignin biosynthesis; *FRAGILE FIBER1 (FRA1)* encodes a kinesin-like protein and regulates the oriented deposition of cellulose microfibrils; and mutant of gene *FRA2* is attributable to altered fiber cell elongation and expansion (Jones et al. 2001; Zhong et al. 2002; Burk et al. 2001).

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ncidentally, identification of some brittle mutants in rice caused by *Tos17* also revealed three catalytic subunits essential for cellulose synthesis, OsCesA4, OsCesA7, and OsCesA9, which are homologous to AtCesA8, AtCesA4 and AtCesA7, respectively (Tanaka et al. 2003). Up to now, at least 25 mutants exhibiting brittle leaves and/or culms have been reported in rice, some of which turned out to be different mutant alleles of *CesAs*, such as *bc7*, *bc11*, *Bc6* and *fc17* (Yan et al. 2007; Zhang et al. 2009; Kotake et al. 2011; Li et al. 2018). In total, 12 genes responsible for brittle traits are cloned, and they directly or indirectly participate in cellulose biosynthesis and cell wall formation (Kotake et al. 2011; Wu et al. 2012; Vega-Sánchez et al. 2012; Wang et al. 2016b). *Brittle Culm1 (BC1)* encoding a cobra-like protein regulates cellulose assembly by modulating cellulose crystallite size (Li et al. 2003; Liu et al. 2013). *BC3* is suggested to be essential for proper SCW construction, and its encoded protein belonging to a dynamin protein family functions in membrane dynamics (Hirano et al. 2010). A dual-targeting kinesin protein encoded by *BC12*, which is localized in the nucleus, cytoplasm and mitotic microtubule arrays in dividing cells, is involved in cell-cycle progression and cellulose microfibril deposition (Zhang et al. 2010). Proteins encoded by *BC10*, *BC14* and *BC15* are all localized in the golgi complex, however they function differently in regulating cellulose synthesis (Zhou et al. 2009; Zhang et al. 2016; Zhang et al. 2011; Song et al. 2011; Wu et al. 2012). Characterization of brittle mutants and functional research of the corresponding genes have uncovered some important biochemical processes in cell wall formation and remodeling, while our understanding regarding to cell wall biosynthesis and modification is still limited.

Up to now, almost all brittle mutants are recessive, such as *bc1*, *bc3*, *bc11* and *fc17*, except one semi-dominant mutant of *OsCesA9* (Li et al. 2003; Hirano et al. 2010; Zhang et al. 2009; Li et al. 2018; Kotake et al. 2011). Meanwhile, except brittle plant bodies, most of them were also aberrant in multiple morphologies, interfering their application in rice breeding. For example, obviously decreased plant height and shorter roots were often seen in these mutants, such as *bc3*, *bc10*, *bc11* and *bc12* (Hirano et al. 2010;

Zhou et al. 2009; Zhang et al. 2009; Zhang et al. 2010). Fertility and/or tillering were even severely affected in some mutants, including NE1031, *S1-24*, *S1-60* and *bc10* (Tanaka et al. 2003; Wang et al. 2016a; Wang et al. 2012; Zhou et al. 2009). In the present study, we isolated a semi-dominant brittle mutant *Brittle culm19* (*Bc19*) through chemical mutagenesis. Cellulose, hemicellulose and lignin contents were all reduced in culms and leaves of *Bc19*, and its SCW was much thinner than the wild type plant, while neither apparent morphologies nor grain yield was altered in the mutant. Through map-based cloning, we confirmed that the mutant gene *Bc19* was allelic to *OsCesA4*, encoding one of the CesAs, and the resulting P507S point mutation within the second cytoplasmic region of *OsCesA4* was responsible for the brittle phenotype. Taken together, we suggest that the semi-dominant mutant gene *Bc19* could be a potential genetic resource for implement of high grain yield, heavy biomass and their efficient utilization in breeding of grain-straw dual-purpose hybrid rice.

Materials And Methods

Plant Materials and Growth Conditions

The *Brittle culm19* (*Bc19*) mutant was obtained from the *japonica* cv. Nipponbare through ethyl methanesulfonate (EMS) mutagenesis. Then Nipponbare, the *indica* cv. Minghui 63 and G46B were crossed with *Bc19* to obtain three F₁ hybrids, respectively. Due to the better polymorphism between Nipponbare and Minghui 63, the F₂ mapping population was generated from selfing F₁ plants of *Bc19* and Minghui 63. Rice plants were cultivated in the local fields in Wenjiang District (Latitude 30°42'N, Longitude 103°50'E, and Altitude 539.3 m), Chengdu City, Sichuan, China (Wang et al. 2010).

Measurement of Major Agronomic Traits

Bc19, Nipponbare, and transgenic plants were cultivated according to randomized complete block design with three replications. Each block contained 40 plants, and the planting density was 16.6 · 25 cm. Field management followed local rice production. Breaking force of flag leaves and the top second internodes were measured two weeks after heading with a digital force testing device (FGJ-1, SHIMPO). The force to break apart a leaf or culm segment was recorded accordingly. Other agronomic traits and yield per plant were investigated after maturation. All of the data were calculated in the software IBM SPSS Statistics 22, and the statistical significance of differences between *Bc19* and the wild type plant was conducted using Student's t-test.

