

Linking differential domain functions of the GS3 protein to natural variation of grain size in rice

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Grain yield in many cereal crops is largely determined by grain size. Here we report the genetic and molecular characterization of *GS3*, a major quantitative trait locus for grain size. It functions as a negative regulator of grain size and organ size. The wild-type isoform is composed of four putative domains: a plant-specific organ size regulation (OSR) domain in the N terminus, a transmembrane domain, a tumor necrosis factor receptor/nerve growth factor receptor (TNFR/NGFR) family cysteine-rich domain, and a von Willebrand factor type C (VWFC) in the C terminus. These domains function differentially in grain size regulation. The OSR domain is both necessary and sufficient for functioning as a negative regulator. The wild-type allele corresponds to medium grain. Loss of function of OSR results in long grain. The C-terminal TNFR/NGFR and VWFC domains show an inhibitory effect on the OSR function; loss-of-function mutations of these domains produced very short grain. This study linked the functional domains of the *GS3* protein to natural variation of grain size in rice.

grain weight | *Oryza sativa* L. | protein domain | yield

In recent years, genes for grain and fruit sizes have been isolated from several plant species (1–8), providing opportunities for understanding genetic and molecular mechanisms regulating these traits. In rice, yield per plant is determined by three component traits: number of panicles (tillers) per plant, number of grains per panicle, and grain weight. Extensive genome mapping studies have identified hundreds of QTLs (quantitative trait loci) for yield traits (9). Although a number of QTLs for tillering (10), grain number (11, 12), grain size (8, 13–15), and panicle size and plant architecture (16–18) have been cloned, molecular characterization of these and many more genes is needed to understand the genetic and molecular bases of yield (9).

A major QTL for grain size (*GS3*) in rice was previously identified on chromosome 3 in a number of studies across different genetic backgrounds and environments (19–21). Fan et al. (8) identified the candidate gene for *GS3*. By comparative sequencing analysis, they found a nonsense mutation in the second exon of the putative *GS3* shared among all of the large-grain varieties tested in comparison with varieties with smaller grains. This mutation caused a 178-aa truncation in the C terminus of the predicted protein, which was widespread in the global rice germplasm collections (22, 23), indicating that this mutation had an ancient origin and played an important role in grain size variation and domestication of the cultivated rice.

Here we report the genetic and molecular analysis of *GS3*, which revealed several important structural and functional features of the *GS3* protein in grain size regulation.

Results

Validation of *GS3* on Grain Size Regulation. We validated the effect of *GS3* on grain size by transformation. A construct CT9.8 (Fig. S1A), containing a 9.8-kb genomic DNA fragment encompassing *GS3* amplified by PCR from rice cultivar Chuan 7 (short grain) in the binary vector pCAMBIA1301, was transformed into rice cultivar Minghui 63 (long grain). Thirty or more seeds were produced

from 19 independent T₀ transformants, and shorter and slightly wider grains were observed in all of the 19 plants (Fig. 1A). We then field-examined two T₁ families randomly selected from T₀ plants with single-copy transferred DNA insertion confirmed by Southern blot and observed perfect cosegregation between the transgene and the phenotype. All transgene-positive plants produced shorter and slightly wider grains than the negative segregants (Fig. S1B–D). We further field-tested T₃ homozygous lines derived from three independent single-copy transformants. Compared with the wild-type Minghui 63, all of the transgenic lines showed a highly significant decrease in grain length, together with a slight but significant increase in grain width, resulting in a large reduction in grain weight (Table 1).

We also used RNA-interference (RNAi) to suppress the expression of *GS3* in Chuan 7 (Fig. S1A). Because it is very difficult to transform Chuan 7 (an *indica* cultivar), we obtained only two positive transgenic plants, both of which showed significantly increased grain length (Fig. 1B), without obvious change in plant morphology. One of the two T₁ families was analyzed for cosegregation between the grain traits and the transgene (Fig. S1E and Table S1). The grain length of the positive segregants was longer than that of negative plants. The correlation between grain length and *GS3* expression level was -0.78 , significant at $P < 0.01$ ($n = 14$). In addition, the grain weight of the positive plants also significantly increased compared with the negative segregants.

Overexpression of *GS3* Conferred Pleiotropic Effects to Rice Plants.

