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DISTRIBUTION OF SEVEN GRAIN GENES AND EVALUATION OF THEIR GENETIC EFFECTS ON GRAIN TRAITS

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Abstract

Grain size is one of the most important factors determining rice yield. Several major QTLs for grain size control have been molecular characterized, and their roles in the regulation of grain size or weight have been explored but still remained obscure. In this study, we systematically examined the distributions of *GW2*, *GS3*, *qSW5*, *qGL3*, *GS5*, *GW8*, and *TGW6* in 240 RIL of TD70 and Kasalath, compared the phenotypic differences between functional (additive) and non-functional alleles of the seven genes by monogenic lines and analyzed the interaction effect of *gs3*, *gw2*, and *qgl3*. The results showed that the 7 genes could be randomly combined, and an individual gene has its specific roles in grain length, grain width, and grain thickness, the seven functional genes regulating grain weight follows the order as *qgl3 >gw2>gs3> GS5>qsw5> GW8>TGW6*, and the combination effect among *gs3*, *gw2*, and *qgl3* were revealed. These findings provide novel insight into grain size regulation in rice and are likely to be useful for marker assisted breeding in rice grain size.

Key words: Distribution, Genetic effect, QTL, *Japonica*.

Introduction

Rice is one of the most important cereal crops and a staple food in Asia (Rabbani *et al*., 2010). Grain weight is closely associated with rice yield. Grain shape, a typical complex quantitative trait, is a major determinant of grain weight and usually measured by grain length, grain width, grain thickness. The identification of major QTLs for grain shape is an important objective of rice genetic research and breeding programs (Masood *et al*., 2005; Bai *et al*., 2012; Huang *et al*., 2013). Several major QTLs for grain size, *GS3* (Fan *et al*., 2006; Mao *et al*., 2010), *GW2* (Song *et al*., 2007), *qGL3*(Hu *et al*., 2012; Qi *et al*., 2012; Zhang *et al*., 2012), *GS5* (Li *et al*., 2011), *GW5*/*qSW5* (Shomura*et al*., 2008; Weng *et al*., 2008), *GW8* (Wang *et al*., 2012), *TGW6* (Ishimaru *et al*., 2013), *GW6a* (Song *et al*., 2015), and *GS6* (Sun *et al*., 2013) have been molecularly characterized by using various mapping populations. These genes were cloned from numerous varieties with different background, such as Zhenshan 97, Minghui 63, Nipponbare, 9311, WY3, HJX74, Basmati, and N411 (Zuo & Li, 2014).The role in the regulation of grain size or weight of one gene has been explored. As for *GW2*, a significant increase (+49.8%) in 1,000-grain weight in NIL (*GW2*) compared with the control of FAZ1 isogenic line was observed (Song *et al*., 2007). Filled grain of NIL-*qgl3* showed 37.03% weight than those of 93-11 (Zhang *et al*., 2012). Additionally, seeds of over-expressed *GS5* (NIL-ZS97) were 8.7% wider and 7.0% heavier than NIL-H94 (Li *et al*., 2011), while *GW8* generated a 13.9% advantage for NIL-*GW8* with respect to 1,000-grain weight (Wang *et al*., 2012). Comparing grain traits of independent homozygous transgenic lines of *GS3* with Minghui 63, *GS3* shows 23%-30 % reduction in 1 000-grain weight (Mao *et al*., 2010). All of these genes contributed significant variations to grain size, but it was difficult to directly sort effects of single gene under different genetic background. To precisely assess each gene's function on grain size regulation and the potential interaction of these genes, we should characterize them under the same genetic background. Yan *et al.* (2011) studied the relationship between two grain size genes of rice, *GS3* and *GW2*, via examining the gene expression based on *GS3*-RNAi and *GW2*-RNAi lines of rice variety Zhonghua 11, respectively. Lu *et al.* (2013) compared the rice (*Oryza sativa*) grain size among haplotypes of *GW2*, *GS5*, *qSW5* and *GS3* of in the genetic background of Zhanshan 97. These studies have advanced our understanding of grain size regulation in rice. However, it is much better if we can examine all grain size regulatory genes in a stable and identical or almost identical genetic background.