Analysis of Cell Wall Components

Leaves and the top second internodes were collected two weeks after heading, and were dried first at 105°C for 1h, then at 65°C for 24h. Afterwards, the materials were ground into fine powder for measurement. Contents of cellulose, hemicellulose and lignin were measured according to the methods previously described by Van Soest et al (1991). For measurement of cell wall sugars, the powdered materials were soaked in 80% acetonitrile, and incubated in 65°C ultrasonic oscillation water bath for 30

min. The supernatants were collected through centrifuge, followed by the analysis of monosaccharides with HPLC system (Agilent 1260A) according to the methods previously reported (Zhao et al. 2020).

Electron Microscopy Analysis

An environmental scanning electron microscope (ESEM, FEI-Q450) was used to observe structure of sclerenchyma and parenchyma tissues in leaves and culms. Flag leaves and the top second internodes were cut into small pieces and then immediately placed on the object stage for observation.

For transmission electron microscope analysis, sections of leaf tissues at seedling stage were treated as previously described (Wang et al. 2010). Generally, these sections were fixed in 3% (w/v) glutaraldehyde overnight and then in 1% osmium tetroxide. After that, a gradient of ethanol series were used to dehydrate the samples, followed by washing with the Epon812 medium. Then, the samples were cut into ultra-thin sections and stained with uranyl acetate and Reynolds' lead citrate. Finally, the slices were observed using the transmission electron microscope (H-600IV, Hitachi).

Fine Mapping and Marker Development

Nine hundred and eighty-three normal plants selected from a F_2 population up to 5000 plants from the cross between *Bc19* and Minghui 63 were used for gene mapping. Genetic linkage analysis was determined using simple sequence repeat (SSR) markers (McCouch et al. 2002) taking genomic DNA as templates. Two DNA pools (normal/brittle), each mixed with 10 individuals, were constructed to screen those SSR markers, which would be further confirmed by the F_2 segregation population.

Insertion/deletion (InDel) markers were designed using Primer Premier 5.0 software based on genomic DNA sequence polymorphism between *japonica* and *indica* from the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST>).

Vector Construction and Transgenic Experiment

The cDNA sequence of the mutant *Bc19* gene was amplified from the mutant with primers 5'-GGGTCTAGAATGATGGAGTCGGGGGTC-3' and 5'-AAAGTCGACTCAGCAGTCGAAGTTGGC-3' (containing a *Xba*I site and a *Sal*I site, respectively), which was inserted into the pMD19-T vector (TaKaRa). After sequencing confirmation, the plasmid was double digested with enzymes *Xba*I and *Sal*I, subsequently the resulting *Bc19* fragment was inserted into the binary vector pCambia2300 behind the rice *Actin1* promoter. The construct *pC2300-Bc19* was introduced into the wild type plant Nipponbare by *Agrobacterium tumefaciens*-mediated transformation (Kumar et al. 2005). Primers for identifying the transgenic plants were 5'-GAATCCCTCAGCATTGTTC-3' and 5'-TCAAATGTGAGCATAGCC-3', with annealing sites on the rice *Actin1* promoter and cDNA of *Bc19*, respectively. Two homozygous lines (TG1 and TG2) from eleven positive transgenic lines were used for test of cell wall components and for qRT-PCR.

Protein Structure Prediction and Sequence Alignment

Encoded amino acid sequences of *CESAs* in rice and *Arabidopsis* were downloaded from GenBank (<http://www.ncbi.nlm.nih.gov>) through the BLAST program. Protein structures of *CesAs* were predicted using the TMHMM program (<http://www.cbs.dtu.dk/services/TMHMM>). Multiple sequence alignments were conducted using DNAMAN software (Lynnon Biosoft).

qRT-PCR Analysis

Total RNA from leaves and roots of two-week-old seedlings, leaves, culms of plants two weeks after heading, and young panicles at the beginning of heading stage was extracted with a TRIzol reagent (Invitrogen). First-strand cDNA was produced using a Superscript [®] Reverse Transcription Kit (Invitrogen). Rice *Actin1* gene served as an internal control. Quantitative RT-PCR (qRT-PCR) was performed using a SYBR Premix Ex Taq2 kit (TaKaRa) according to the following program: 95°C for 5 min, then 40 cycles of 95°C for 10 s and 58°C for 30 s. Specific primers used for qRT-PCR could be seen in Additional file 1: Supplementary Table S3. For each experimental group, qRT-PCR was operated with three technical replicates for each of the three biological replicates.

Results

Characterization of the *Bc19* Mutant

Bc19 was a brittle mutant obtained from the *japonica* rice cv. Nipponbare by chemical mutagenesis. Like most of the brittle mutants reported, leaves and internodes of *Bc19* could be easily broken and the breakpoints were smooth (Fig. 1a, b). The brittle phenotype was milder at seedling stage and became more severe after heading. Not only culms and leaves, the weak mechanical strength went through the whole plant body of *Bc19*, including leaf sheaths, panicle branches, glumes and roots. However, different from most brittle mutants reported, apparent morphology and major agronomic traits were not affected in the *Bc19* mutant, including plant height, number of productive panicles per plant and spikelets per panicle, seed setting rate, 1000-grain weight, and grain yield (Fig. 1c-i). Strengths for breaking the leaves and culms were measured then, which showed that breaking forces of *Bc19* leaves and culms were reduced by approximately 90% and 70% of those in the wild-type plant, respectively (Fig. 1j, k).

