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Genetic analysis and gene cloning of a *triangular hull 1* (*tri1*) mutant in rice (*Oryza sativa* L.)

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Grain shape and size are two key factors that determine rice yield and quality. In the present study, a rice triangular hull mutant (*tri1*) was obtained from the progeny of *japonica* rice variety Taipei 309 treated with ^{60}Co γ-rays. Compared to the wild type, the *tri1* mutant presents a triangular hull, and exhibits an increase in grain thickness and protein content, but with a slight decrease in plant height and grain weight. Genetic analysis indicated that the mutant phenotype was controlled by a recessive nuclear gene which is stably inherited. Using a map-based cloning strategy, we fine-mapped *tri1* to a 47-kb region between the molecular markers CHR0122 and CHR0127 on the long arm of chromosome 1, and showed that it co-segregates with the molecular marker CHR0119. According to the rice genome sequence annotation there are six predicated genes within the mapped region. Sequencing analysis of the mutant and the wild type indicated that there was a deletion of an A nucleotide in exon 3 of the *OsMADS32* gene, which could result in a downstream frameshift mutation and premature termination of the predicted polypeptide. Both semi-quantitative and real-time RT-PCR analyses showed that this gene expressed highly in young inflorescences, while expressed at very low levels in other tissues. These results implied that the *OsMADS32* gene could be a candidate of *TRI1*. Taken together, the results of this study lay the foundation for further investigation into the molecular mechanisms regulating rice caryopsis development.

Oryza sativa* L., *triangular hull 1* mutant (*tri1*), genetic analysis, gene cloning, *OsMADS32

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Grain size and shape are two key determinants of cereal grain yield and quality, and are also important target traits for either domestication or genetic improvement. Rice is one of the most important foods for human beings, and also an important model for genetic studies of monocotyledonous plants. Therefore, it is quite important for both theoretical and practical reasons to elucidate the molecular mechanism of grain development. In recent years, many genes controlling rice grain size and shape have been cloned. For example, *GS3* was identified as a major QTL (quantitative trait locus) controlling rice grain weight and length, and also as a minor QTL for grain width and thickness. *GS3* might also function as a negative regulator for controlling both grain and organ size [1,2]. *GS5*, encoding a putative serine carboxypeptidase, plays an important role in controlling grain width, filling and grain weight. Highlevel expression of *GS5* is correlated with promotion of cell division, causing an increased cell number and finally resulting in a larger grain size [3,4].

The rice spikelet consists of rudimentary glumes, the lemma, palea, lodicules, stamens and pistil. Among these floral organs, development of the lemma and palea is one of the key factors controlling grain shape and size, and ultimately effect grain yield and quality. *Fon* (*floral organ*

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number) is a gene controlling rice floral organ number, which plays an important role during the development of shoot apical meristems, the inflorescence and floral meristems [5–8]. The *fon* mutant presents an enlargement of the floral meristem, and parts of the flowers have extra lodicules or plaea/lemma-like organs [5–8]. In 1943, Morinaga et al. [9] were the first to describe the triangular hull (TH) phenotype, subsequently, several other reports followed [10,11]. The triangular hull usually exhibits curved lemma, which results in the triangular phenotype of both spikelet and grain. Li et al. [11] identified a *th1* (*triangular hull 1*) mutant from a population of EMS (ethyl methane sulphonate)-induced mutants, and the *TH1* gene was then mapped to a 60-kb region on chromosome 2. The cloned *TH1* gene, belonging to the DUF640 domain-containing family, is mainly expressed in the young inflorescence, the lemma and palea, and has proven to be one of the most important genes controlling lemma and palea development in rice.

In the present study, we identified a *tri* mutant from the progeny of the *japonica* rice variety Taipei 309 treated with ^{60}Co γ-rays. The *tri* mutant is able to set seeds normally, and the mutation is stably inherited. Genetic analysis showed that the triangular hull phenotype is controlled by a single recessive nuclear gene. By using a map-based cloning strategy, the *TRI1* gene was located within a 47-kb region on the long arm of chromosome 1. The results from sequence verification and gene expression revealed that *OsMADS32*, a gene encoding a transcription factor important for inflorescence development, could be the candidate gene for *TRI1*.