In this study, TD70, a large grain rice carrying with additive (functional) genes of *GW2*, *GS3*, *qGL3*, *GS5*, *qSW5*, and *GW8* (Zhang *et al*., 2015), and Kasalath, a small grain *Indica* rice carrying with a functional gene of *TGW6*(Ishimaru *et al*., 2013), and 240 recombined inbred lines (RIL) deriving from these two parents at F_9 and F_{10} generation were used to examine the distribution of seven genes and evaluate effect of seven genes on seed size and their interactions. Our findings have critical reference value for the understanding of grain size regulation and for molecular designed breeding in grain size of rice.

Materials and Methods

Plant materials and growth condition: *Japonica* variety TD70 (an large grain derived from Tian-e-gu///9520// (72-496/Yu-nuo)), *Indica* variety Kasalath (a small size grain) and 240 recombined inbred lines (RILs) developing from a these two parents at F_9 and F_{10} generation population were used as research materials (Zhang *et al*., 2013). They were grown with two replicated plots in the experimental field of Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, China, during the natural growing season in 2012 and 2013, respectively. All plants were transplanted at the 30th days after sowing, and each line was planted into a plot of four rows with ten plants. 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, was followed with the normal agricultural practice. Seeds were harvested immediately once they developed to maturation, then they were dried by wind for 30 days to maintain the water content of them around 14%.

Phenotype characterization: Length, width, thickness, and 1 000-grain weight of rice grain were investigated in this study. Ten full seeds of each single plant from parents and 240 lines were selected randomly and measured for length, width and thickness by vernier caliper (to 0.01 mm). The mean value of each trait from five plants was defined as its phenotypic value. Total weight of one hundred grains from each plant was measured and converted to its 1000-grain weight using electronic balance (to 1/10000 gram).

Genotype determination: Three derived cleaved amplified polymorphic sequences (dCAPS) markers for *GW2*, *qGL3* , and *GW8*, two derived cleaved amplified polymorphic sequences (CAPS) markers for *GS3* and *TGW6*, a SSR marker for *GS5* genes, and an Indel marker for *qSW5*(*GW5*) were used to identify the differences of the seven genes sequences among TD70, Kasalath and their RILs. Products generated by PCR or endonuclease digestion are listed in Supplemental Table 1. The method to identify functional and non-functional grain size gene was following the reported procedure (Zhang *et al.*, 2015).

Results

Distribution of grain genes in RILs: TD70 was accumulated 6 additive functional genes of *GW2*, *GS3*, *qGL3*, *GS5*, *qSW5*, and *GW8*, and Kasalath was accumulated a functional gene of *TGW6* in previous work (Zhang *et al.*, 2015). We developed some molecular markers to identify these functional polymorphisms of seven genes and used these markers to test the distribution of seven grain genes in RILs. In theory, seven additive genes were classified eight kinds of combinations with the feature of numbers ranging from 0 to 7 of additive genes and assembled 128 kinds of combinations with the randomly separation of some functional genes, and the corresponding statistically segregation ratios were estimated to be 1: 7: 21: 35: 35: 21: 7: 1. Our results showed that the number of lines in these combinations is 4, 22, 69, 54, 53, 30, 5, 3 (Fig. 1A), which converts into the actual segregation ratio of 1: 7: 17: 24: 23: 15: 3: 1 (Fig. 1B), respectively. Compared with estimated segregation ratios, the types of combinations carrying with three and four additive genes missed more than other combinations, which had the number of 11 and 12, respectively. Both of them stand for more than 50% in total number of loss combinations. Loss of genotype and disparity in the number of different genotypic lines might result from the limitation of population size. However, at least the result had indicated that $GW2^{TD70}$ (*gw2*), $GS3^{TD70}$ (*gs3*), $qGL3^{TD70}$ (*qgl3*), $GS5^{TD70}$ (*GS5*), $GW8^{TD70}$ (*GW8*), $qSW5^{TD70}$ (*qsw5*), and $TGW6^{Kasalath}$ (*TGW6*) were randomly separated and assembled in RILs.