To investigate possible effect of *GS3* at the whole-plant level, we overexpressed the gene by transforming Minghui 63 with a construct 35SFL (Fig. S1A) containing the full-length cDNA (fl-cDNA, Osigcea013f09t3) of *GS3* from the plumule of an *indica* rice cultivar, Guangluai 4 (medium grain) (8). Twenty-three independent transformants were obtained, each with 30 or more seeds. Overexpressing *GS3* greatly reduced plant size, including shortened height, leaves, and panicles, in addition to short grains (Fig. 1C–F). We field-tested two T₁ families randomly selected from T₀ plants. All of the positive segregants showed reduced grain length compared with negative segregants (Fig. S1F–H). Two homozygous transgenic lines were further compared with the wild-type Minghui 63 for yield traits by field test. The transgenic lines showed a large reduction in grain length and weight, as well as number of grains per panicle, with a significant increase in tillers, whereas *GS3* overexpression did not affect flowering time (Table S2).

Plant-Specific Unique Domain in the *GS3* Protein. The *GS3* protein was previously predicted as consisting of four putative domains

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Fig. 1. Effect of *GS3* on grain size and plant morphology exhibited by transgenics. (A–C) Grains of positive (Left) and negative (Right) segregants from T_1 family by (A) transforming Minghui 63 with CT9.8 containing the genomic fragment of Chuan 7; (B) transforming Chuan 7 with the RNAi-*GS3* construct; and (C) overexpressing *GS3* using the 35SFL construct. (D–F) Positive (Left) and negative (Right) segregants from T_1 family overexpressing *GS3* using the 35SFL construct: (D) whole plants; (E) panicles and flag leaves; and (F) main culms, with arrowheads indicating the internodes.

(8): a phosphatidylethanolamine-binding protein (PEBP)-like domain of 54 aa at the N terminus, a transmembrane region at amino acids 97–117, a TNFR (tumor necrosis factor receptor)/NGFR (nerve growth factor receptor) family cysteine-rich domain at sites 116–155, and a von Willebrand factor type C (VWFC) 60–80 aa in length in the C-terminal region. Later on, however, the PEBP domain was no longer predicted by the same analysis of the *GS3* sequence (<http://www.ebi.ac.uk/Tools/InterProScan/>), whereas the other domains still remained. Moreover, *GS3* was not identified in the recent analyses of rice PEBP protein family by other groups (24, 25). We performed an alignment analysis of the previously identified putative PEBP domain of *GS3* with the 19 proteins identified as PEBP family members (24) and found that the putative *GS3* PEBP was only approximately one third of the length of the predicted PEBP domain in those proteins, sharing poor similarity (20.3–28.4%) in the PEBP region. Thus the putative PEBP domain is not found in *GS3*.

We searched databases for homologous sequences of the *GS3* N-terminal region and found an N-terminal motif 66 aa in length (sites 7–72) of *GS3* not reported as a conserved domain in the literature but sharing high similarity with the N-terminal regions of proteins from a number of plants, ranging from angiosperm to

gymnosperm (Fig. S2), including *DEP1* identified as a gene for panicle architecture in rice (17). Interestingly, a *GS3* ortholog in maize was found to be involved in kernel development (26). *GS3* exhibited the highest similarity with *Zea mays* *ZmGS3-1* (95.8%) and the lowest with *Arabidopsis* *AtGS3* (49%) in this region. However, this domain was not found in animals or other organisms, thus is highly likely plant specific. To reflect the function of this protein domain (see below), we tentatively named it the organ size regulation (OSR) domain.

Unique Allele from Chuan 7 Had a Strong Function in Grain Size Regulation.