1 Materials and methods

1.1 Plant materials

Two *japonica* rice varieties, Taipei 309 and Nipponbare, and one *indica* variety, Nanjing 11, were used in this study. The *tri1* mutant was derived from the rice variety Taipei 309 treated with γ-rays from a ^{60}Co source.

1.2 Investigation of agronomic traits and grain quality

The *tri1* mutant and the wild type (Taipei 309) were grown under normal cultural conditions during the regular rice-growing season in the experimental field at Yangzhou University in 2010 and 2011. At maturity stage, the major agronomic traits, including plant height, tiller number, 1000-grain weight, grain length, grain width and grain thickness, were carefully investigated and compared between the mutant and the wild type. The mature grains were milled and air-dried at room temperature for physical and chemical quality measurements. Total crude protein content was determined by the Micro-Kjeldahl method, and the amylose content was determined by an iodine colorimetric method. Starch viscosity was analyzed with a Rapid Visco Analyzer (RVA) (Newport Scientific, Narrabeen, Australia) according to the manufacturer's instructions. ANOVA (an analysis of variance) was performed by using SPSS16.0 to investigate whether there was a significant difference between/among the tested samples.

1.3 Genetic analysis

The *tri1* mutant was crossed with the *indica* cultivar Nanjing 11 in August 2009, and the F_1 hybrid was planted in Lingshui County at Hainan Province in November of the same year. The F_2 populations were grown in the experimental field at Yangzhou University in the summers of 2010 and 2011. When the plants were mature, segregation of normal vs. triangular hull grains was determined, and the ratio was analyzed by the χ^2-test.

1.4 DNA extraction and molecular markers analysis

A total of 1153 plants showing triangular hull grains and several indivduals with normal grains from the above F_2 population were used for gene mapping. DNA was extracted from the leaves by the method described by Dellaporta et al. [13]. Some SSR (simple sequence repeat) marker sequences were obtained from Gramene web (http://www.gramene.org/). Also, based on the published complete genome sequences of *indica* cultivar 93-11 and *japonica* cultivar Nipponbare, several molecular markers were designed using Primer Premier 5.0 software (Figure 1). All primers were synthesized by Shanghai Generay Biotech Co., Ltd. PCR products of SSR markers were electrophoresised on 3% agarose gels, and subsequently photographed by a Gel Imaging System after staining with ethidium bromide. For the CAPS (Cleaved amplified polymorphic sequence) marker CHR0135, the PCR product was digested with the restriction enzyme *Dde* I prior to electrophoresis.

1.5 Gene mapping

Using the principle of bulked segregant analysis (BSA), 10 normal plants and 10 mutant plants were randomly selected from the above F_2 population to construct the normal and mutant pools, respectively. Polymorphic markers were first selected between the parental lines, Nanjing 11 and Taipei 309, and were then used to identify markers linked to the *TRI1* gene. Based on the linked marker and the newly developed markers around the primary restricted region, fine mapping of the target gene was performed with all selected mutant plants.

1.6 Identification of the candidate gene

Based on the predicted gene sequences (http://blast.ncbi.nlm.nih.gov) in the fine-mapped region, specific primers were designed to amplify the puptative genes from both

wild type and the *tri1* mutant. The resulting PCR products were cloned into the pMD18-T vector for sequencing analysis.