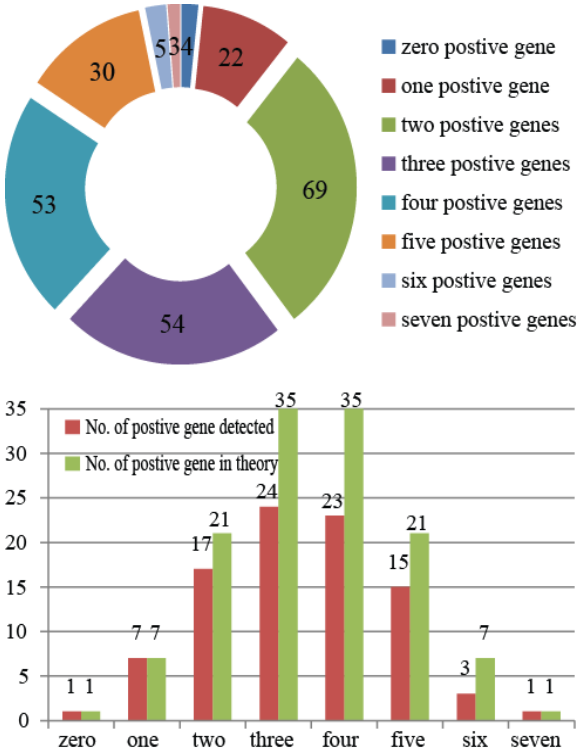

Fig. 1. Distribution of additive grain size genes, *gw2*, *gs3*, *qgl3*, *GS5*, *qsw5*, *GW8*, and *TGW6*, and their combinations in RIL. (A)The number of lines with zero to seven additive grain size gene, respectively. (B) The comparison of combination types number of lines with zero to seven additive grain size gene in theory and in practical in RIL, respectively. Zero to seven on the abscissa represents the number of additive grain size gene.

Supplemental table 1. Restriction endonuclease, marker type, and size of fragment used in this work.

Gene	Marker type	Restriction endonuclease	Size of fragment	
			TD70	Kasalath
GS3	CAPs	PstI	168bp	110bp,58bp
qGL3	dCAPS	AccI	105bp	84bp,21bp
GW2	dCAPS	ApoI	180bp,21bp	201bp
GW8	dCAPS	SpeI	128bp	20bp,108bp
TGW6	CAPs	BssHII	589bp	217bp,372bp
GS5	SSR	-	216bp	210bp
GW5	Indel	-	759bp	-

Phenotypes of monogenic lines: We obtained seven types of monogenic lines containing each functional gene and one type of lines without these functional additive genes in combinations with zero or one additive gene. In detail, the number of *gw2*, *gs3*, *qgl3*, *GS5*, *qsw5*, *GW8*, and *TGW6* plants were 3, 2, 1, 1, 1, 7, 1, 6, respectively. Typical grain size of each gene is shown in Figure 2. Compared with control (CON), additive (functional) *gs3* increases grain length, width and thickness of 1.36mm, 0.03mm and 0.2mm, and it increased 3.26g weight in 1000-grain weight in 2013. For *gw2*, it considerably increased grain width and thickness of 0.65mm and 0.28mm, which resulted in an enhanced 1000-grain weight of 10.27g. Furthermore, the average grain length of *qgl3* monogenic lines was 10.47mm, which longer than the seeds of *gs3* plants. At the meantime, it increased grain thickness by0.43mm, which immediately resulted in an increase of 1000-grain weight by 12.54g in 2013. It contributes more on grain length than *gs3*. *GS5* was a gene mainly controlling grain width and increases grain width and length by 0.37mm and 0.35mm, respectively, resulting in an increase of 1000-grain weight by 2.26g. *qsw5* only increased grain width by 0.1mm, but increases grain length by 0.37mm which increased the 1000-grain weight by 1.94g. The additive role of single *GW8* and *TGW6* was weaker than other genes in 1000 grain weight. *GW8* increased grain weight by 1g and *TGW6* reduced grain weight by 1g as to control line in 2013.