The grains of Chuan 7 are lighter than other varieties, primarily because of shorter length. For example, the 1,000-grain weight of Minghui 63 is usually 26–28 g, and that of Zhenshan 97 is 22–24 g, whereas 1,000 grains of Chuan 7 only weigh 12.5 g (8, 19, 20, 27). Grains of transgenic plants harboring the Chuan 7 genomic segment (CT9.8) in Minghui 63 background obtained in this study were also smaller than those of Zhenshan 97, although larger than Chuan 7 (Table 1 and Table S3). Such grain size differences indicate that *GS3* encoded by Chuan 7 may be a stronger allele than that of Zhenshan 97 in negatively regulating grain size. We thus conducted comparative sequencing of the entire *GS3* region of several varieties (Fig. 2): Minghui 63 (long grain), Zhenshan 97 and Nipponbare (medium grain), and Chuan 7 (short grain). The predicted protein of Zhenshan 97, whose coding region was identical to Guangluai 4, was 231 aa in length. Relative to Zhenshan 97, Minghui 63 had a C→A substitution at 165 bp downstream from the predicted translation start site (ATG), causing premature termination of the predicted protein and resulting in long grain, which was also observed in several studies (8, 22, 23). Chuan 7 had a 1-bp deletion at 357 bp (C) downstream from the predicted translation start site, which caused a frameshift mutation in the C terminus that yielded a truncated 149-aa protein (Fig. 2). Interestingly, Nipponbare had a 3-bp (TCC) insertion at the same site, resulting in an in-frame addition of a serine residue. Therefore, *GS3* has at least four different alleles according to the coding sequence: *GS3-1* (Zhenshan 97), *GS3-2* (Nipponbare), *GS3-3* (Minghui 63), and *GS3-4* (Chuan 7).

To investigate the effects of these variants and their abundance in the rice germplasm, we sequenced the *GS3* genomic region and measured grain length for a total of 82 accessions, mostly from a core collection (28) of the Chinese cultivated rice germplasm (Table S3). Eleven of the 82 accessions contained the C→A substitution as in Minghui 63; all of them produced long grain. The remaining 70 accessions (with Chuan 7 excluded) were resolved into two distinct groups using STRUCTURE analysis (29) based on data of 16 simple sequence repeat markers, essentially an *indica* group and a *japonica* group (Table S3). There was no significant difference in grain length between the two genotypes (TCC insertion/deletion) within the groups (Table S4), suggesting that it is unlikely that this insertion/deletion has a large effect on grain size, although the numbers of accessions were small in two of the classes.

Table 1. Comparison of grain traits of three independent homozygous transgenic lines with Minghui 63

Trait	No. of plants	Minghui 63	C6	C10	C15
Grain length (mm)	10	9.93 ± 0.03	7.69 ± 0.03	7.61 ± 0.05	7.83 ± 0.05
<i>P</i>			1.09 × 10 ⁻²¹	1.92 × 10 ⁻¹⁹	2.31 × 10 ⁻¹⁹
Grain width (mm)	10	2.87 ± 0.01	3.09 ± 0.01	3.09 ± 0.02	3.07 ± 0.02
<i>P</i>			1.71 × 10 ⁻¹¹	3.72 × 10 ⁻¹⁰	2.51 × 10 ⁻⁹
1,000-grain weight (g)	30	25.73 ± 0.15	18.38 ± 0.15	18.24 ± 0.25	19.85 ± 0.10
<i>P</i>			2.47 × 10 ⁻⁴¹	7.50 × 10 ⁻³⁴	4.73 × 10 ⁻⁴⁰

All data are given as mean ± SEM. Each *P* value for each trait was obtained from a *t* test between Minghui 63 and the transgenic line.

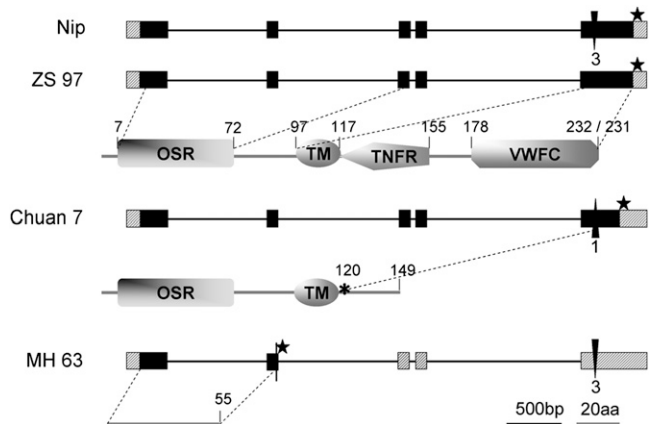


Fig. 2. Comparison of the genomic DNA and predicted protein sequences of *GS3* in Zhenshan 97 (ZS97, *GS3-1*), Nipponbare (Nip, *GS3-2*), Minghui 63 (MH63, *GS3-3*), and Chuan 7 (*GS3-4*), relative to ZS97. Genomic DNA: hatched boxes represent 5' and 3' UTR, and five exons are indicated by black boxes. The thin lines between black boxes represent introns. Black triangles represent a 3-bp insertion (Nip) and a 1-bp deletion (Chuan 7). Stop codons are marked by stars. Protein structure (underneath each genomic DNA fragment): the domains encoded by Nip/ZS97 allele are shown, with the numbers indicating the positions of the domains. The 1-bp deletion in Chuan 7 causes frameshift from site 120 (marked with asterisk), resulting in the loss of TNFR and VWFC domains. The putative protein encoded by Minghui 63 allele contains no functional domain because of premature termination.