1.7 RNA extraction and expression analysis

Different tissues, including the root, culm, leaf and leaf sheath at the elongation stage, young panicles before heading date, and developing seeds at the filling stage, were collected. Total RNAs were extracted using a total RNA extraction kit (Tiangen) according to the manufacturer's instructions. After digestion with DNase (RNase-free), the first strand cDNA was synthesized. The rice housekeeping gene *OsActin1* (LOC_Os03g0718100) was chosen as the internal control and was amplified with the primer pair ACT-F (5′-CCAAGGCCAATCGTGAGAAGA-3′) and ACT-R (5′-AATCAGTGAGATCACGCCCAG-3′). Semi-quantitative RT-PCR was perfomed with 25 cycles of amplification. Real-time PCR was performed using the SYBR® *Premi Ex Taq*™ (Perfect Real-Time) Kit (TaKaRa) and the ABI 7500 Real-Time PCR system (Applied Biosystems). Each 20 μL PCR reaction mixture contained 10 μL of SYBR® Premi Ex Taq™II (2X), 8 nmol of each specific forward and reverse primers, 10 ng of the synthesized cDNA, and 0.4 μL of ROX Reference Dye (50×).

2 Results and analysis

2.1 Agronomic performance and grain quality of the *tri1* mutant

As shown in Figure 1(a) and Table 2, plant height of the *tri1* mutant was somewhat reduced compared to the wild type Taipei309. During panicle development, the top of the *tri1* lemma curved inward toward the palea, some spikelets could not close completely, and the hulls exhibited the characteristic triangular shape (Figure 1(b)). After stripping off the lemma, it was seen that the palea of the wild type was much smaller, but the palea of the mutant was laterally elongated and triangular, and wrapped around most of the embryo tightly (Figure 1(d)). At maturity, the *tri1* grains were irregular and triangular in shape (Figure 1(b)), and the brown rice was also triangular (Figure 1(c)).

Data for seed shape and size of the *tri1* mutant and the wild type are given in Table 2. It is obvious that, compared with the wild type, grain thickness of the *tri1* mutant increased significantly ($P < 0.05$), while the grain weight decreased significantly ($P < 0.05$). There was no significant difference observed in grain length between wild type and the mutant. Although the crude protein content of mutant grains (8.5%±0.1%) was significantly higher than in wild type (6.9%±0.2%) ($P < 0.05$), there was no difference in the amylose content, gelatinization temperature and gel consistency (data not shown), or the RVA profile between the mutant and wild type (Figure 1(e)). This implies that mutation of the target gene had a large effect on grain shape and size, but little effect on rice grain qualities.

2.2 Genetic analysis and mapping of the mutated gene

F_2 seeds derived from selfing F_1 plants of the cross between Nanjing11 and the mutant *tri1* were all normal and basically identical to those of the wild type, which suggested that the triangular hull is a recessive trait. Among the 200 plants randomly selected from the F_2 population, 157 and 43 plants exhibited the normal or *tri1* mutant phenotypes, respectively.