Every gene has its special function, resulting variations in the corresponding grain trait(s). The *qgl3* and *gs3* were major genes that increased grain length, and *gw2*, *GS5* and *qgl3* were major genes that increased grain width. All genes had slightly increases on grain thickness and significant differences on increasing 1000-grain weight (Fig. 3).The influence effect of gene on grain length was illustrated as *qgl3 >gs3> gw2>TGW6> qsw5>GS5>GW8*. For grain width, the gene order was the following as *gw2> GS5>qgl3>qsw5>GW8>TGW6> gs3*, and it was the order of *qgl3 >gw2>gs3>GW8>GS5> qsw5>TGW6* for grain thickness. For the grain weight, we found that *qgl3* and*gw2*were the two major genes with most significant effects. The order of the seven genes for their effect on grain weight was followed as *qgl3 >gw2>gs3>GS5>qsw5>GW8>TGW6*. A similar trend could also be observed from the data in 2012 (Fig. 3).

Fig. 2. Grains size of monogenic line with one additive grain gene. Scale bar, 10 mm.

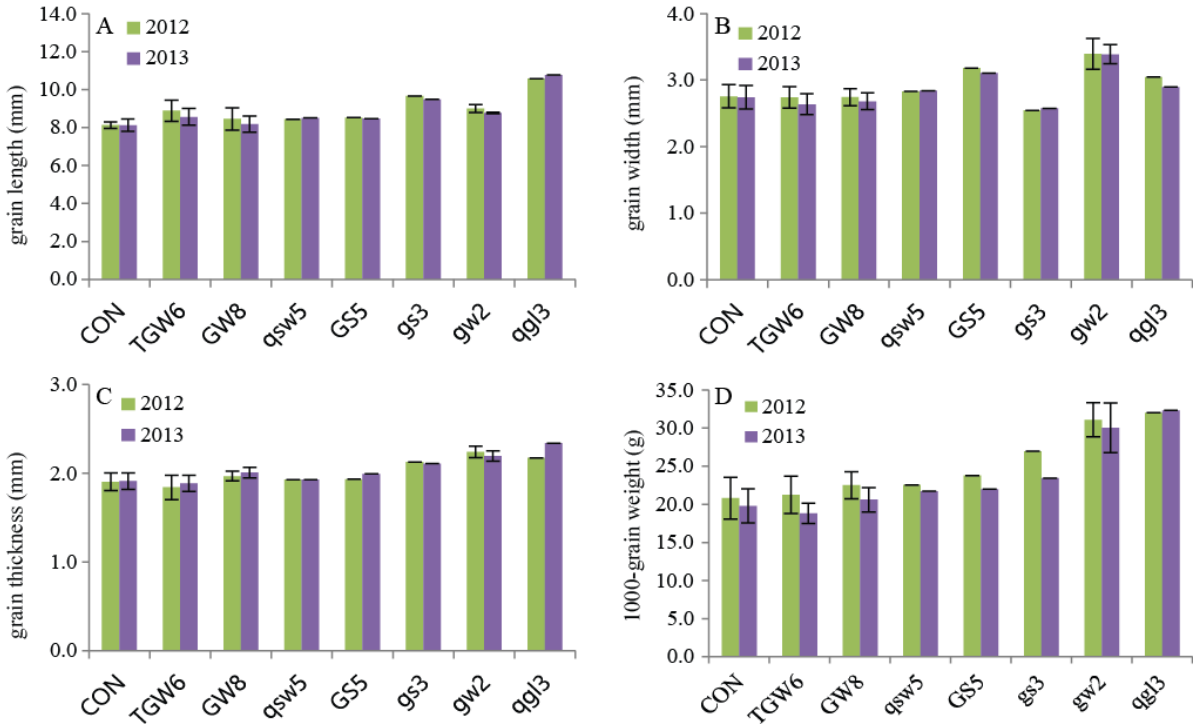

Fig. 3. Genetic effect of monogenic lines. Mean comparison of grain traits among seven functional grain size gene and control (CON). (A-D) Mean comparison of grain length, grain width, grain thickness and 1,000-seed weight among *qgl3*, *gs3*, *gw2*, *TGW6*, *qsw5*, *GS5*, *GW8*, and CON, respectively. CON stands for some lines without anyone of the seven additive genes.

