Genetics and Molecular Research

Haplotypes of *qGL3* and their roles in grain size regulation with *GS3* alleles in rice

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Genet. Mol. Res. 15 (1): gmr.15017587
Received September 3, 2015
Accepted November 5, 2015
Published January 29, 2016
DOI http://dx.doi.org/10.4238/gmr.15017587

ABSTRACT. Grain size is an important trait that directly influences rice yield. The *qGL3* and *GS3* genes are two putative regulators that play a role in grain size determination. A single rare nucleotide substitution (C→A) at position 1092 in exon 10 of *qGL3* might be responsible for variations in grain size. However, little is known about the haplotype variations of *qGL3* and their interactions with *GS3* during the regulation of grain length and grain weight. In this study, *qGL3* haplotype variations were examined in 61 *Indica* varieties, and the effects of *qGL3* and *GS3* on grain trait variation in 110 lines were evaluated. Six *qGL3* haplotypes were identified, and *qGL3*-2 was a major haplotype in *Indica* varieties. Moreover, *qGL3*-6, a reported key single nucleotide polymorphism, was validated. Our results showed that the mutants *qgl3* and *gs3* (loss-of-function mutation types of *qGL3* and *GS3*, respectively) had significant effects on grain length and grain weight. However, no significant effects associated with differences in the regulation of grain thickness were observed. The genetic effects of *qgl3* on grain phenotypes were stronger than those of *gs3*. In addition to increased grain length, *qgl3* had an evident role in grain width increases. In contrast, *gs3*

played an opposite role in grain width regulation. These results provided novel insights into grain size control and the functions of *qgl3* and *gs3* in rice yield improvement.

Key words: Rice; Haplotype; Grain size; *qGL3*; *GS3*

INTRODUCTION

Rice is one of the most important food crops, and it provides daily diet nutrition for more than half of the global population. Therefore, the stability and increase of rice output is vital to the elimination of hunger and the improvement of quality of life. Grain weight is closely associated with rice yield (Xing et al., 2002). Grain shape, as the major determinant of grain weight, is characterized by a combination of four parameters: grain length, grain width, grain thickness, and filling degree (Xing and Zhang, 2010; Huang et al., 2013; Ikeda et al., 2013; Zuo and Li, 2014). In recent years, several key genes that regulate grain shape were identified (Fan et al., 2006; Shomura et al., 2008; Weng et al., 2008; Li et al., 2011; Zhang et al., 2012; Ishimaru et al., 2013; Song et al., 2007, 2015; Wang et al., 2012, 2015a,b). For instance, *qGL3*/*qGL3*.1 is a newly identified QTL associated with grain length, which exhibited the highest logarithm of the odds value and the greatest phenotypic variation compared to other markers, and it also significantly contributed to grain thickness and grain width (Hu et al., 2012; Qi et al., 2012; Zhang et al., 2012). A comparison of functional and non-functional sequences revealed a single nucleotide substitution (C→A) at position 1092 in exon 10, resulting in the replacement of *Asp* (D) (a non-functional allele) with *Glu* (E) (a functional allele) at the 364th amino acid. *qGL3*/*qGL3*.1 encodes a putative protein phosphatase with a Kelch-like repeat domain (OsPPKL1), which directly dephosphorylates its substrate (CyclinT1,3) to regulate cell division (Qi et al., 2012). Furthermore, genetic and transgenic studies revealed that *qGL3*/*qGL3*.1 acts as a negative regulator of grain length. The discovery of functional single nucleotide polymorphisms (SNPs) in *qGL3* was expected to improve selection for grain weight in rice-breeding programs.

Previous results indicated that *GS3* was a negative regulator of grain size, and that it had major effects on grain length and weight as well as minor effects on grain width and thickness (Fan et al., 2006). The complete GS3 protein contains a plant-specific organ size regulation (OSR) domain at the N-terminus, a transmembrane domain, and both a tumor necrosis factor receptor/nerve growth factor receptor family cysteine-rich domain and a von Willebrand factor type C at the C-terminus (Mao et al., 2010). The OSR domain is the key negative regulator of grain length, whereas the two C-terminus domains have inhibitory effects on OSR function. A *GS3* allele, characterized by a C→A substitution at 165 bp, which caused premature termination of the predicted protein, resulted in the complete loss of the functional protein domain. Moreover, this allele is common in rice with long grains (Mao et al., 2010).