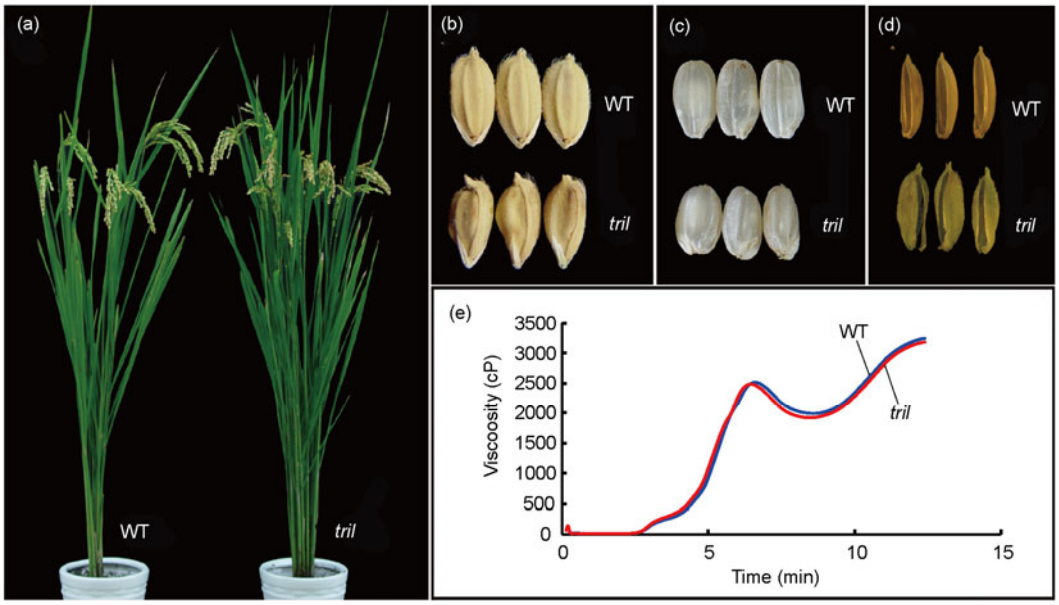

Figure 1 Phenotypic comparisons between the *tri1* mutant and the wild type. (a) Plant phenotype; (b) mature grain; (c) brown rice; (d) palea; (e) RVA profile of milled rice. WT, wild type; *tril*, mutant.

This ratio correlated well with the expected ratio of 3:1 (χ^2 = 1.31 < $\chi^2_{0.05}$ = 3.84). Therefore, the mutant phenotype is probably caused by a recessive nuclear gene.

Among the 150 SSR markers distributed evenly on 12 chromosomes, 80 markers were identified that were polymorphic between the two parental lines, Nanjing 11 and Taipei 309. The polymorphic markers were then analyzed within both the normal and *tri1* mutation DNA pools, and the results showed that the SSR marker CHR0101, which maps to a locus on the long arm of chromosome 1, could be linked to the target gene *TRI1*. Subsequently, we found that the CHR0101 marker showed significant segregation distortion among 46 mutant (*tri1*) plants from the F_2 population, but do high exchange frequency among 20 normal plants. These results implied that the CHR0101 marker is linked to the target gene.

For fine mapping, 16 new SSR or CAPS markers, from the region around the marker CHR0101, were developed by using the genome sequences of the *japonica* line Nipponbare and the *indica* line 93-11; 8 of the markers (Table 1) showed polymorphisms between Nanjing 11 and Taipei 309. By using another 83 mutant plants from the F_2 population, *TRI1* was then mapped within a 705-kb region between the molecular markers CHR0101 and CHR0111, and it co-segregated with the markers CHR0115, CHR0116 and CHR0117 (Figure 2(a)). To further map the *TRI1* gene, we generated a large F_2 population and screened 1153 mutant plants, and developed 11 new polymorphic markers (Table 1) that mapped to the interval between marker loci CHR0101 and CHR0111. The *TRI1* gene was finally narrowed down within a 47-kb region between markers CHR0122 and CRH0127 in the BAC clone AP003343 on chromosome 1 (Figure 2(c)), and it co-segregated with the marker CHR0119 (Figure 2(b)).

2.3 Cloning and sequence analysis of the candidate gene

Based on the Rice Genome Annotation Project, six candidated genes identified within the mapped 47-kb region (Figure 2(c)) are *LOC_Os01g52680*, *LOC_ Os01g52690*, *LOC_Os01g52700*, *LOC_Os01g52710*, *LOC_Os01g52720* and *LOC_Os01g52730*. Because the partial sequences of the predicted gene *LOC_Os01g52730* were not included in the region, only the other five intact annotated genes were analyzed. The five candidate genes encode *OsMADS32*, a putative retrotransposon protein, a hypothetical protein, a putative glycosyl transferase 8 domain-containing protein, and a putative complex I intermediate-associated protein 30 domain-containing protein. Among these, *OsMADS32* belongs to the MADS-box family and has a MIKCc type-box; this gene has been reported to be a transcription factor with a function in flower development.