Genetic interaction effects of *gs3, gw2,* and *qgl3*: Comparing genetic effects of monogenic, we found *gs3*, *gw2*, and *qgl3* were major genes on improving grain size gene. To study the interaction among *gs3*, *gw2*, and *qgl3*, 240 RIL were divided into eight groups, one control group carrying without anyone of the three functional genes, three groups carrying with one of one of the three additive genes, three groups carrying with two of the three genes and one group carrying with all the three additive genes. The number of lines in each group was listed in Figure 4. The mean value of grain length, grain width, and 1000-grain weight of each group was used for comparison. Substantial differences were observed among different groups (Fig. 5A-D). The 1000-grain weight effects of these three genes were in following order: *qgl3*>*gw2*>*gs3*. In addition, the three genes demonstrated additive effect in all the four tested traits. The more functional genes a plant carried, the higher grain weight, grain length, grain width, and grain thickness the plant generated. Further, the interaction effects of these three genes were also revealed. The RIL lines carrying with the *qgl3* (*gs3*) and *gw2* possessed higher weight than those of *qgl3* and *gs3* (Fig. 5D).

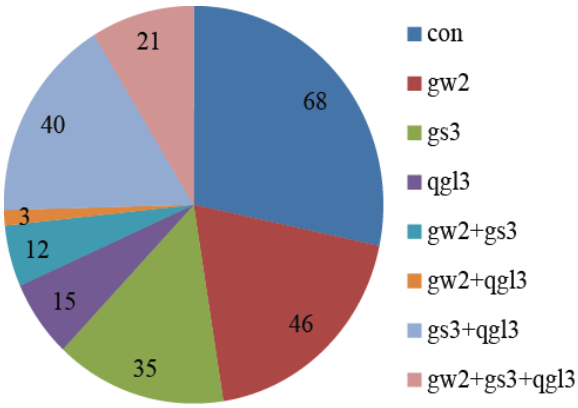

Fig. 4. The number of lines with the grain gene(s) of con, *gs3*, *gw2*, *qgl3*, *qgl3* + *gs3*, *qgl3* + *gw*, *gw2* + *gs3*, and *qgl3* + *gw2* + *gs3*, respectively. Con stands for Control lines with no functional genes, *gw2*, *gs3*, *gw2* represent that only one itself is a functional gene and the other two are non-functional genes. *qgl3+gs3*, *qgl3* + *gw2* and *gw2+gs3* represent that only two genes themselves are two functional gene and another is non-functional genes. *qgl3+gw2+gs3* represents that three genes themselves are functional genes assembled.

Discussion

Randomly separation of functional genes: TD70, a variety gathering six functional genes and a normal *TGW6*, and Kasalath, a small size grain and only having a positive gene *TGW6* were two suitable materials to study the genetic effect of grain size genes (Zhang *et al.*, 2015). In this study, we tested distribution of 7 grain genes in RILs, and found the randomly separation of these functional genes. Theoretically, seven functional genes could be assembled into 128 kinds of combinations, but only 91 kinds of combinations were identified by molecular markers. The combinations with three and four additive genes of seven genes missed more than other combinations, which stand for the total loss combinations number by 23. However, we had successfully identified that 22 lines were only accumulated with one single additive gene, 4 lines were found to accumulate without anyone of seven additive genes and 3 lines were assembled with all seven genes, which may explain the loss of genotype and disparity in the number of different genotypic lines may result from the limitation of population size. Seven genes were located on chromosome 2, 3, 5, 6, 8, respectively, and our work show that these genes did not have the characteristics of being closely linked genetically. Except for TD70, we obtained some stable materials with many grain size genes, which also indicated that donor materials of functional genes could be gathered into one genetic material. Therefore, seven genes of TD70 and Kasalath could be independently pyramided. This was a good example for pyramiding many grain genes.

Evaluating genetic effect of additive grain size genes by monogenic lines: The research on the genetic effect of grain size genes always limit on explaining the genetic effect between functional and non-functional genes. Seven known genes were cloned from various genetic materials and controls, so it was difficult to directly compare the difference effect of seven genes. During the process of evaluating the genetic effect of seven genes, the expected gene resources should come from common parents being accumulated by functional and non-functional genes, which could ensure the genetic effect not being interrupted by the haplotypes of genes. Only this could exactly evaluate the differences of genetic effect on functional and non-functional alleles of grain genes. Lots of agronomic mutants exist in natural, like dwarf genes D1 (Ashikari *et al.*, 1999; Ishikawa *et al.*, 1995), D2 (Hong *et al.*, 2003) and D11 (Tanabe *et al.*, 2005), and they can influence grain size in an indirect pathway, but the grain size of normal agronomic plants is mainly regulated by grain size genes. TD70 and Kasalath were two stable germplasm with normal agronomic traits, so their grain size should be completely dependent on their own genotype of grain gene. In this study, we selected some monogenic lines with one additive gene to analyze the genetic effect generated by the corresponding gene in their RILs. Though we all knew that some novel genes not being cloned in rice germplasm might cause some variations on grain traits, we still can preliminary evaluate the genetic effects of existing seven genes in a single genetic background, at least our results had counteracted the effects of known genes. It is a viable method to evaluate and compare the genetic effects of one gene by some monogenic lines.

