**BEAK-SHAPED GRAIN 1/TRIANGULAR HULL 1, a DUF640 gene, is associated with grain shape, size and weight in rice**

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Grain shape and size both determine grain weight and therefore crop yield. However, the molecular mechanisms controlling grain shape and size are still largely unknown. Here, we isolated a rice mutant, *beak-shaped grain1 (bsg1)*, which produced beak-shaped grains of decreased width, thickness and weight with a loosely interlocked lemma and palea that were unable to close tightly. Starch granules were also irregularly packaged in the *bsg1* grains. Consistent with the lemma and palea shapes, the outer parenchyma cell layers of these *bsg1* tissues developed fewer cells with decreased size. Map-based cloning revealed that *BSG1* encoded a DUF640 domain protein, TRIANGULAR HULL 1, of unknown function. Quantitative PCR and GUS fusion reporter assays showed that *BSG1* was expressed mainly in the young panicle and elongating stem. The *BSG1* mutation affected the expression of genes potentially involved in the cell cycle and *GW2*, an important regulator of grain size in rice. Our results suggest that *BSG1* determines grain shape and size probably by modifying cell division and expansion in the grain hull.

**cell division and expansion, DUF640 protein, gene expression, grain size, lemma and palea**

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for normal palea formation [15]. In the rice open beak (oph) mutant, the lemma bends towards the palea and the lateral growth of the lemma and palea is suppressed such that they are unable to close [16].

The basic cell cycle machinery that regulates cell division for growth and development is highly conserved in eukaryotes [17–19]. Different cyclin-dependent kinase (CDKs) complexes phosphorylate different substrates at the G1-to-S and G2-to-M transition points to induce DNA replication and mitosis to control the cell cycle. For example, CDKA is essential at both the G1-to-S and G2-to-M transitions of the cell cycle in plants [20,21]. CDKB1 is expressed during the S to M phases while CDKB2 is mainly expressed in the G2 to M phases [22–24]. CDK-activating kinases (CAKs) and cyclin-dependent kinase inhibitors (CKIs) modulate the level of CDK activity together to control cell cycle progression [25–28].

In this study, we isolated two allelic mutants, beak-shaped grain1-1 (bsg1-1) and bsg1-2, whose palea and lemma develop incompletely and whose grain width, thickness and weight are all significantly reduced. BSG1 encodes a protein containing a DUF640 domain of unknown function. Our results show that BSG1 regulates the expression of cell cycle genes and floral homeotic genes in the grain hull. This work demonstrates that BSG1 controls grain hull development and provides further evidence for the functional exploration of DUF640 proteins.

1 Materials and methods

1.1 Plant materials and mutants isolation

The bsg1-1 and bsg1-2 mutants were identified from a 60Co γ-ray-mutagenized population of cultivar Zhejing22 (Oryza sativa ssp. japonica). The mutants were crossed with Zhejing22 for genetic analysis and with 93-11 (ssp. indica) for genetic mapping. All of the parents, F1 hybrids and corresponding F2 individuals were grown in paddy fields at Shanghai and Hainan under natural conditions.

1.2 Phenotyping of grain traits

Fully filled and air-dried grains were used for measurements. Thirty grains of each genotype were measured for grain length and width, and 3000 grains were weighed for 1000-grain weight. The cell lengths in the hulls were measured using the ImageJ software.

1.3 Map-based cloning

Mutant individuals showing the bsg1 phenotype in the F2 population derived from bsg1-1 and 93-11 were used for map-based cloning. An initial screening for molecular markers linked to BSG1 was performed using markers from publicly available rice databases, including Gramene (http://www.gramene.org) and the Rice Genomic Research Program (http://rgp.dna.affrc.go.jp/). Fine mapping was performed using new markers developed from genomic polymorphisms between 93-11 (http://rise.genomics.org.cn/rice/index2.jsp) and Nipponbare (ssp. japonica, http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl). The main markers are listed in Table S1.