Based on the gene annotation sequences of Nipponbare, specific primers were designed to amplify and clone all five genes from both the *tri1* mutant and the wild type. The results from sequence alignment revealed that there was no difference in the sequences of four genes, *LOC_Os01g52690*, *LOC_Os01g52700*, *LOC_Os01g52710* and *LOC_Os01g52720* between the mutant and the wild type. However, a single nucleotide A deletion was identified in the third exon of the gene *LOC_Os02g56610* (*OsMADS32*) in the mutant compared with the wild type (Figure 2(d)). This deletion is predicted to result in a frameshift at the 107th codon and then premature termination at the 109th codon (Figure S1), which could cause a change or loss of

Table 1 Primer sequences for the molecular markers developed in this study

Molecular marker	Forward primer (5′→3′)	Reverse primer (5′→3′)
CHR0101	ATAGTTCGCCATCGTCAT	ACACGCCATAGCAAGGAA
CHR0102	CATATCTCTACAAAACAAACAAAAA	CATCAATGGTGGTGACCTTTT
CHR0103	CACATTTTCTGTCCCAAAGTTCA	ATTTAGAGCGTGTTGTGTTTTGG
CHR0107	AACCCCTCCCTTCTCCTTCTTC	CACAGGACGTGAGGCTCGG
CHR0111	TCCTCCTCATTCGGACACTTTT	CCAAACAGTGGATGGAGCAGTAG
CHR0112	AGATTTTCTCATGTGTTATGCGA	GTGGTTTTGTTTTCTCTGGGACT
CHR0114	GCACATTTTATTTCGATTCGACCA	ATCTTGCCCCTTTCTCGCCT
CHR0115	AATCTGTATGGTCAACTCTTCAC	AGCAGCCTAACTTATGTTCCA
CHR0116	AGAGGAAATGATGGTCTGGAG	GTACATAGGCAGAAGCTCTAAAGTC
CHR0117	GTGTTAAGGCTTGTCCCGAGT	AATCTCCCAATCTCGTAGGCT
CHR0119	CCAATGGTAGGTGAAACAAAGAATG	TACAGGCTCTCGTCTTCAACACACT
CHR0120	AGGAGAGGAGAGACGCTGGCT	TACAGCAACCCCCCAAATCAT
CHR0121	CAATTCTTGAATATGGCGATGGTCT	TGAGATCATCACGCCCAGATTTC
CHR0122	CTCTTGCCGCAAACACTTCT	CAGATTTCCTTTATTGCTGCTTAG
CHR0127	TGTGGGTAAGTTCTGACGGGATT	GCACGCAAACGACGAAACAG
CHR0128	TTGATGTAGCGGATGATGGCGTTA	CAGCCAATGCCTGCCGAAAT
CHR0135	CGTAGTAATCAGTTGTTTGAGCGTT	CACAGGTATGAATCGTATCAGAAGT