Both *qGL3* sequence variations and functional relevance have been explored, but the information remains obscure. In addition, the interactions with *GS3*, a similar gene that regulates grain length, have not been investigated. In this study, *qGL3* haplotypes in 61 *Indica* rice varieties were identified, and the phenotypes of 110 lines with different *qGL3* and *GS3* genotypes were analyzed. Our study validated the reported SNPs and insertions and deletions (indels) in *qGL3*, identified additional SNPs in the functional domain, and evaluated the genetic effects of *qGL3* in combination with different *GS3* genotypes. Our findings provided insight into the control of grain length in rice and will be of value for the improvement of rice grain yield.

MATERIAL AND METHODS

Plant material and phenotypic data collection

A total of 61 *Indica* varieties were examined, including 49 *Oryza sativa* L. accessions containing a wide range of *Indica* (mainly from China), six rice varieties from the International Rice Research Institute (IRRI), and four varieties from other countries. Most varieties were developed through crossbreeding, and some were landraces (**Table S1**). A total of 110 inbred lines, which were developed from a cross between TD70 (a long grain *Japonica* variety carrying *gs3* and *qgl3* loss-of-function mutations) and Kasalath (a short grain *Indica* variety carrying *GS3* and *qGL3* wild-type alleles) were used to study the combined effects of *qgl3* and *gs3*. In 2013, the 61 varieties and 110 lines were grown within two replicated plots in the experimental field located at the Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, China. Seeds were immediately harvested once they developed to maturation, and they were then wind-dried for 30 days to maintain a water content of approximately 14%. The grain length and grain width of 10 full seeds were measured using vernier calipers, and the mean values were recorded. The total weight of 100 grains from each plant was measured and converted to its 1000-grain weight using an electronic balance (measured to the nearest 0.0001 g).

DNA extraction, PCR, and sequencing

The fresh young leaves of growing plants from each rice variety were harvested, and the cetyl-trimethyl ammonium bromide method was used to extract genomic DNA. The *qGL3* sequences were downloaded from the National Center for Biotechnology Information (accession Nos. EF447275 and AK288069). Primer sets, based on the downloaded sequences, were designed to amplify genomic DNA regions containing all *qGL3* exons and introns, and Prime STAR HS DNA polymerase (TaKaRa-Bio Inc.) was used. The following primers in Table 1 were used in this study.

Table 1. Primer sequences used for *qGL3* gene cloning.

Primer name	Sequence
qGL3g1R2	5'-CCTCCATTCGTATGGCTT-3'
qGL3g2F	5'-TGATGGTAAGCAATTGCTCC-3'
qGL3g2R	5'-TCCTGGGAGTTGTGACAAC-3'
qGL3g3F	5'-CTCATCACTCTGTGATGACATTG-3'
qGL3g3F2	5'-GAGGTATAGGTAATTGTTGGTTTCC-3'
qGL3g3R	5'-TCTTAACAGGAGCTTTAAGTTGC-3'
qGL3g3R2	5'-AACTCACGCAATGTCTCCTG-3'
qGL3g4F	5'-CTGCTGCGATACTTATCAGGT-3'
qGL3g4R	5'-TGTTACCGTGTTACGAGGAAGG-3'
qGL3g5F	5'-CACAGGTACTGCAAATAATGC-3'
qGL3g5R	5'-TTCGGAGCCAAACAAGATAT-3'

PCR was performed using a standard three-step protocol with 2X PrimeSTAR GC buffer. PCR products were purified using an Axyprep DNA gel extraction kit (Axygen), and were then sequenced on an ABI 377 sequencer using the same primer sets and a BigDye Terminator Cycle sequencing kit. The gene sequences were assembled, and the consensus sequences were used for analyses. Sequences were aligned using CLUSTALX 2.1 (Larkin et al., 2007), and the results were saved in the TASSEL input format. SNPs and indels were detected using TASSEL 4.0 (Bradbury et al., 2007).