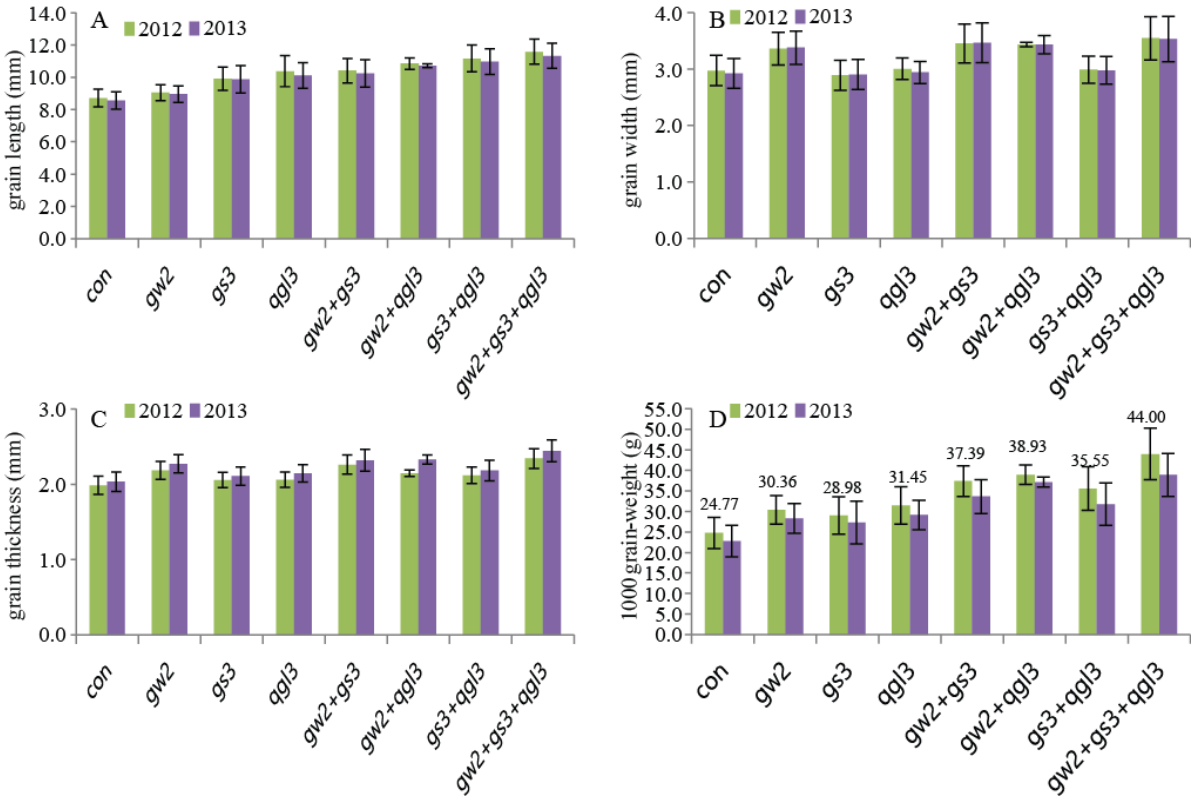

Fig. 5. Comparison of grain traits among eight combinations came from three grain size gene. *gw2*, *gs3*, *gw2* represent that only one itself is a functional gene and the other two are non-functional genes. *qgl3+gs3*, *qgl3+gw2* and *gw2+gs3* represent that only two genes themselves are two functional gene and another is non-functional genes. *qgl3+gw2+gs3* represents that three genes themselves are functional genes assembled. (A-D) mean comparison of grain length, grain width, grain thickness and 1,000-seed weight among *gw2*, *gs3*, *gw2*, *qgl3+gs3*, *qgl3+gw2*, *gw2+gs3*, *qgl3+gw2+gs3* and control (con), respectively. Con represents that three genes themselves are non-functional genes.