1.4 Histological analysis

Young spikelets before heading were fixed in FAA (70% ethanol, 5% glacial acetic acid, 3.7% formaldehyde), embedded in Epon812 resin, cut into 2-μm sections and stained with toluidine blue. Sections were examined under a microscope (BX51, Olympus, Tokyo, Japan) and photographed. For scanning electron microscopy (SEM), the samples were fixed overnight at 4°C in FAA and dehydrated in a graded ethanol series. The samples were then critical-point dried in liquid carbon dioxide and coated with gold, followed by visualization with a scanning electron microscope (JSM-6360LV, JEOL, Tokyo, Japan).

1.5 GUS reporter assay

The GUS coding sequence was released by digesting the vector pBI101 with Hind III and EcoR I, ligated with a 1950-bp promoter fragment of the BSG1 gene, and inserted into the binary vector pCAMBIA1300 to generate the fusion reporter plasmid pBSG1-GUS, which was then transformed into O. sativa cv. Nipponbare by an Agrobacterium tumefaciens-mediated method [29]. Different tissues and organs of the transgenic plants were collected for histochemical assays of GUS activity.

1.6 RNA isolation and expression assay

Total RNA was isolated from different tissues using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and treated with DNase I using the DNA-free kit (Ambion, Austin, TX, USA). Real-time PCR (quantitative PCR) was performed in triplicate using the SYBR Premix Ex Taq kit (TaKaRa, Otsu, Japan) and the Mastercycler ep realplex detection system (Eppendorf, Hamburg, Germany). The amplification program was as follows: 95°C for 3 min, followed by 40 cycles at 95°C for 20 s, and 60°C for 1 min. ACTIN2 was amplified as an internal control for loading normalization. Relative transcript levels were obtained using the 2−ΔΔCt quantification method [30]. All primers are listed in Table S1.

2 Results

2.1 Grain phenotypes of the bsg1-1 mutant

The bsg1-1 mutant was isolated from the M2 population of
a Zhejing22 mutant pool treated with $^{60}$Co γ-rays. There was no obvious difference between the mutant and the wild-type during the vegetative stage. In adult plants, bsg1-1 showed no significant differences in architecture traits such as plant height and tiller number, compared with the wild-type. However, the hull of the mature grain in bsg1-1 was bend and not completely closed, making it look like a beak (Figure 1A). Phenotypic analysis showed that grain width and thickness in bsg1-1 were reduced by 25.1% and 17.1%, respectively, compared with the wild-type (Figure 1A and D), whereas grain length did not change significantly (Figure 1B). The conical seeds of bsg1-1 had dark brown coats and were also smaller than the seeds of the wild-type (Figure 1A).

To investigate the influence of BSG1 on grain weight, we compared bsg1-1 with the wild-type and observed a significant decrease (50.2%) in 1000-grain weight in bsg1-1 (Figure 2A). The bsg1-1 grain also showed markedly abnormal development and irregular packaging of starch granules (Figure 2B). These results together indicated that BSG1 affected not only grain shape but also grain weight.

2.2 Abnormality of lodicules in bsg1-1

Besides the lemma and palea, the lodicules in bsg1-1 were also abnormal compared with those of the wild-type (Figure 3A, B, D, and E). The upper regions of the lodicules were elongated and a few awn-like protuberances were formed on their top in bsg1-1 (Figure 3A and B). SEM images showed that the cells in the elongated parts of the lodicules differed to those of wild-type lodicules; they lacked the striation of the wild-type epidermal cells (Figure 3C and F). This implied that the differentiation of specific cells in the lodicules might be changed in bsg1-1 and suggested that BSG1 was involved in lodicule development.

2.3 Map-based cloning of the BSG1 gene

The bsg1-1 mutant was crossed with the wild-type (Zhejing22) and all F1 hybrids displayed the wild-type phenotype. In the F2 population, the segregation ratio of wild-type to bsg1-1 plants showed typical Mendelian segregation (3:1, $\chi^2=1.59 < \chi^2_{0.05,1}=3.84$). This result indicated that the mutant phenotype was controlled by a single recessive gene. An F2 population with 560 individuals displaying the bsg1 mutant phenotype from a cross between bsg1-1 and 93-11 was used for mapping. BSG1 was finally narrowed down to a region of 194-kb between markers J59 and J95, which contained partial sequences of three BAC clones: AP004081, AP005303 and AP005691 (Figure 4A).