Functional markers for the identification of *qgl3* and *gs3*

The *qGL3*-derived cleaved amplified polymorphic sequence (dCAPS) markers resulted in wild-type products that were cleaved into two fragments (21 and 84 bp) via endonuclease *Acc*I digestion, whereas those associated with the mutants exhibited a single 105-bp fragment. After PCR, the *GS3* dCAPS marker products were specifically cleaved using *Pst*I. The mutant produced a 168-bp fragment, and the wild type produced 110- and 58-bp fragments (Zhang et al., 2015). The following *qGL3* and *GS3* primer pairs were used: *qGL3*-F: 5'-GATTCTATCTGGTTCAGTGGTAGA-3'and *qGL3*-R: 5'-CCTGCTGCATCTGCACTATAT-3'; *GS3*-F: 5'-CCCATCTCCCTCGTTTACTT-3' and *GS3*-R: 5'-GTAAAGACGAGAAGAAATGG-3'. The PCR conditions were as follows: 4 min at 94°C; 33 cycles at 94°C for 30 s, 49°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min.

RESULTS

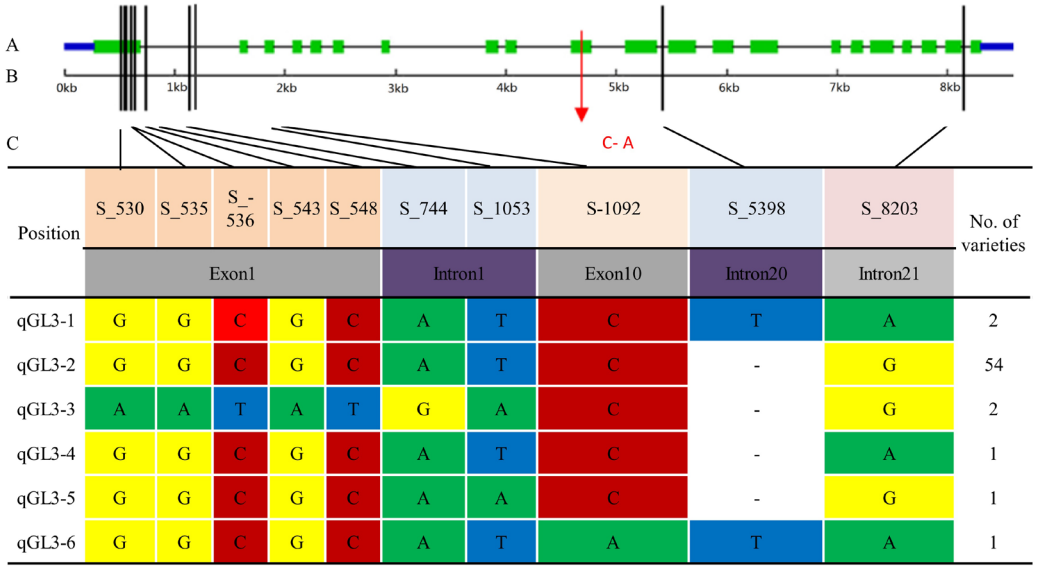
qGL3 haplotypes

qGL3 was recently identified as a regulator of grain length, and analyses of its gene structure, SNPs, and indels indicated that the variations were conserved in exons and introns (Figure 1A). Only eight SNPs and one indel in 8047 bp were newly identified in the 61 rice varieties, despite the presence of 21 exons and 20 introns (Figure 1B). There was only one SNP for every 1 kb, and 87.5% of the SNPs were found in exon 1 and intron 1. A total of 54 rice varieties had the same sequences identified as *qGL3*-2, and only seven accessions shared the other five haplotypes (Figure 1C). A single nucleotide substitution (C→A) was identified as the replacement of arginine with glycine. The S_1092 SNP, which was the same as that found in the extra-large grained *Japonica* N411 variety (Zhang et al., 2012), was identified as *qGL3*-6 in this study (Figure 1). Five SNPs in the first exon caused two amino acid changes in *qGL3*-3, including S_535 and S_536 (cysteine to tyrosine) and S_543 (glycine to arginine) (Figure 1). The other two differences in exon 1, the S_530 and S_548 SNPs, did not cause amino acid residue changes. By analyzing the phenotypes of the two varieties with the two amino acid changes, we found that there was no significant phenotypic correlation between the two *qGL3*-3 SNPs and grain length (**Table S1**). With the exception of *qGL3*-6, five other *qGL3* haplotypes were short grained, and no significant differences associated with grain length (Figure 2A), width (Figure 2B), or thickness (Figure 2C were observed.