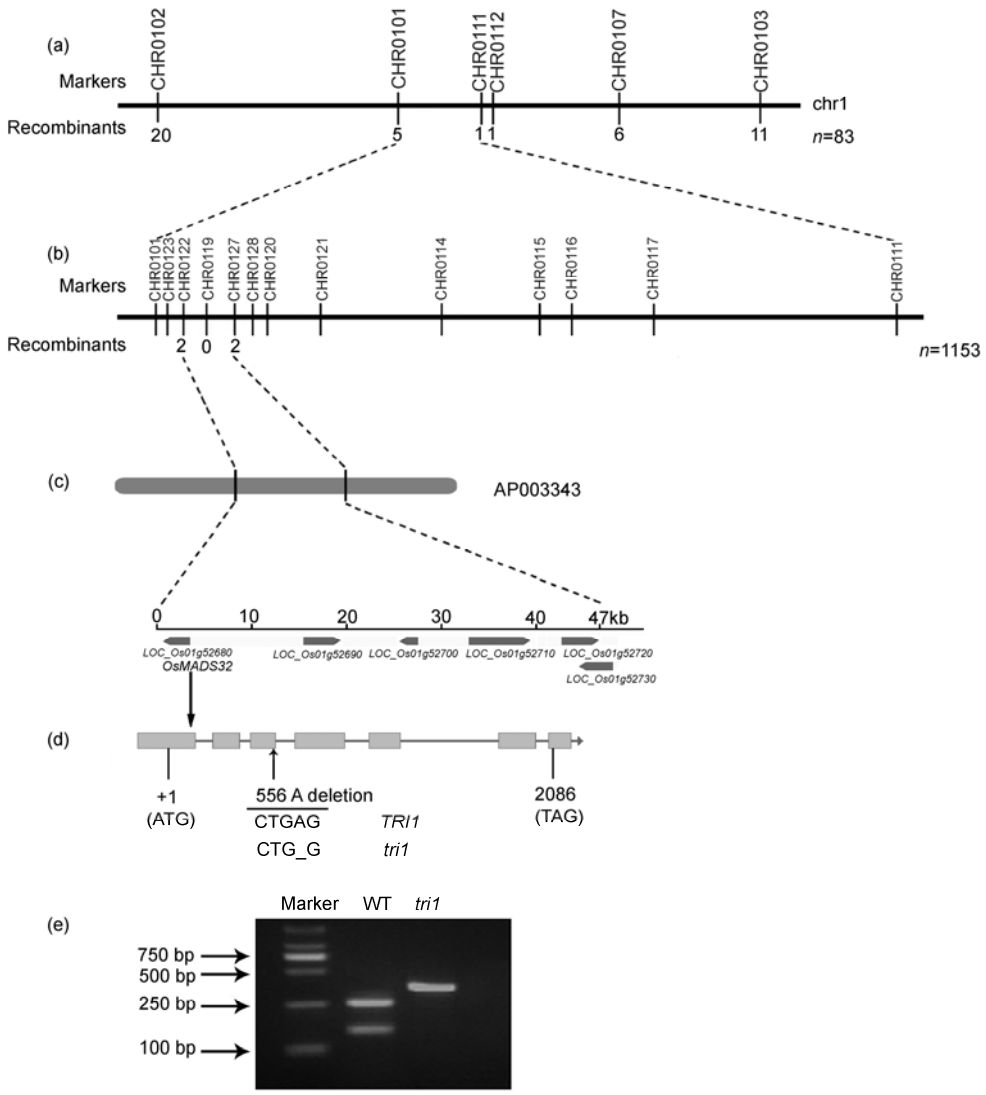

Figure 2 Gene mapping of *TRI1* on the long arm of chromosome 1 and candidate gene prediction. (a) Primary mapping of *TRI1* on chromosome 1 between the markers CHR0101 and CHR0111; (b) fine mapping of *TRI1* between markers CHR0122 and CHR0127 in a 47-kb region; (c) the mapped region is located in the BAC clone AP003343, which contains six predicted genes; (d) the genomic structure of *LOC_Os01g52680* (*OsMADS32*); deletion of an A nucleotide (C556), which disrupts a *Dde* I restriction site, was observed within exon 3 in the *tri1* mutant; (e) detection of the mutated site by using a CAPS marker CHR0135.

Table 2 Comparison of some characteristics between the *tri1* mutant and the wild type Taipei 309[a)]

Year	Line	Plant height (cm)	Effective tillers	Grain length (mm)	Grain width (mm)	Grain thickness (mm)	1000-grain weight (g)
2011	Mutant	108.82±2.77**	8.80±1.77*	7.27±0.25	3.95±0.20**	2.71±0.07*	26.11±0.34*
	Wild type	122.48±4.34	6.40±1.95	7.75±0.14	3.62±0.07	2.36±0.12	27.39±0.06
2010	Mutant	109.86±3.08*	7.80±1.30	7.16±0.15	3.59±0.15	2.84±0.10**	23.89±0.25**
	Wild type	115.70±1.39	8.70±1.59	7.48±0.31	3.53±0.10	2.30±0.10	26.97±0.08

a) * Significance at $P < 0.05$ by *t*-test; ** Significant at $P < 0.01$ by *t*-test.

gene function.