Based on the available sequence annotation database (http://rapdblegacy.dna.affrc.go.jp/), 28 predicted genes were located in the 194-kb target region. We compared the genome sequences of these candidate genes in bsg1-1 and Zhejing22 and a 5-bp deletion in the genomic sequence of the predicted gene Os02g0811000 was identified in bsg1-1 (Figure 4B) and verified by restriction enzyme digestion patterns. According to the annotation database, the full-length cDNA of Os02g0811000 is 1587 bp, containing a 747-bp coding sequence that encodes a putative protein with an unknown-function domain called DUF640 (Figure 4B).

Besides bsg1-1, we identified an additional beak-shaped
grain mutant from the same M2 population, bsg1-2 (Figure 4C), which was also controlled by a single recessive gene. Sequencing revealed a transition from A to T in the exon of the same Os02g0811000 gene, resulting in a change of...
amino acid from Asp to Val (Figure 4B). The deletion in bsg1-1 and amino acid transition in bsg1-2 both occurred in the DUF640 coding sequence (Figure 4B). Recently, another mutant, triangular hull1-1 (th1-1) in the same gene was reported with a similar phenotype [31]. Taken together, these results demonstrated that the Os02g0811000 gene was responsible for the phenotype of bsg1/th1-1.

Real-time PCR analysis of the BSG1 expression pattern showed that transcripts of BSG1 were accumulated most strongly in the young panicles (inflorescence length from 0.1–2 cm), followed by stems and older panicles (inflorescence length from 8–10 cm) (Figure 4D). Almost no expression of BSG1 was detected in roots, seedlings and mature leaves. These results were consistent with the expression patterns detected by the BSG1::GUS fusion reporter in transgenic plants (Figure 4E).

2.4 Histological characterization of the lemma and palea

To study the histological phenotype of the hull, we examined cross-sections of the central part of the lemma and palea of young spikelets. The floret of bsg1-1 was open and lacked interlocking between the lemma and palea (Figure 5C), compared with the wild-type that was hooked closely (Figure 5A). The outer parenchyma cell layers of the lemma and palea (Figure 5B and D) in bsg1-1 were both shorter and contained fewer cells than the wild-type (Figure 5E and F). These features demonstrated that the reduced width of the bsg1-1 spikelet hull resulted mainly from the decrease in cell number and size. Moreover, in wild-type plants, the lateral margin tissue of the palea (MTP), which differed from the rest of palea, was membranous with a smooth surface and lacked epicuticular and silicified thickening (Figure 5B). However, the bsg1-1 palea lacked distinctive marginal tissue structure and had more parenchyma cell layers than the wild-type (Figure 5D). These morphological characteristics suggested that the cell identity of the lateral margin tissue of the palea was lost and that the cell specification was disturbed in bsg1-1.

2.5 Effect of BSG1 on the expression of cell-cycle genes

Since cell identity and cell specification were disturbed in bsg1-1, we investigated whether cell-cycle genes were regulated by BSG1. We analyzed the expression levels of 25 rice genes, identified by querying sequences of previously published core cell cycle regulators [11], in the hulls of young panicles in the bsg1-1 mutant and the wild-type (Figure 6A). Among them, the transcript levels of 11 genes were greatly reduced in bsg1-1. Moreover, the expression level of CDC20, an activator of the anaphase-promoting complex (APC) for mitosis exit via destruction of cyclin subunits [17,32], was elevated in bsg1-1. Therefore, it appears that BSG1 likely acts as a positive regulator of cell division by regulating downstream cell-cycle genes to control grain shape and size.

Previous studies have reported that reduced expression of GW2 increased grain width, whereas overexpression of GW2 decreased grain size [5], and higher expression of GS5 was correlated with larger grain size owing to an increase of cell number in glumes [11]. We analyzed the transcript levels of GW2 and GS5 in the hulls of bsg1-1 and the wild-type, and found that GW2 expression was significantly upregulated in 8-cm young panicles of bsg1-1 compared with the wild-type, whereas GS5 expression changed only slightly in young panicles (Figure 6B). These observations suggested that BSG1 might be involved in the regulation pathway of GW2.