Grain length differences between *qgl3* and *gs3* wild types and mutants

To analyze the phenotypic differences between the *qGL3* mutants and wild types, we divided 61 accessions into two groups. According to the sequencing results, one group included lines containing *qGL3* mutants, and the other group included lines with *qGL3* wild types. Moreover, one group had a single species (TD70) associated with *qGL3*-6, and another group contained 60 species associated with five *qGL3* haplotypes. The average grain length of the wild-type *qGL3* grain was 8.75 mm. Regarding grain length, highly significant differences were found between the *qgl3* mutants and wild types (Figure 3A). Furthermore, significant differences were detected between grain length in 60 accessions carrying the *qGL3* wild type

and those with the *GS3* mutant and wild type (Figure 3B). These accessions were divided into the *GS3* mutant and wild-type varieties based on the *GS3* dCAPS markers (Figure 4A), which resulted in the numbers 29 and 31 in **Table S1**, respectively. To compare the grain length of the *GS3* wild type, the grain length of the *gs3* mutant was measured at 9.71 mm, which indicated a 1.8 mm increase (Figure 3B). This result indicated that the *gs3* mutant had strong genetic effects on grain length. TD70 (S1), which carried *qGL3*-6, was found to contain the *gs3* mutant (Figure 4A and **Table S1**).



Position	S_530	S_535	S_-536	S_543	S_548	S_744	S_1053	S-1092	S_5398	S_8203	No. of varieties
	Exon1					Intron1		Exon10	Intron20	Intron21	
qGL3-1	G	G	C	G	C	A	T	C	T	A	2
qGL3-2	G	G	C	G	C	A	T	C	-	G	54
qGL3-3	A	A	T	A	T	G	A	C	-	G	2
qGL3-4	G	G	C	G	C	A	T	C	-	A	1
qGL3-5	G	G	C	G	C	A	A	C	-	G	1
qGL3-6	G	G	C	G	C	A	T	A	T	A	1

Figure 1. Gene structure, positions of SNPs and indels, and haplotypes of *qGL3*. **A.** *qGL3* gene structure. Blue boxes indicate upstream/downstream, green boxes indicate exons, and black lines represent introns. **B.** Gene sequence and positions of SNPs and indels. The vertical lines of the gene structure indicate the positions of polymorphic sites. **C.** *qGL3* haplotypes.

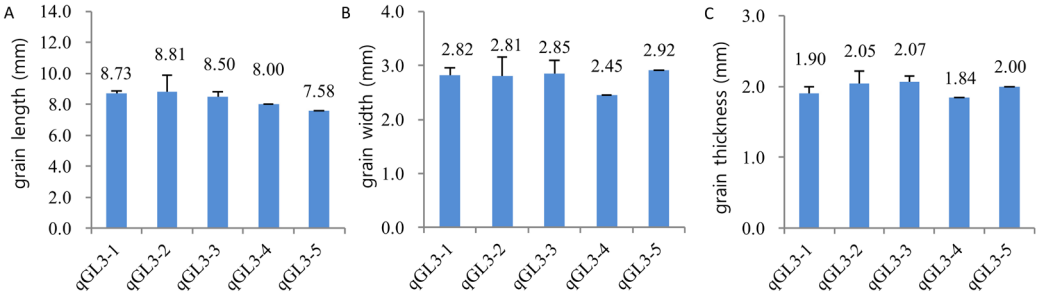


Figure 2. Comparisons of grain phenotypes among varieties carrying different *qGL3* haplotypes. **A.**-**C.** Grain length, grain width, and grain thickness of different haplotypes, respectively.