Decreased mechanical strength in the *Bc19* mutant leaves and culms may be a result of irregular cell arrangement, cell wall structure and thickness. Therefore, we examined cross sections of leaves and internodes of the wild-type plant and *Bc19* mutant using environmental scanning electron microscope and transmission electron microscope (Fig. 2). Obviously, cell walls of sclerenchyma tissues of the wild-type plant were much thicker than those of *Bc19* in both leaf veins and internodes, while cell walls of parenchyma tissues showed no obvious differences between them (Fig. 2a-d). In addition, structures of sclerenchyma tissues, parenchyma tissues, and vascular bundles were not obviously affected in the mutant (Fig. 2a-d). Moreover, SCW of *Bc19* was found particularly thinner and the layered structure of SCW was not distinct when compared with the wild-type plant (Fig. 2e-h). These results suggested that the mutation traits of *Bc19* were very likely associated with decrease in cell wall thickness, especially the SCW thickness in sclerenchyma tissues which provided the basal structural support of the plant body.

Alterations in Cell Wall Composition

As alterations in content and percentage of cell wall compositions influence wall structure and mechanical strength of plant bodies, we compared contents of cellulose, hemicellulose, and lignin between the *Bc19* mutant and the wild-type plant. As shown in Fig. 3, the amounts of cellulose, hemicellulose and lignin were all reduced in leaves and culms of the *Bc19* mutant, when compared with the wild-type plant. Cellulose reductions of 24.1% in leaves and 28.5% in culms were observed in *Bc19*, and lignin contents decreased 20.1% in leaves and 25.9% in culms compared to those in the wild type (Fig. 3). Relatively slight decrease of hemicellulose content occurred in the mutant, 12.5% in leaves and 20.9% in culms (Fig. 3). Subsequently we performed quantitative analysis of monosaccharides in culms of *Bc19* and wild type plant through HPLC assay. As expected, glucose which make up the cellulose, and xylose which is the main component forming hemicellulose, were all decreased in the *Bc19* mutant (Table 1). These results indicated that poor mechanical strength of *Bc19* was probably connected with reduction in components of cell wall, and mutation in gene *Bc19* affected cell wall biosynthesis through alterations of these major components.

Table 1
Comparison of monosaccharides composition between the wild type (WT) and *Bc19*.

Sugar	WT	<i>Bc19</i>	Compared with WT (%)
Glucose	9653 ± 617	7963 ± 75	-17.5*
Fructose	8568 ± 455	8135 ± 259	-5.1
Xylose	321 ± 38	251 ± 24	-21.8*
Arabinose	8.7 ± 0.6	8.4 ± 1.4	-3.4
Galactose	11.3 ± 0.9	12.2 ± 1.1	8.0
Rhamnose	119 ± 11	123 ± 3	3.4
Mannose	48.1 ± 4.2	61.0 ± 2.4	26.8**
The data are given as means ± SE of three independent repeats. Each monosaccharide was formulated as mg g fresh weight ⁻¹ . Single and double asterisks signify statistical differences compared to the wild type at <i>P</i> < 0.05 and <i>P</i> < 0.01, respectively.			

Map-based Cloning of the *Bc19* Mutant Gene

The *Bc19* mutant was crossed with three rice cultivars, including the wild-type Nipponbare, and two *indica* cultivars, Minghui 63 and G46B. All of the F₁ plants from the three crossings tended to be brittle. Meanwhile, milder phenotypes of weak mechanical strength and of decrease in breaking force were observed in F₁ plants producing from crossing between *Bc19* and the wild type (Fig. 1a, b, j, k), indicating that *Bc19* should be a semi-dominant mutant. Consistent with the slight brittle phenotype of the F₁

plants, reduction of cell wall components in F_1 were also milder than those in the homozygous mutant of *Bc19* (such as 18.9% and 18.1% of cellulose decrease in the F_1 leaves and culms, respectively) (Fig. 3). Therefore, we assumed that the mutant gene acted in a semi-dominant manner rather than completely dominant effect. Furthermore, three quarters of F_2 plants derived from the three crosses between *Bc19* and normal cultivars were also brittle plants (Additional file 1: Supplementary Table S1), which indicated that the *Bc19* mutant phenotype was controlled by a semi-dominant nuclear gene.

In order to fine map the *Bc19* locus, an F_2 population up to 4000 plants from a cross between *Bc19* and Minghui 63 was generated. Initially, we analyzed 140 normal plants from this F_2 population with more than 300 SSR markers and located the *Bc19* gene on the long arm of rice Chromosome 1, with 9.7 cM to RM212 (Fig. 4a). Then several InDel markers on both sides of RM212 were developed based on sequence polymorphism between *japonica* cv. Nipponbare and *indica* cv. 93 – 11, and the *Bc19* gene was mapped between RM212 and InDel marker C1, with 5.1 cM to C1 (Fig. 4a, Additional file 1: Supplementary Table S2). To narrow the *Bc19* locus, we designed more InDel markers within this region, analyzed 983 normal plants in total, and finally located *Bc19* gene into a 145-kb interval between InDel markers C3 and C4, with the genetic distance of 0.05 cM and 0.10 cM, respectively (Fig. 4b, c, Additional file 1: Supplementary Table S2).