The *GS3-4* allele encoded by Chuan 7 occurred only once in the 82 accessions (Table S3). It was neither found with additional sequences of 60 accessions of *Oryza rufipogon* and *Oryza nivara* from Bangladesh, China, India and Myanmar, Nepal, Sri Lanka, and Thailand, nor observed among the 54 diverse accessions published previously (22). Database searching of the predicted polypeptide sequence of the frameshift C terminus found no match with any conserved motif.

We evaluated the effects of these alleles with near isogenic lines (NILs) in the background of Minghui 63: NIL(zs97) carrying an introgressed *GS3-1* from Zhenshan 97, and NIL(c7) with the *GS3-4* from Chuan 7 (Fig. S3A). NIL(zs97) was obtained by three successive backcrosses, using Minghui 63 as the recurrent parent, with the F₁ from a cross between Minghui 63 and RIL236, a recombinant inbred line containing 55% of Minghui 63 background from a cross between Zhenshan 97 and Minghui 63. NIL(c7) was obtained by six backcrosses, using Minghui 63 as the recurrent parent, with the F₁ from a cross between Minghui 63 and Chuan 7. The two NILs were crossed, and self-pollination of the F₁ produced three genotypes: NIL(c7), NIL(zs97), and NIL(het). As expected, NIL(c7) produced the shortest grains, and grains of NIL(zs97) were also significantly shorter than Minghui 63 (Table S5). Grain length of the heterozygote, NIL(het), was not significantly different from the mean of the two homozygotes (Fig. S3B and Table S5), indicating that there is no genetically dominant-recessive relation between the two alleles. Grain weight essentially followed the same trend, which was also reflected in grain yield per plant. Small but statistically detectable differences were observed among the four genotypes in number of grains per panicle and number of tillers per plant (Table S5).

Expression Pattern of *GS3*. RNA in situ hybridization showed that *GS3* was highly expressed in young panicle, and the signal gradually decreased with panicle development (Fig. 3A–C). Weak signals were observed in other tested tissues, including embryo, shoot apical meristem, leaf, and stem (Fig. 3E–H). Interestingly, *GS3* was also highly expressed in root tips (Fig. 3D). Real-time PCR analysis of Minghui 63 and NIL(c7) showed that *GS3*

mRNA preferentially accumulated in panicles less than 5 cm in length in both genotypes, whereas the transcript level was low in other tissues assayed (Fig. 3J). This observation was consistent with the result from in situ hybridization analysis. These suggested that the expression level and pattern were not the cause of the loss of function of *GS3* in Minghui 63.

Differential Functions of the Domains. The fact that Minghui 63 with premature termination in the OSR domain produced long grain, whereas the frameshift mutant lacking TNFR and VWFC domains produced the shortest grain, suggested that these domains may exert antagonistic effects in grain size regulation. To investigate the roles of these domains in regulating grain size, seven constructs were made by domain deletions (Fig. 4A), in which the coding sequences were driven by the *GS3* native promoter from Chuan 7 and again delivered to Minghui 63. On the basis of grain length of field-planted T₁ plants (Fig. 4B and Table 2), it is clear that the OSR domain was both necessary and sufficient for reducing grain size; all of the constructs with this domain produced significantly shorter grains, whereas constructs without this domain did not significantly affect grain size. The construct of fl-cDNA (*GS3-1*) had the mildest effect on grain size reduction (9.5%), whereas the one (OM) with only the OSR domain had the strongest effect (29.1%), even larger than that of C7 (24.5%). Thus the TNFR and VWFC domains could to some extent inhibit the effect of OSR in grain size reduction. Moreover, the frameshift C terminus encoded by *GS3-4* allele also seemed to have some effect on grain size regulation. Comparison of numerical values of size reduction indicated that the VWFC domain seemed to have a larger effect of inhibiting OSR than did TNFR and that these two domains added to each other in regulating OSR for grain size.