The *OsMADS32* gene is predicted to consist of six introns and seven exons with a 591-bp coding region (Figure 2(d)). The mutated locus in the wild type Taipei 309 encompasses the recognition site for the restriction endonuclease *Dde* I (5′-CTGAG-3′). Thus, the mutated region in the *tri1* mutant could not be digested by *Dde* I due to the deletion of nucleotide A in the recognition sequence. Therefore, we designed a CAPS marker CHR0135 (Table 1), and a 415-bp DNA fragment was amplified from the genomic DNA of both the wild type Taipei 309 or the *tri1* mutant. After digestion with *Dde* I, two small fragments of

sizes 149-bp and 266-bp, respectively, were obtained in the wild type, but the 415-bp fragment was not digested in the *tri1* mutant (Figure 2(e)). This further confirmed that the predicted mutation was present in the *OsMADS32* gene in the *tri1* mutant. Taken togather, the gene sequencing and the CAPS marker test strongly indicate that the *OsMADS32* gene is the candidate gene for *TRI1*.

2.4 Expression pattern of the *OsMADS32* gene in rice

Total RNAs were extracted from different organs, cDNA was synthesized, and semi-quantitative RT-PCR was performed. The results showed that the *OsMADS32* gene is highly expressed in the inflorescence, but is barely detectable in other organs including the root, culm, leaf, leaf sheath and seeds. Real-time PCR analysis further confirmed the relatively high level of expression of the *OsMADS32* gene in the inflorescence, and the very low level of expression in other tissues (Figure 3(b)).

3 Disscussion

To date, several genes controlling rice panicle development have been reported, and most of these were identified as mutants; examples are *leafy head* (*lhd*) [14,15], *branched floretless 1* (*bfl1*) [16], *short panicle 1* (*sp1*) [17], *aberrant spikelet and panicle 1* (*asp1*) [18], *floral organ number* (*fon*) [6,18,19], and *enclosed panicle 2* (*esp2*) [20]. Also, other genes have been reported to be associated with rice hull development, including *frizzy panicle* (*fzp*) [21], *abnormal hull* (*ah*) [22], *long sterile lemma* (*gl*) [23], *elongated empty glume* (*ele*) [24], *leafy hull sterile 1* (*lhs1*) [25], *depressed palea* (*dp*) [26], and *triangular hull* (*th1*) [11]. Some of these genes have been cloned and their functions were analyzed; for example, the *th1* mutant controlled by a recessive gene has an apophysis, tortuous and narrower lemma and palea [11].

In the present study, we identified a *tri1* mutant displaying triangular hull with an apophysis lemma and palea. The phenotype of the *tri1* mutant is similar, but not identical, to that of the *th1* mutant [11]. The hull of the *th1* mutant is tortuous and slender, while that of the *tri1* mutant is wide, short and thick, and the middle sections of some glumes do not close completely at the stage of inflorescence development. The top of the *tri1* lemma is severely curved inwards toward the palea, and the transverse side of the *tri1* palea is extended somewhat. Therefore, the *tri1* mutant displays the triangular hull phenotype, which wraps around most of the embryo and results in an abnormal grain and brown rice kernels. The results of the present study showed that, compared with the wild type, the *tri1* mutant presents increased grain thickness, reduced plant height and 1000-grain weight, suggesting that there is a definite effect of the mutation in the target gene on plant development and grain shape.

The present study also showed that there was no obvious difference in grain length, grain width and effective tiller number between the mutant and the wild type, which suggested that the *tri1* mutation had little effect on these three traits. The results from grain quality analyses also showed that the RVA profiles and other propterties of the *tri1* grains were similar to those of the wild type, suggesting that the target mutation had little effect on major determinants of grain quality. While the grain protein content of the *tri1* mutant is significantly higher than in the wild type, it could perhaps be that the abnormal hull results in an abnormal grain and affects grain filling.