According to the gene annotation information provided by the MSU Rice Genome Annotation Release 7 (<http://rice.plantbiology.msu.edu>), this region totally contains 23 genes (Fig. 4c), of which 8 are transposons. Based on putative functions of the rest 15 genes, gene *LOC_Os01g54620* encoding OsCesA4 is most likely the candidate gene, which was previously identified essential for cellulose synthesis (Tanaka et al. 2003). Therefore, we amplified this gene from genomic DNA of *Bc19* and wild-type plants, respectively. Sequencing and comparison of them showed one base-pair C-to-T substitution at position 3439 (Fig. 4d). Through reverse transcription (RT)-PCR, the corresponding C-to-T substitution at position 1519 of the gene's cDNA sequence was revealed, turning CCA into TCA and resulting in the 507th amino acid residue, proline, replaced with serine.

Sequence analysis revealed that *BC19* gene contained 13 exons and 12 introns with 5743-bp genomic DNA and 2970-bp cDNA. Multiple alignment indicated that the missense mutation from proline to serine at the 507th residue of *Bc19* is strictly conserved among CesaA family members in rice, Arabidopsis and several other species, which may affect the proper function of OsCesA4 (Fig. 5, Additional file 2: Supplementary Fig. S1).

Functional Identification of Bc19

Transgenic experiment was conducted to confirm that mutation in gene *LOC_Os01g54620* is responsible for the brittle phenotype of *Bc19*. The plasmid *pC2300-Bc19* containing the coding region of the mutant gene *Bc19* and the rice *Actin1* promoter, was introduced into the wild-type plant. As a result, 13 independent transgenic plants were obtained, of which 11 plants were identified as positive transgenic plants by PCR with a pair of primers extending from the plasmid to the coding sequence of *Bc19* (Fig. 6a,

showing 2 among 11 lines). As expected, independent transgenic lines carrying the mutant gene *Bc19* showed very obviously brittle phenotype. Moreover, decrease in cell wall components of transgenic lines TG1 and TG2 were severer than those of mutant *Bc19* (Fig. 6b, c), probably because of their higher transcript level of mutant gene *Bc19* guided by the strong promoter of *Actin1* in the recombinant plasmid (Fig. 6d). These results suggested that the brittle phenotype of *Bc19* mutant was due to the P507S substitution of the OsCesA4 protein, and the mutant *Bc19* gene blocked synthesis of essential components of cell wall in a dominant way.

qRT-PCR Analysis of BC19 and the Related Genes

To analyze the function of *BC19* gene and in which way the mutant gene impacted its cell wall synthesis, expression patterns of *BC19* and other cell wall biosynthesis related genes were assessed in both wild-type plant and the *Bc19* mutant by qRT-PCR. We included *BC1*, *OsCesA7*, *OsCesA9*, and *OsPAL* (*Os02g41630*) for the analysis. *OsCesA4*, *OsCesA7* and *OsCesA9*, which encode three catalytic subunits of CesA, are essential for the synthesis of β -1,4-glucan and are not redundant during formation of SCW (Tanaka et al. 2003; Doblin et al. 2002). *BC1* encodes a COBRA-like protein which participates in the formation of SCW, and it was reported that expression of COBRA-like genes closely connected with expression of *CesA* genes in Arabidopsis (Li et al. 2003; Roudier et al. 2005). *OsPAL* was reported to regulate lignin synthesis in the phenylpropanoid pathway (Vanholme et al. 2008).

As shown in Fig. 7a, three *CesA* genes, *BC1* and *OsPAL* were mainly expressed in roots of seedling, culms of plants two weeks after heading and young panicles at the beginning of heading stage, and very low transcript levels of the five genes were detected in leaves at seedling stage and after heading. The only difference was the expression level of *OsPAL* in young panicles, which was higher than that in culms after heading. Similar expression patterns of *CesAs* and *BC1* in different tissues suggested that they might be co-expressed in SCW synthesis, which were consistent with the results reported in the study of an *OsCesA9* mutant (Kotake et al. 2011). However, they only verified the relationship between *OsCesA9* and *BC1*. Additionally we here confirmed the co-expression pattern between three *CesA* genes and *BC1*.

To seek if there was any effect on these related genes' expression caused by the mutated *OsCesA4* in the *Bc19* mutant, we then examined and compared these genes' expressions between *Bc19* and the wild type. It turned out that expressions of the five genes were down-regulated in leaves and roots of the mutant, while up-regulated in the *Bc19* culms and panicles (Fig. 7b). However, changes of transcription levels of these genes were less than two folds in *Bc19*, indicating that the point mutation in *Bc19* didn't necessarily affect expression of gene *Bc19* or other related genes. A similar case occurred in the *bc1* mutant, where the expression levels of the three *CesA* genes were not affected by the mutation of gene *BC1* (Liu et al. 2013).

In addition, we compared transcript levels of *OsCesA4* in the homozygous mutant *Bc19*, the hybrid F₁ plant, the wild type and the two particularly brittle transgenic lines TG1 and TG2. As a result, the expression levels in *Bc19* and F₁ plants (the according transcripts probably including both the wild type

and mutant gene, *BC19* and *Bc19*) were comparative to those in the wild type. However, sharply raised transcript levels of *Bc19* gene were detected in the transgenic lines TG1 and TG2 (Fig. 6d), showing inverse correlation with sharply decreased contents of cellulose, hemicellulose and lignin in them (Fig. 6b, c).