To further evaluate the effect of the VWFC domain, we overexpressed a truncated cDNA sequence (OMT) with the

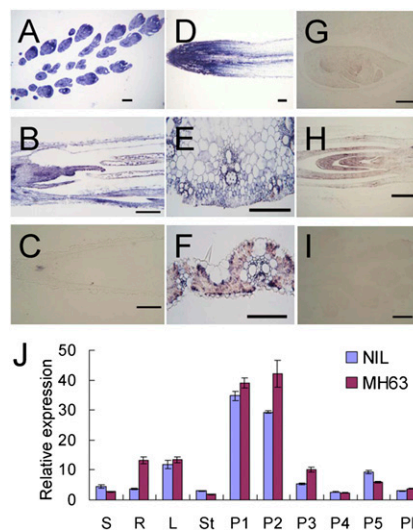


Fig. 3. Expression pattern of *GS3*. (A–H) Expression of *GS3* in various tissues revealed by in situ hybridization: (A) panicle at stage of pollen mother cell formation, (B) panicle at pollen mother cell meiosis, (C) panicle at pollen grain filling, (D) root at trefoil stage, (E) stem at heading stage, (F) leaf at tillering stage, (G) endosperm and embryo 14 d after flowering, and (H) longitudinal section of 7-d-old seedling. (I) Negative control by hybridizing the young panicle with the sense probe. All tissues were sampled from Zhenshan 97 (medium grain). (Scale bars, 200 μ m.) (J) Comparative expression pattern of *GS3* in Minghui 63 and NIL(c7) in various organs using real-time RT-PCR analysis: S, seedling at trefoil stage; R, root at trefoil stage; L, leaf at tillering stage; St, stem at heading stage; P1, young panicle <1 cm in length; P2, young panicle <5 cm in length; P3, young panicle 5–10 cm in length; P4, panicle before heading >10 cm in length; P5, panicle 5 d after heading; PI, plumule.