In this study, the *TRI1* gene was mapped to the long arm of chromosome 1, which showed that it was not an allele of the *th1* gene. Previously, no gene related to rice hull development had been reported in this region of the rice genome; we, therefore, concluded that *TRI1* was a new gene regulating lemma and palea development. Sequence verification indicated that the *tri1* mutant harbored a nucleotide A deletion within the third exon of *OsMADS32*, leading to a predicted translational frameshift at the 107th codon, which in turn results in premature termination at the 109th codon. The results from semi-quantitative RT-PCR and real-time PCR analyses showed that the expression of *OsMADS32* exhibited organ-specificity, and that the *OsMADS32* gene was mainly expressed temporally during the period of inflorescence development, while the expression in root, culm leaf, leaf sheath and seed was almost undetectable. Therefore, we suggest that the *OsMADS32* gene could participate

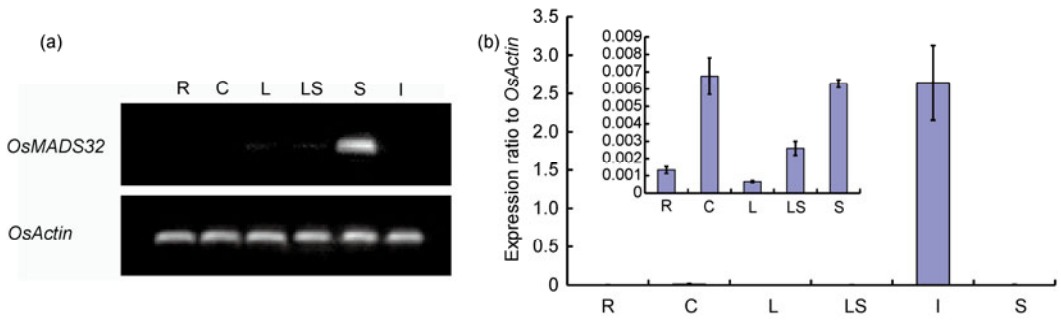

Figure 3 Expression pattern of the *OsMADS32* gene in rice. (a) and (b) show the semi-quantitative RT-PCR and real-time RT-PCR results, respectively. R, root; C, Culm; L, leaf; LS, leaf sheath; I, inflorescence; S, seed.

in the development of the rice panicle, and it is the candidate gene for *TRI1*.

Previous research has shown that many of the genes involved in floral organ development in plants belong to the MADS-box family, such as *OsMADS3*, *OsMADS7*, *OsMADS8*, *OsMADS13*, *OsMADS58*, etc. The MADS-box genes comprise a large family, and exist widely in animals, plants and fungi. Each member has a highly conserved MADS-box domain sequence. Arora et al. [28] found that there were more than 75 MADS-box genes in rice, and most of them were categorized into the MIKC groups based on a genome-wide molecular characterization and microarray-based expression profiling analyses. Some studies have indicated that the MADS-box genes play an important role in rice growth and floral organ development. Kim et al. [29] reported that *OsMADS51* acts to promote flowering, especially under short-days (SD), and functions upstream of *Ehd1*, *OsMADS14*, and *Hd3* and downstream of *OsGI*, transmitting a signal from *OsGI* to *Ehd1*. Ryu et al. [30] showed that, under long days (LD), *OsMADS50* serves as a flowering activator by controlling various floral regulators such as *OsMADS1*, *OsMADS14*, *OsMADS15*, *OsMADS18* and *Hd3a.* These observations also implied that *OsMADS50* and *OsMADS56* function antagonistically through a combination of the *OsLFL1* and *Ehd1* genes in regulating LD-dependent flowering, thus it could be inferred that *OsMADS56* was an LD-specific floral repressor.

Until now, little has been reported concerning the function of the *OsMADS32* gene. Zhao et al. [31] identified a novel *OsMADS32*-like clade in monocotyledons, consisting of *TaAGL14* and *TaAGL15* in wheat and *OsMADS32* in rice, and these three genes are the only identified members of this clade to date. Arora et al. [28] reported that there is no ortholog of the *OsMADS32* gene in *Arabidopsis*, and confirmed that the *OsMADS32* transcripts were only restricted to the early stages of panicle and seed development. Taken together, this suggests that the *OsMADS32*-subgroup genes are monocot-specific genes that play a role in regulating floral and seed development.

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Supporting Information

Figure S1 Predicted coding region and deduced amino acid sequences of *OsMADS32* in the mutant (*tri1*) and the corresponding wild type (*TRI1*).

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