Discussion

Bc19 Alters Mechanical Strength and Cell Wall Composition but Has No Distinct Influence on Growth and Grain Yield

Researches on brittle mutants have showed several genes underlying cellulose synthesis and cell wall formation, while mutation of these genes not only affected mechanical strength of plant bodies, but also resulted in other pleiotropic abnormalities in rice. The *bc3* mutant showed easily snapped culms, dwarf plant bodies, and short roots (Hirano et al. 2010). Besides brittle culms and leaves, *bc10* also exhibited yellowish seedlings, fewer and shorter roots, decreased plant height, and fewer tillers (Zhou et al. 2009). The other brittle mutant, *bc12*, also showed abnormal growth and morphogenesis, including dwarfism and reduced root length (Zhang et al. 2010). In addition, alterations in cell wall components of these mutants were varied. Briefly, cellulose contents in *bc3* and *bc10* were decreased but not affected in *bc12*; lignin content was increased in both *bc10* and *bc12* (Hirano et al. 2010; Zhou et al. 2009; Zhang et al. 2010).

In case of *CesA* mutants in rice, different mutation types and sites also resulted in varied phenotypes. NE1031, NC0259, ND8759, and ND2395 were four brittle mutants generated by *Tos17* insertion in *OsCesA* genes (NE1031 relative to *OsCesA4*, NC0259 and ND8759 relative to *OsCesA7*, and ND2395 relative to *OsCesA9*), and growth and development of them were all aberrant, mainly in plant height, leaf size, culm thickness, and fertility. Moreover, cellulose contents of the four mutants were sharply decreased to 8.9% – 25.5% of that of the wild type plant (Tanaka et al. 2003). *bc7* was another mutant allele of *OsCesA4* obtained through 60Co- γ radiation, resulting in the premature transcription due to the 7-bases deletion in the junction of exon 10 and intron 10 (Yan et al. 2007). Besides weak mechanical strength, plant height of *bc7* was slightly shorter than wild type, and cellulose content and cell number of the parenchymatous tissues were all reduced. Other 7 mutants of *OsCesA* genes were point mutations as reported, including 2 of *OsCesA4* (*bc11* and *fc17*), 1 of *OsCesA7* (*S1-24*), and 4 of *OsCesA9* (*Bc6*, *S1-60*, *bc13*, and *bc-s1*) (Zhang et al. 2009; Li et al. 2018; Wang et al. 2016a; Kotake et al. 2011; Wang et al. 2012; Song et al. 2013; Jin et al. 2016). In addition to prominent brittle phenotype, 3 mutants (*bc11*, *S1-24*, and *S1-60*) among them also showed shorter plant bodies, and/or other abnormal morphologies, such as poor fertility, fewer tillers and shorter roots. In most of those mutants, there were reductions in cellulose contents and compensatory increases in hemicellulose and lignin contents, which was different from the brittle mutant in this study (Yan et al. 2007; Wang et al. 2016a; Kotake et al. 2011; Wang et al. 2012; Li et al. 2018). Here, we reported another brittle mutant *Bc19*, in which cellulose, hemicellulose and lignin contents were reduced by 12.5% – 28.5%, but apparent morphology, growth and grain yield were not affected (Figs. 1, 3). Additionally, different from most recessive brittle mutants such as *fc17*, we verified

Bc19 as a new semi-dominant mutant allele of gene *OsCesA4*, and the mutant gene influenced mainly the SCW synthesis (Figs. 1, 2, 3, 4).

In recent years, constantly raising grain yield attracts breeders' increasing concern of lodging resistance in rice cultivars. Enhancing culm strength can increase the lodging resistance in rice and confrontation capacity against natural disasters such as strong wind and rain (Li et al. 2014a). On the other hand, reduced proportion of components in cell walls could make rice straw easily degraded after harvest, improving its utilization efficiency either as animal feed or biofuels (Wang et al. 2005; Johnson et al. 2006). In addition, engineering improvement of rice straw as biofuel resources will be a sustainable solution to solve environmental problems owing to straw burning (Himmel et al. 2007; Ragauskas et al. 2014). In this study, cellulose content was reduced while growth and yield were not affected in *Bc19* mutant, thus the mutant gene could be a potential genetic resource for rice straw recycling, which could maintain grain yield as well (Figs. 1, 3). What's more, the specific semi-dominant characteristic of brittle gene *Bc19* makes it more convenient to implement of high grain yield, heavy biomass and their efficient utilization in breeding of grain-straw dual-purpose hybrid rice (Peng et al. 2010; Li et al. 2019).

The Point Mutation in *Bc19* Could Partially Abolish *OsCesA4* Function

OsCesA4, together with *OsCesA7* and *OsCesA9*, encoding CesAs, are indispensable and irredundant during biosynthesis of cell wall in rice (Tanaka et al. 2003). The three *OsCesA* subunits share strong similarity in amino acid sequences, and they possess similar function domains including a Zinc Finger domain, a D,D,D,QXXRW motif, a Plant-conserved Region (P-CR), a Class-specific Region (C-SR) and totally 8 transmembrane domains (TMDs) (Somerville 2006; Li et al. 2014b) (Fig. 8). Recently it was visually identified that the PttCesA8 subunit in poplar (*Populus tremula* × *tremuloides*) possessed 7 TMDs, but none of such discoveries was found in rice or other species (Purushotham et al. 2020). Nevertheless, the functional cellulose synthase complex (CSC) has been identified as a hexamer, the so-called rosette structure which is anchored to the plasma membrane (Atanassov et al. 2009). Through interactions induced by the Zinc Finger domain with each other, single CesA subunit first forms homodimers and then six homodimers are polymerized into the rosette structure (Kurek et al. 2002; Timmers et al. 2009; Hill et al. 2014; Nixon et al. 2016). Although formation of the rosette structure relies on what motif of CesA subunits remains unclear, it is predicted that the D,D,D,QXXRW motif as well as the P-CR in the second cytoplasmic region between TMD2 and TMD3 are essential for the catalytic activity during cellulose synthesis (Taylor et al. 2000; Doblin et al. 2002; Arioli et al. 1998; Rushton et al. 2017). In another word, a functional CSC must possess three elements, correct Zinc Finger motif to form the rosette, correct second cytoplasmic region for the catalytic activity, and correct TMDs for locating to the plasma membrane.