A great amount of research progress is made for every gene, especially genetic effect of different haplotypes of *GS3*, *qSW5*, *GW2*, *GS3*, *GS5* and *TGW6* were been revealed, and all experiments had been tested that only functional polymorphic sites contributes more significantly to grain traits than other haplotypes (Dixit *et al.*, 2013; Lu *et al.*, 2013; Mao *et al.*, 2010; Xu *et al.*, 2015; Yan *et al.*, 2011; Zhang *et al.*, 2012). The additive alleles of *GS3*, *qSW5*/*GW5*, *GS5* and *GW8* for grain size are common in modern rice varieties, but the beneficial allele *qGL3* was rare, which was similar to *GW2* (Zuo & Li, 2014). We confirmed *qgl3* and *gw2* were two genes with the most significant effect, which are followed with *gs3* and *GS5*. The order of the seven genes controlling grain weight was as follows *qgl3*>*gw2*>*gs3*>*GS5*>*qsw5* >*GW8*>*TGW6* in RILs.

Comparing the effect of grain genes: Many genes associated with rice grain development have been cloned in recent years and will be cloned in the near future. However, the successful cloning of a locus was not the end of the quest for that genetic element, but the start of a new journey to determine how the gene works (Huang *et al.*, 2013). Clarifying the genetic effect of *GS3*, *GW2*, *GS5*, *qSW5* (*GW5*), *qGL3*, *GW8*, *TGW6* in a single genetic background is the theoretical basis for the molecular design breeding for rice yield. Therefore, only on the basis of understanding genetic information about background material and owning genes sources of the same donor functional genes, we can accurately evaluate the effect of different genes.

The gene function change of *gs3*, *gw2*, and *qgl3*, is determined by a single base substitution or deletion in gene encoding region, which leads to different protein of three major genes. In this study, we used 240 RIL lines to analyze the genetic interaction pattern of the *gs3*, *gw2* and *qgl3*. We found that the combination carrying the *qgl3* (*gs3*) and *gw2* possessed higher weight than those of *qgl3* and *gs3*.

Guiding significance for rice breeding: The aim of rice grain size study was to improve grain related trait and apply them on breeding with high efficiency (Bai *et al.*, 2012; Huang *et al.*, 2013). Before the application on breeding, we need to have the knowledge on the effect of each grain size gene. We found that grain size genes in RIL could combine randomly, so we could pyramid different combinations of grain genes to obtain different grain weights. Cloned grain gene *GS3* controlling grain length in most of *Indica*, has been proved to have relatively great breeding value (Takano-Kai *et al.*, 2011; Takano-Kai *et al.*, 2009; Wang *et al.*, 2011). *GW2* and *qGL3* did not exist in varieties under natural selection, whereas they had stronger effect on increasing grain

weight (Ding *et al*., 2014; Zhang *et al*., 2014). We compared the combination effect of *qgl3*, *gs3*, and *gw2*, which will provide a guiding significance for rice breeding. Once the interactions among these genes are clearly identified, we believe that the ultimate goal of integrating multiple favorable genes in one rice variety will become possible.

Conclusions

To compare the phenotypic effect of the seven grain size genes *qgl3*, *gw2*, *gs3*, *GS5*, *gw5* (*qsw5*), *GW8*, and *TGW6*, some monogenic lines were used to evaluate the genetic effect. The seven functional genes regulating grain weight followed the order as *qgl3 >gw2>gs3>GS5>qsw5> GW8>TGW6*. Analyzing the combination effect of the *gs3*, *gw2* and *qgl3*, we found that the single rare allele of *gw2* and *qgl3* had stronger effects than *gs3* had on grain size, and the combination carrying the *qgl3* (*gs3*) and *gw2* possessed higher weight than those of *qgl3* and *gs3*. Our results would provide a meaningful guide toward the molecular design for yield improvement.

Acknowledgments

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