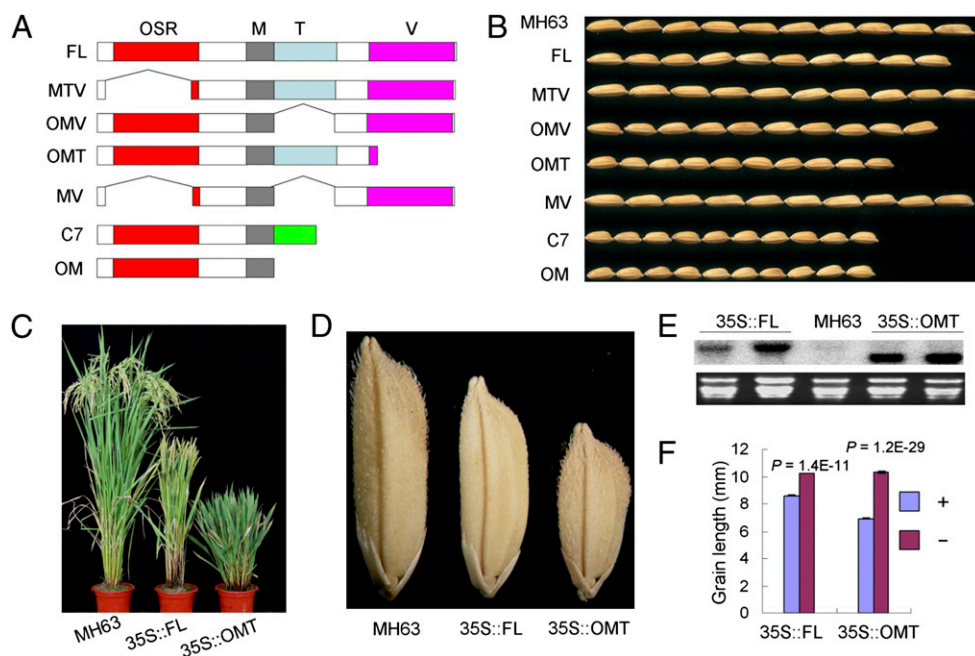


Fig. 4. Effects on grain size of the various domains assessed using transgenics. (A) Schematic representation of the coding sequences of the constructs with one or more domains deleted; (B) grains of the T₁ generation transgenic plants compared with Minghui 63. OSR, organ size regulation domain; M, putative transmembrane domain; T, TNFR/NGRF domain; V, VWFC domain. Green box represents the polypeptide sequence resulting from frameshift. (C and D) Effect of VWFC in inhibiting OSR in regulating growth of plant and grain size. From left to right: Minghui 63, transgenic plants overexpressing the fl-cDNA (FL), or the truncated cDNA (OMT). (E) Northern blot analysis of *GS3* transcripts in wild-type plants and in two positive segregants from respective T₁ generations. (F) Grain length of T₁ plants overexpressing fl-cDNA (FL) or the truncated cDNA (OMT). + and -, positive and negative segregants. Data are given as mean \pm SEM. Student's *t* test was used to generate the *P* values.

VWFC domain deleted. Transformants produced much shorter grains and highly dwarfed plants compared with those overexpressing the fl-cDNA (Fig. 4 C–F), providing further evidence that VWFC has a general role of inhibiting the effect of OSR in regulating plant growth and grain size.

Discussion

This study has revealed several structural and functional features of the *GS3* protein in grain size regulation. First, the plant-specific domain OSR identified in the N terminus is the most important one, which is both necessary and sufficient for *GS3* to function as a negative regulator of grain size as well as organ size. Loss of function or deletion of this domain would result in long grain. Second, there is an intramolecular interaction between the OSR domain and the C-terminal domains, TNFR/NGFR and VWFC. The C-terminal domains inhibit the function of OSR on grain and organ size reduction, independently of tissues or cell types. This phenomenon is similar to previously reported autoregulation in

the plant steroid receptor *BRI1* (30) and autoinhibition in the human platelet-derived growth factor β -receptor (31), both of which were membrane proteins and whose functions involved kinase activity. However, the biochemical function of the *GS3* protein remains to be determined.

Because grain size has been the target of both natural selection and breeding (32), the *GS3* locus provides an informative system for studying the evolutionary processes underlying rice domestication and breeding. The *GS3-1* allele is probably the prototype and corresponds to medium grain length, which is the genotype for the majority of widely grown medium grain *indica* varieties (e.g., Zhenshan 97). Relative to *GS3-1*, an in-frame insertion of 3 bp (*GS3-2*) in the fifth exon did not change the grain length, which is the case for most of the temperate *japonica* varieties (e.g., Nipponbare). One base pair deletion at the same site caused frameshift of the C-terminal domains of the protein and thus generated an allele (*GS3-4*) with very strong function as a negative regulator producing small grain. However, this allele is

Table 2. Grain length of transgene-positive and transgene-negative segregants in one of the T₁ families for each construct

Constructs	Positive (mm)	Negative (mm)	<i>P</i>	Reduction (%)*
C7	7.35 \pm 0.07 (<i>n</i> = 16)	9.74 \pm 0.03 (<i>n</i> = 14)	1.48 \times 10 ⁻²²	24.5
FL	9.05 \pm 0.02 (<i>n</i> = 23)	10.00 \pm 0.04 (<i>n</i> = 7)	1.12 \times 10 ⁻¹⁸	9.5
OMT	7.96 \pm 0.03 (<i>n</i> = 21)	9.95 \pm 0.06 (<i>n</i> = 8)	8.83 \times 10 ⁻²³	20.0
OMV	8.77 \pm 0.03 (<i>n</i> = 20)	10.23 \pm 0.04 (<i>n</i> = 9)	1.72 \times 10 ⁻²¹	14.3
OM	7.12 \pm 0.07 (<i>n</i> = 25)	10.05 \pm 0.10 (<i>n</i> = 5)	5.50 \times 10 ⁻¹⁷	29.1
MTV	9.66 \pm 0.04 (<i>n</i> = 21)	9.77 \pm 0.03 (<i>n</i> = 9)	0.0520	
MV	9.79 \pm 0.05 (<i>n</i> = 23)	9.87 \pm 0.08 (<i>n</i> = 11)	0.1656	

All data are given as mean \pm SEM. *P* values were obtained by *t* tests between the positive and negative plants in the same family.