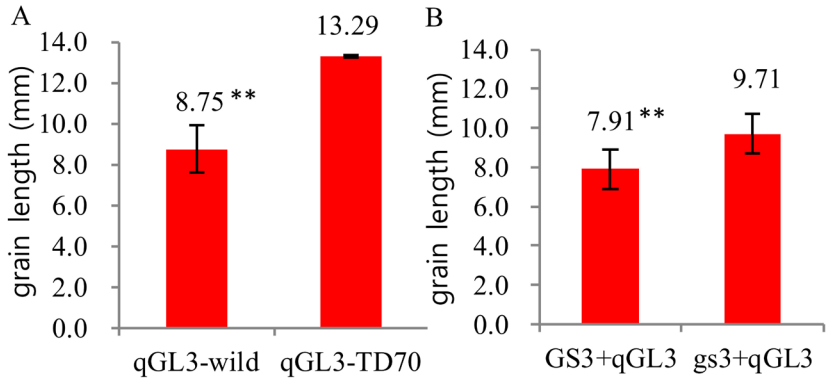


Figure 3. Comparisons of grain length between the mutant and wild-type genotypes of two genes associated with grain length that were found in 61 varieties. **A.** Grain length of *qGl3* and *qgl3*. **B.** Grain length of *GS3* + *qGL3* and *gs3* + *qGL3*. **Significant differences at the 0.01 level.

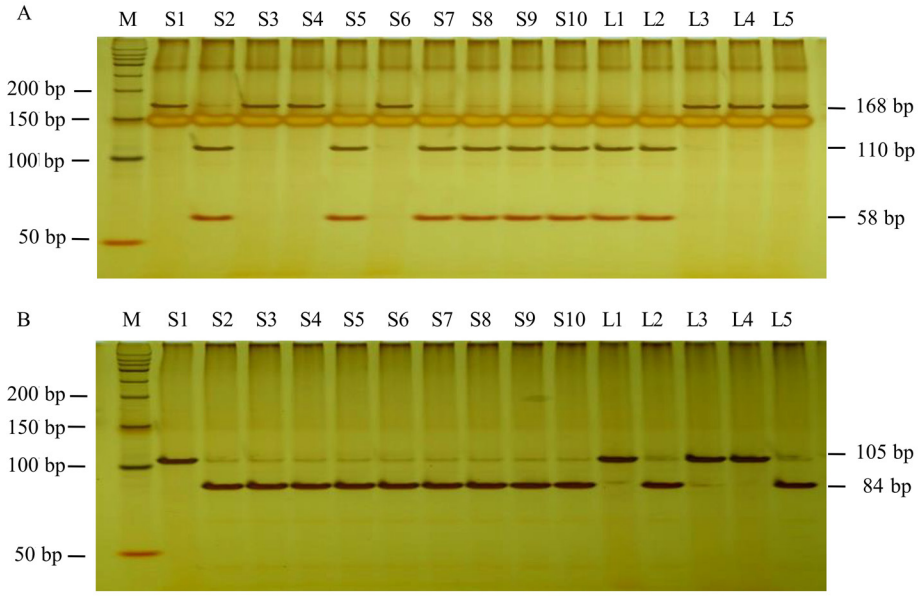


Figure 4. *GS3* PCR amplification and enzyme reaction products. **A.** *qGL3*. **B.** Examples of some of the 61 rice variety samples (S1 to S10) and some RILs lines from S1 and S2. *Lane M*: DNA size marker; *lane S1*: TD70; *lane S2*: Kasalath; *lanes S3* to *S10*: some samples of the 61 rice varieties; *lanes L1* to *L5*: some RIL lines carrying different *GS3* and *qGL3* combinations. A 21-bp fragment is not shown in B, because it was too far from the main fragment.

Effects of *qgl3* and *gs3* combinations

Of the 61 varieties, a variety containing a *qgl3* mutant and a *GS3* wild type was not detected, but a variety containing two mutant genes was detected in the 61 varieties (**Table S1**). To analyze the genetic effects of a *qgl3* mutant, we used the effects of two mutant genes to replace those of the *qgl3* mutant, and this may have magnified its genetic effect because the influence of *gs3* was