2.6 BSG1 regulates genes in flower development

It has been established that MADS-box genes play critical roles in the specification of floral organ identity in plants,
including rice [33]. Thus, we assayed the expression levels of MADS-box genes involving in rice lemma/palea and lodicule development. The genes that showed significant differences in expression levels between bsg1-1 and the WT are shown in Figure 7. The expression levels of E-class genes OsMADS1 and OsMADS6 were only down-regulated in 0.5-cm young panicles, while OsMADS16 was down-regulated in both 0.5-cm and 8-cm panicles (Figure 7). The transcript level of OsMADS55 was increased in 0.5-cm young panicles but reduced in 8-cm panicles of the bsg1-1 mutant (Figure 7). Moreover, we found that expression of the C-class gene OsMADS58 and the D-class gene OsMADS13 was up-regulated in 8-cm panicles, and OsMADS13 was also greatly up-regulated in 0.5-cm panicles (Figure 7). These results implied that BSG1 was also involved in floral organ formation and development.

3 Discussion

The shape and size of rice grains is controlled by cell division and differentiation, as well as by grain filling. The TH1 gene (BSG1 in our study) was previously reported to function in the control of rice seed shape and size [31]. However, it was unknown how TH1 influences the shape and size of the hull. The bsg1 mutants developed small grains with reduced weight and thickness (Figure 1). Consistent with the grain phenotypes, histological observation showed that bsg1-1 exhibited fewer and smaller cells in the outer parenchyma cell layers of the lemma and palea compared with wild-type plants. Moreover, gene expression assays indicated that most of the positive regulatory genes predicted to be involved in cell cycle control were down-regulated in the bsg1-1 mutant. Therefore, BSG1 most likely functions as a positive upstream modulator of cell division during rice floral organ development. Supporting this function, BSG1 was most strongly expressed in the young panicles (Figure 4D and E).
A database search indicated that there are 10 genes that encode proteins with DUF640 domains in both rice and Arabidopsis [34] (Figure 8A and B). LIGHT-DEPENDENT SHORT HYPOCOTYL1 (LSH1), which mediates light regulation of seedling development via phytochrome, is the only DUF640-domain gene functionally elaborated in Arabidopsis [35]. In rice, the homeotic gene LONG STERILE LEMMA (G1) was shown to be involved in transcriptional regulation and specification of sterile lemma identity [34]. LSH1 and G1 are both localized in the nucleus, similar to BSG1. In addition, we found that BSG1 has transcriptional activation ability using a yeast-two-hybrid assay (Figure S1). Thus, it is likely that BSG1 functions at a specific regulatory node controlling cell division and expansion in the rice grain hull. Further studies will be required to clarify the underlying mechanisms by identifying the BSG1-interacting protein(s).

OsMADS55 functions as a negative regulator of brassinosteroid responses and stem elongation and senescence processes [36]. The expression level change of OsMADS55 in bsg1 suggests the possible implication of BSG1 in OsMADS55-mediated inflorescence elongation and floral organ augmentation. Previous studies have shown that OsMADS58 functions in the establishment of floral meristem

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**Figure 8** Phylogenetic tree of proteins containing the DUF640 domain. A, Protein and cDNA sequences of BSG1. Dashed lines indicate the DUF640 domain. Black boxes indicate the mutation sites in the bsg1 mutants. B, Phylogenetic tree of DUF-640 proteins in rice and Arabidopsis. The tree was constructed by the neighbor-joining method. Numbers indicate bootstrap values.
determinacy and carpel development, while OsMADS13 is involved in ovule specification [37,38]. Thus, we speculate that BSG1 might also participate in the regulation pathways of carpel and ovule development besides those of lemma/palea and lodicule development. However, detailed expression pattern changes in different floral organs need to be analyzed and the mechanism of floral homeotic gene regulation by BSG1 remains to be elucidated.

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Supporting Information

Table S1  Primers used in this study

Figure S1  Subcellular localization of BSG1 and transcriptional activation assay. A, BSG1 was localized in the nucleus. B, Transcriptional activation analysis of BSG1 in a yeast-two-hybrid system.

The supporting information is available online at life.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.