NE1031, *bc11* and *Bc19* are allelic mutants of *OsCesA4* with different mutation sites, while they exhibit different morphological abnormalities. NE1031 was found the Tos17 insertion in the sixth exon, which resulted in the premature transcription of *OsCesA4* (Tanaka et al. 2003). The point mutation in *bc11* was located in the end of TMD5, resulting in decreased abundance of *OsCESA4* in the plasma membrane

(Zhang et al. 2009). Despite weak mechanical strength, these two mutants also displayed dwarfed plant bodies; and NE1031 even exhibited small leaves, thinner culms and low fertility. Moreover, cellulose contents were all sharply reduced in NE1031 and *bc11*, by 79.6% and 57%, respectively. In this study, the P507S mutation within the second cytoplasmic region of OsCesA4 only influenced the mechanical strength of *Bc19*, accompanied by 28.5% reduction in cellulose content and thinner SCW (Figs. 1, 2, 3). Meanwhile, transcript levels of *Bc19* were not apparently affected in the mutant (Figs. 6d, 7b). Considering normal Zinc Finger domain and TMDs of OsCesA4, the rosette probably could still be secreted and trafficked to the plasma membrane in *Bc19*. Evidence was that in the *irx1* (*AtCesA8*) mutant in Arabidopsis, antibodies specifically combining to IRX3 (*AtCesA7*) and IRX5 (*AtCesA7*) could still coprecipitate IRX1, suggesting that the point mutation in the third Asp residue of the D,D,D,QXXRW motif in mutant *irx1* didn't affect interactions of the three kinds of CesA subunits (Taylor et al. 2000; Taylor et al. 2003). Therefore, different from complete loss of function of OsCesA4 in NE1031 due to its premature transcription termination, catalytic efficiency of the mutated OsCesA4 in *Bc19* might just be reduced to some extent, instead of thoroughly abolished. After all, cellulose content in *Bc19* was not sharply reduced as it was in NE1031 (Fig. 3) (Tanaka et al. 2003).

Possible Explanation for the Dominant Negative Effect of the Point Mutation in *Bc19*

Although more than 20 brittle mutants have been reported in rice, almost all of them were recessive, and the only exception was *Bc6*, a semi-dominant mutant allele of *OsCesA9* in rice (Kotake et al. 2011). In this study, we characterized another semi-dominant brittle mutant *Bc19*, which was allelic to *OsCesA4*. Till now, 8 single-base substitution mutant alleles of CesA proteins have been located on different functional domains of them (Fig. 8). *S1-24*, mutated on the Zinc Finger domain of protein OsCesA7, was presumed to affect interactions of OsCesA7 with other CesA subunits, and the mutant phenotype was recessive (Wang et al. 2016a). Similar events occurred in mutant *bc13* and *bc-s1*, of which the missense mutations were mapped closely before and behind the Zinc Finger domain, respectively. Recessive mutants *bc11* and *S1-60*, possessing point mutations at the beginning and the end of TMD5, respectively, were speculated to impede enrichment of the corresponding CesA subunits (OsCesA4 and OsCesA9) on the plasma membrane (Fig. 8) (Zhang et al. 2009; Wang et al. 2012). Furthermore, over-expression of the mutated cDNA of *bc11* in the *bc11* mutant background unexpectedly rescued the brittle phenotype, suggesting that excessive amount of the mutant version of OsCesA4 could make up the abundance of OsCesA4 in the plasma membrane, which implied that this point mutation did not change its normal catalytic activity, and might only affect secretion of the complex from the endomembrane to the plasma membrane (Zhang et al. 2009). As for the two semi-dominant mutants, *Bc6* reported before and *Bc19* in this study, the former one carrying a R588G mutation in *OsCesA9* and the latter one with a P507S mutation in *OsCesA4*, both of them mutated in the second cytoplasmic domain between TMD2 and TMD3 (Kotake et al. 2011). Multiple alignment of CesA subunits from rice and other species showed that the interval between the P507 of *OsCesA4* and the R588 of *OsCesA9* was only one amino acid (Fig. 5). It's worth noting that the P507 residue is highly conserved among CesA family members, and it exactly corresponds to P557 of *AtCesA7*, which was changed to a threonine residue in the semi-dominant mutant *fra5* in Arabidopsis (Figs. 5, 8) (Zhong et al. 2003).