not ignored. We used a dCAPS marker for *qGL3* to distinguish the *qGL3* genotype, and the results indicated that only TD70 carried the mutant *qgl3* in 61 accessions (Figure 4B). To accurately assess the combinatory effects of *qGL3* and *GS3*, 110 lines were established (without selection for *qGL3* and *GS3*) from the F_9 generation of the hybridization between TD70 (S1) (a variety carrying two mutant genes) and Kasalath (S2) (a variety carrying two wild-type genes), which were selected to detect the genetic effects of *qgl3* and *gs3* (Figure 4). We divided the 110 lines into the following four classifications based on *qGL3* and *GS3* molecular markers: 62 lines with *qgl3* and *gs3* wild types, 10 lines with one *qgl3* mutant, 28 lines with one *gs3* mutant, and 10 lines with *qgl3* and *gs3* mutants (which contained 62, 10, 28, and 10 lines, respectively). In order to analyze the combined effects of the two genes, the grain length and grain weight phenotypes were investigated in the following genetic groups: *qgl3* + *gs3*, *GS3* + *qgl3*, *gs3* + *qGL3*, and *GS3* + *qGL3* (Figure 5). The grain length of the *qgl3* + *gs3* combination was significantly higher than that of the *gs3* + *qGL3* (+1.23 mm) and *GS3* + *qGL3* (+2.44 mm) combinations (Figure 5A). The ascending order of the grain width phenotypes was *GS3* + *qgl3*, *GS3* + *qGL3*, *qgl3* + *gs3*, and *gs3* + *qGL3*, but a significant difference was not detected (Figure 5B). Furthermore, the same results were found following the analysis of grain thickness (Figure 5C). The results of comparisons of the grain weight phenotypes of lines carrying the *GS3* + *qGL3* genotype combinations (*gs3* + *qGL3*, *qgl3* + *GS3*, and *qgl3* + *gs3*) indicated increases of 4.02, 7.76, and 8.92 g, respectively (Figure 5D). The single mutants, *qgl3* (*qgl3* + *GS3*) and *gs3* (*qGL3* + *gs3*), had significant effects on the regulation of grain length and grain weight, but no significant differences associated with the regulation of grain thickness were detected between the two mutants. The genetic effects of *qgl3* on grain traits were greater than that of *gs3*. In addition to the role of increasing grain length, the *qgl3* mutant played a specific role in increasing grain width. In contrast, the *gs3* mutant had partially opposite effects on grain width.

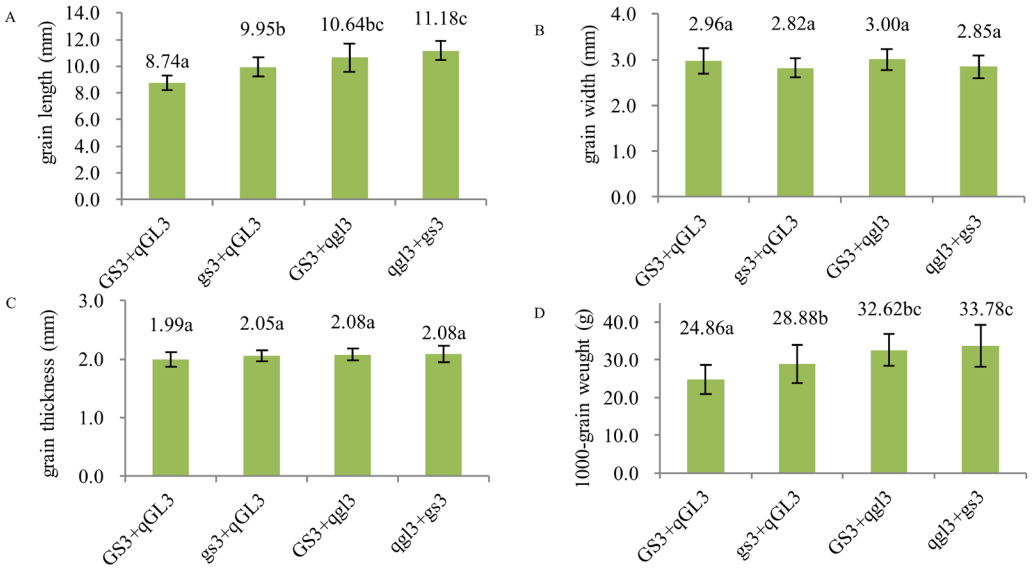


Figure 5. Comparisons of grain traits among different genotype combinations of *qGL3* and *GS3*. **A.-D.** Grain length, width, thickness, and 1000-grain weight of different combinations, respectively. a, b: ranked using the Duncan test at $P < 0.05$.