*Extent of grain length reduction of the transgene positive plants relative to the negative plants.

extremely rare in rice germplasm collections, indicating that such small grain has not been favored by natural selection or artificial breeding. In contrast, premature termination of the OSR domain by a single base substitution in the second exon greatly increased grain length, which is the case for long grain varieties widely cultivated in the world, suggesting that this allele has been highly favored in breeding programs. These results are directly useful in breeding programs to modify grain size and increase grain yield using both molecular marker-assisted selection and genetic transformation techniques (33, 34).

Methods

Field Growth of Rice Plants. The rice plants examined under natural field conditions were grown in normal rice growing seasons at the Experimental Station of Huazhong Agricultural University, Wuhan, China. Seeds were planted in a seed bed in mid-May and transplanted to the field in mid-June. The planting density was 16.5 cm between plants in a row, and the rows were 26 cm apart. Field management, including irrigation, fertilizer application, and pest control, followed essentially the normal agricultural practice.

Measurements of Grain Traits. Harvested paddy rice was air-dried and stored at room temperature before testing. Ten randomly chosen, fully filled grains from each plant were lined up length-wise along a vernier caliper to measure grain length and then arranged by breadth to measure grain width. Grain weight was calculated on the basis of 200 grains and converted to 1,000-grain weight.

Generation of Constructs and Transformation. For preparing the *GS3* construct of the genomic DNA from Chuan 7, a 9,868-bp fragment spanning from 2,780 bp upstream of the translation start codon to 1,371 bp downstream of the termination codon of *GS3* was amplified by two-step PCR from rice cultivar Chuan 7 with two pairs of primer, 1512f-9247r and U9EcoRI-RBP (Table S6). The overlapped region of the two PCR products contained an *EcoRI* site, which was used to cut and ligate the two fragments. The fragment was cloned into the binary vector pCAMBIA1301.

Two cDNA clones were used for the overexpression constructs. The first was the fl-cDNA (*Osigcea013f09t3*) from the plumule of Guangluai 4, cloned in pBluescript SK2 (Stratagene). The second was the fl-cDNA with 3'-deletion prepared by PCR from the full-length cDNA with primers F1Sacl and VWFKcpnl (Table S6). These two clones were digested with *SacI* and *KpnI* and inserted into pCAMBIA1301s under the control of the Cauliflower mosaic virus CaMV-35S promoter.

To construct an RNAi vector for *GS3*, a 790-bp fragment of *GS3* was digested with *BamHI* and *KpnI* from the above-mentioned fl-cDNA clone

and inserted into the pDS1301 vector, which was a modified version of pCAMBIA1301 (35).

All of these constructs were independently introduced into the *Agrobacterium* strain EHA105, and transformation was done as described previously (36).

DNA Blot Analysis. The experimental procedures for Southern blot analysis, including DNA extraction, enzyme digestion, electrophoresis, and hybridization, were essentially as described previously (37). The PCR product of *GUS* (β -glucuronidase) gene using GUS1.6F and GUS1.6R as primers was purified and prepared as probe for Southern blot analysis.

Expression Analyses. We isolated total RNA from the tissues/organs using an RNA extraction kit (TRIZOL reagent; Invitrogen) according to the manufacturer's instructions. For quantitative real-time PCR, approximately 3 μ g total RNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen) in a volume of 100 μ L to obtain cDNA. A pair of primers, G53F and G53R, was used to amplify the transcript of *GS3*, with Actin1F and Actin1R for β -Actin1 as the internal control (Table S6). We carried out quantitative real-time PCR in a total volume of 25 μ L containing 2 μ L of the reverse-transcribed product above, 0.25 mM gene-specific primers, and 12.5 μ L SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions. The measurements were obtained using the relative quantification method (38).

For Northern blot analysis, 15 μ g of total RNA from leaves was separated on 1.2% (wt/vol) denaturing agarose gel and transferred to nylon membrane. A *GS3* fragment amplified with gene-specific primers CFPXbaIF and F-ExHindIIIIR (Table S6) was labeled with 32 P-dCTP using Random Primer kit (Invitrogen) and hybridized to the RNA blots.

In Situ Hybridization. The *GS3* probe was amplified with the full-length cDNA clone as the template using the gene-specific primers F1Sacl and VWFKcpnl (Table S6). A 485-bp PCR fragment was then inserted into the pGEM-T vector (Promega) for RNA transcription. The sense and antisense RNA probes were produced by T7 and SP6 transcriptase labeled with digoxigenin (Roche), respectively. RNA in situ hybridization and immunological detection were done as described previously (39).

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