Controlling mechanism of whether a particular mutated CesA subunit acts in a dominant or recessive way might be associated with different mutation sites in different function domains of them. In the study of *fra5*, it was hypothesized that the second cytoplasmic domain might participate in interactions of CesAs with other components in the rosette complex, and the missense mutation in *fra5* perturbed this kind of interaction and then affected the normal function of CSC, which resulted in the dominant negative effect (Zhong et al. 2003). However, Kotake et al. (2011) speculated that the P-CR of the second cytoplasmic domain might be involved in interactions between CesA subunits during the formation of higher order CesA oligomerization, so that the mutation in *Bc6* might interfere the formation of functional CSC. In this study, overexpression of *Bc19* in the wild type caused more severe brittle phenotype than the *Bc19* mutant, while the mutant phenotype of F₁ plants was milder than *Bc19* (Figs. 6b-d, 3, 1a, b, j, k). It seems that the severity of the mutant phenotype is tightly related to the level of *Bc19* expression, implying that despite the dominant-negative effect, *Bc19* might also act in a dosage way. Considering these characteristics of mutant gene *Bc19*, we raise the possibility here that in the hybrid F₁ plants, the encoded product of the mutant gene *Bc19* can randomly bind to the CSC and can be secreted to the plasma membrane together with the normal CSCs, but the abnormal CSCs cannot catalyze cellulose synthesis as efficiently as the normal CSC, resulting in milder cellulose deficiency compared to the homozygous mutant. Similarly, in the *Bc19* overexpression plants, even more abnormal CSCs carrying the mutated OsCesA4 subunit gather onto the plasma membrane, thus severely impacting efficiency of cellulose synthesis, which fits the dominant-negative effect of a dose-dependent fashion. In cases of hybrid plants of point mutations in Zinc Finger domain or TMDs, abnormal subunits encoded by mutated genes either cannot form the CSC or cannot be secreted to the plasma membrane, thus CSCs transferred onto the plasma membrane are all functional CSCs compromising of normal subunits encoded by the correct genes, which can efficiently catalyze cellulose synthesis, and that's why these mutated genes act in recessive ways. Intensive exploration on action mechanism of P-CR of CesA subunits and on activities between components within the CSC complex will help to uncover how these P-CRs participate in cellulose biosynthesis.

Conclusions

Bc19, a semi-dominant brittle mutant allele of gene *OsCesA4*, was identified using map-based cloning approach. Breaking force and cellulose content of the *Bc19* mutant were decreased, while its apparent morphology, growth and grain yield were not affected. The product encoded by *Bc19* possessed a P507S missense mutation located in the second cytoplasmic region, causing the dominant-negative effect of gene *Bc19*. The relationship between expression levels of *Bc19* and severity of brittle phenotype further revealed that gene *Bc19* might affect cellulose synthesis in a dosage-dependent manner. Moreover, we presumed that the *Bc19* gene could be a potential genetic resource in breeding of grain-straw dual-purpose hybrid rice.

Abbreviations

CesA: cellulose synthase A; PCW: primary cell walls; SCW: secondary cell walls; P-CR: Plant-conserved Region; C-SR: Class-specific Region; TMD: transmembrane domains; CSC: cellulose synthase complex

Declarations

Acknowledgments

We are grateful to Prof. Chengcai Chu for kindly providing pCAMBIA2300 vector (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China).

Authors' contributions

XM and CL performed the experiments, and XM wrote the manuscript. RH, KZ , QW and CF conducted the field experiments. WL, CS and PW helped managing the main data analysis. XD and FW designed the study and critically revised the article.

Funding

This study was supported by the National Natural Science Foundation of China (31901531 and 31971869), the Natural Science Foundation of Guangdong Province (2017A030310290, 2019A1515110867 and 2020A1515011052), Guangdong Province Scientific and Technological Plan Project (2018B020202004 and 2020B1212060047), and the Agricultural Competitive Industry Discipline Team Building Project of Guangdong Academy of Agricultural Sciences.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article (and its supplementary information files).

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no potential competing interests.

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Figures

Figure 1

Comparison of phenotypes (a-c), major agronomic traits (d-i) and breaking force (j leaves and k culms). a Leaf. b The second internode of main culm from the top. c Plants two weeks after heading. Bars represent standard deviations of three independent measurements. WT: Nipponbare; F1: Obtained from mutant Bc19 crossing with WT. Double-asterisk signifies statistically significant difference compared to the wild type at $P < 0.01$.

Figure 2

Environmental scanning electron microscope images (a-d) and transmission electron microscope images (e-f) of Bc19 mutant and wild-type plant. a and b Transverse section of the wild-type and Bc19 mutant leaf vein, respectively. c and d Transverse section of the wild-type and Bc19 mutant culm, respectively. e and f Sclerenchyma cells of the wild type and Bc19 mutant, respectively. g and h Single cell in sclerenchyma tissues of the wild type and Bc19 mutant, respectively. sc, sclerenchyma tissues; pc, parenchymatous tissues; pcw, primary cell wall; scw, secondary cell wall. Scale bar equals 40 μm in a-d and 10 μm in e-h.

Figure 3

Contents of cellulose, hemicellulose and lignin in cell walls from leaves (a) and culms (b) of Bc19, the wild type (WT) and F1 plants, in mg g fresh weight⁻¹. F1: Obtained from Bc19 crossing with WT. Double and single Asterisks indicate statistically significant differences compared to the wild type at $P < 0.01$ and $P < 0.05$, respectively.