DISCUSSION

Haplotypes of genes associated with grain size

In recent years, substantial research progress has been made regarding genes associated with grain size, particularly research focused on *GS3*, *qSW5*, *GW2*, *GS3*, *GS5*, and *TGW6* haplotypes (Weng et al., 2008; Fan et al., 2009; Takano-Kai et al., 2009; Mao et al., 2010; Li et al., 2011; Dixit et al., 2013; Ishimaru et al., 2013; Lu et al., 2013; Xu et al., 2015). Zhang et al. (2012) found that only SNP1 in the *qGL3* region in 94 germplasms (the same SNP that was found in TD70) contributed highly to grain length, whereas other polymorphic sites had no significant contributions to grain length. According to the coding sequence, *GS3* has at least four different alleles (Mao et al., 2010). Lu et al. (2013) compared the rice grain characters among *GS5* and *qSW5* genotypes, and the results showed that functional polymorphic sites contributed significantly more to grain traits than other haplotypes. The positive functional alleles of *GS3*, *qSW5*/GW5, *GS5*, and *GW8*, which are associated with grain size, were common in modern rice varieties (Shomura et al., 2008; Weng et al., 2008; Mao et al., 2010; Li et al., 2011; Yan et al., 2011; Wang et al., 2012). However, the beneficial *qGL3* allele was rare (Zhang et al., 2012), and this was similar to *GW2* (Song et al., 2007). In this study, six haplotypes were detected in 61 accessions, and only the *qGL3*-6 allele had great potential for the improvement of grain size. This allele was extremely rare in rice germplasm collections, indicating that grains of this length have not been favored by natural selection or artificial breeding.

Contributions of qGL3 and GS3 to rice breeding

A major goal of studying genes associated with rice grain size is the improvement of crop yield using the obtained knowledge. A thorough understanding of the function of these genes is essential for applications to breeding. We found that a single nucleotide substitution (C→A) at position 1092 in exon 10 resulted in the replacement of *Asp* (D) (a non-functional allele) with *Glu* (E) (a functional allele) at the 364th amino acid, which led to increases in grain length and weight. Premature termination of the OSR domain by a single base substitution (a C→A nonsense mutation in the second exon of *GS3*) greatly increased grain length, and this mutation is associated with long grain varieties that are widely cultivated around the world. Moreover, this *GS3* allele has been highly favored in breeding programs (Mao et al., 2010; Takano-Kai et al., 2011).

Although the location of *qGL3* and *GS3* genes on chromosome 3 was close, they did not exhibit genetic linkage characteristics (Fan et al., 2006; Zhang et al., 2012). We found that *qGL3* can not only be freely combined and separated in the offspring, but it can also be stably integrated with *GS3* in the same varieties. Integration of these two genes did not show any inhibitory effects, but the combination of the two mutant genes exhibited stronger effects than either gene alone.

For each individual gene, the effect of *qgl3* on the improvement of grain length and grain weight was greater than that of *gs3*. Therefore, *gw8* could shorten grain length when it increases grain width (Wang et al., 2012), but *gs3* had the opposite effect. Interestingly, *qgl3* could increase both grain length and grain width simultaneously. Because it is widely used in most *Indica* varieties, the breeding value of the *GS3* mutant was confirmed (Takano-Kai et al., 2009; 2011). We believe that when introduced to existing varieties, *qgl3* will play an important role in the improvement of whole rice grain size.

In summary, *qGL3* variations are conserved in exons and introns, and *GL3*-6 exhibited great potential for grain size improvement. The development of the two markers could accurately distinguish the *qGL3* and *GS3* mutant and wild-type alleles, which could be used for molecular marker-assisted selection of these two genes. The combination of *qgl3* and *gs3* generated significant genetic effects, which led to larger grain sizes than observed in either of the parents. The rare *qgl3* allele had greater effects on the 1000-grain weight than *gs3*. These results will provide critical information for breeding programs that seek to modify grain size and increase grain yield.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (Grant #31271678), the Jiangsu Agricultural Scientific Self-Innovation Fund (Grant #CX[14]5107), and the Key Research and Development Program for Modern Agriculture of Jiangsu Province (#BE2015363-3).

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Supplementary material

Table S1. The 61 rice varieties analyzed in this experiment and their grain shape phenotype, genotypes of GS3, and haplotypes of *qGL3*.

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