Figure 4

Molecular mapping of Bc19 locus. a The Bc19 locus was mapped between InDel marker C1 and SSR marker RM212 on the long arm of chromosome 1 (Chr.1) of rice using 140 recessive F2 plants. b Bc19 was narrowed between InDel markers C3 and C4 depending on analysis of 983 recessive F2 plants. c 23 annotated genes were found in the 145-kb region between markers C3 and C4. d Structure of the candidate gene Bc19 (*LOC_Os01g54620*). Gray boxes indicate exons and lines indicate introns. The C3439T point mutation is marked with a black arrow.

Figure 5

Part of alignments of amino acid sequences of OsCesA4 with similar sequences in other CesA family members of rice and Arabidopsis (a) and in different species (b). Dark and gray backgrounds showed the identical and similar residues, respectively. A red arrow indicates the missense mutation P507S in mutant Bc19, which is strictly conserved and is only one residue away from the R588G mutation in mutant Bc6 (black arrow, Kotake et al. 2011). Accession numbers for these sequences are as follows: *Oryza sativa* OsCesA4 (LOC_Os01g54620, Q5JN63.1), OsCesA1 (LOC_Os05g08370, Q6AT26.1), OsCesA2 (LOC_Os03g59340, Q84M43.1), OsCesA3 (LOC_Os07g24190, Q69V23.1), OsCesA5 (LOC_Os03g62090, Q851L8.1), OsCesA6 (LOC_Os03g62090, Q6YVM4.1), OsCesA7 (LOC_Os10g32980, Q9AV71.1), OsCesA8 (LOC_Os07g10770, Q84ZN6.1), and OsCesA9 (LOC_Os09g25490, Q69P51.1); *Arabidopsis thaliana* AtCesA1 (At4g32410, NP_194967.1), AtCesA2 (At4g39350, NP_195645.1), AtCesA3 (At5g05170, NP_196136.1), AtCesA4 (At5g44030, Q84JA6.1), AtCesA5 (At5g09870, Q8L778.2), AtCesA6 (At5g64740, Q94JQ6.2), AtCesA7 (At5g17420, NP_197244.1), AtCesA8 (At4g18780, NP_567564.1), and AtCesA9 (At2g21770, Q9SJ22.1); *Zea mays* (DAA36974.1); *Hordeum vulgare* (AAR29965.1); *Aegilops tauschii* (EMT23140.1); *Brachypodium distachyon* (XP_003569818.1); *Eucalyptus camaldulensis* (AEK31215.1); *Populus tomentosa* (AEE60894.1); *Setaria italica* (XP_004969957.1); *Sorghum bicolor* (XP_002456361.1); and *Triticum aestivum* (CBH32503.1).

Figure 6

Functional identification of Bc19. a PCR assay of transgenic lines of brittle gene Bc19. M, DL-2000 plus marker; WT, the wild-type parent Nipponbare (the transgenic acceptor, as PCR-negative control); CK, pC2300-Bc19 plasmid (PCR-positive control); TG1 and TG2, positive transgenic lines. b and c Comparison of cell wall components among the wild type plant, mutant Bc19 and two positive transgenic lines, in leaves and culms, respectively. d Transcript levels of OsCesA4 in different tissues of the wild type (WT), the mutant Bc19, the hybrid F₁ plants from a cross between WT and Bc19, and two transgenic lines (TG1 and TG2), respectively. SL, seedling leaves; SR, seedling roots; L and C, leaves and culms from plants two weeks after heading, respectively; P, young spikelets. The expression data of WT were all set to 1.0 and those of other plants were adjusted accordingly. Bars represent standard deviations of three independent experiments. Double asterisks signify statistically significant differences compared to WT at $P < 0.01$.

Figure 7

qRT-PCR analysis. Total RNA was isolated from seedling leaves (SL), seedling roots (SR), leaves and culms from plants two weeks after heading (L and C), young panicles (P). The rice Actin1 was used as a control. Values represent averages of three independent replicates. Vertical bars show standard errors. a Expression of OsCesAs, BC1 and OsPAL relative to Actin1 in different tissues of the wild type plant. b Comparison of OsCesAs, BC1 and OsPAL mRNAs between WT and Bc19. The expression data of WT were all set to 1.0 and those of Bc19 were adjusted accordingly. Bars show standard deviations. Asterisks signify statistically significant differences compared to WT at $P < 0.05$.

Figure 8

The predicted structure of a CesA protein showing the relative locations of reported point mutations of CesAs in rice. The basic domain structure of CesA was adapted from a review on cellulose synthesis in Arabidopsis (Li et al. 2014b). The open box stands for the plasma membrane (PM). A Zinc-finger domain (a blue bar) exists towards the N-terminus in the first cytoplasmic domain, followed by two transmembrane domains (TMDs; gray bars), the second cytoplasmic domain between the second and the third TMDs, and the other six TMDs. The Plant-conserved Region (P-CR, a green bar), the conserved D,D,D,QXXRW residues (black spots), and the Class-specific region (C-SR, a yellow bar) are all located in the second cytoplasmic domain. Red bars indicate point missense mutations as follows: bc-s1 (OsCesA9, G10R, Jin et al. 2016), S1-24 (OsCesA7, C40Y, Wang et al. 2016a), bc13 (OsCesA9, G101K, Song et al. 2013), fc17 (OsCesA4, F426S, Li et al. 2018), Bc19 (OsCesA4, P507S) in this study, Bc6 (OsCesA9, R588G, Kotake et al. 2011), S1-60 (OsCesA9, G905D, Wang et al. 2012), and bc11 (OsCesA4, G858R, Zhang et al. 2009).

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