Analysis of QTL Interaction for Grain Weight using Near Isogenic Lines in Rice

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ABSTRACT Grain weight (GW) is one of the most important targets for grain yield in rice breeding. In previous studies, two quantitative trait loci (QTLs) for grain weight, *tgw2* and *gw8.1*, were identified using progeny derived from crosses between the *japonica* cultivar Hwaseong and *Oryza grandiglumis*, and between Hwaseong and *O. rufipogon* (IRGC 105491), respectively. The wild alleles increased GW at two loci. An F₂ population (186 plants) was developed by crossing two near isogenic lines (NILs) harboring *tgw2* and *gw8.1* to test their interaction. Simple sequence repeat (SSR) markers tightly linked to the two QTLs were used to check the genotype of the F₂ population. Based on the genotype at the two loci, *tgw2* and *gw8.1*, the F₂ plants were classified into 9 groups with a combination of three genotypes at each of the two loci. Two-way ANOVA revealed no interaction between the 2 QTLs in the F₂ population. The 1,000 grain weight (TGW) of homozygous plants with wild alleles at the two loci was significantly higher than that of plants with a single QTL in the F₂ and F₃ lines. These results indicate that two QTLs act additively, in distinct or complementary pathways, to control grain weight. Based on these results, it is desirable to pyramid the two QTLs into a single line, since the double-QTL line displayed further increase of TGW in the Hwaseong background.

Keywords Rice, Grain weight, Quantitative trait loci, Simple sequence repeat, Interaction, Near isogenic line

INTRODUCTION

Rice (*Oryza sativa* L.) is the world's most important cereal food crop. Its yield is determined by three major components: grain weight, grain number per panicle and panicle number per plant. Grain weight shows a positive correlation with grain size, which is controlled by the length, width and thickness of the grain. In addition, grain weight is important for the evolution of cereal crops, because large seed size was selected by humans during crop domestication (Li *et al.* 2004). Large size of the seeds could have a favorable effect on seed vigor and uniform germination, whereas more seeds are able to be produced by smaller seeds (Takeda 1991). Genetic control of grain weight has been extensively investigated over the last decade. Recently, many QTLs affecting grain size have been identified in rice populations based on crosses between divergent cultivars or accessions (Cui *et al.* 2003;

Ishimaru 2003; Kang *et al.* 2011). Several genes involved in grain size control have also been cloned. GS3, a major QTL for grain length, encodes a putative transmembrane protein (Fan *et al.* 2006). *GW2* encodes an E3 ubiquitin ligase, and affects grain width by restricting cell division (Song *et al.* 2007). An unknown protein encoded by qSW5/GW5 is also required to control grain width through limitation of cell proliferation (Shomura *et al.* 2008; Weng *et al.* 2008). GS5 encodes a putative serine carboxypeptidase, which functions as a positive factor for grain width (Li *et al.* 2011). SPL16 (GW8) encodes a protein which acts as a positive regulator of cell proliferation and promotes cell division, leading to increase in grain width (Wang *et al.* 2012). Although such studies have improved our understanding of the genetic control of grain size traits, the interaction among these QTLs remains to be clarified.

To elucidate the genetic basis of the yield-related traits of rice (spikelet number per panicle and 1000-grain weight),

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information about the cloned genes or QTLs has been employed for pyramiding of the QTLs by genomic-assisted selection to accumulate the beneficial QTLs in a single line. Molecular marker-assisted QTL pyramiding has enabled quick and accurate selection of lines with the target traits. The results demonstrated that multiple alleles underlying QTLs contribute to the increase in grain width and grain thickness in rice, and that accumulation of these additive effects allows generation of the large grain shape in rice (Jin *et al.* 2011; Ying *et al.* 2012).

To date, a number of studies reported interactions among genes which are thought to make substantial contributions to the genetic architecture driving diversification, which can be dissected via QTL analysis in rice (Yu *et al.* 1997). In studies employing various primary mapping populations (F_2 , RIL), no clear support was provided for the existence of interactions among QTLs, mainly due to background noise and small population size (Yano and Sasaki 1997). The employment of QTL-NIL has been proposed for the detection of interactions among genes and detailed characterization, as the background genetic effects can be minimized through this method. However, the epistatic interactions between GW QTLs are unclear, and studies on the interaction among QTLs are quite limited (Jin *et al.* 2011). Characterization of the genes underlying GW and their interactions will provide more insights into the molecular mechanisms underlying seed size development in rice, and will likely be useful for gene pyramiding aimed at improving rice grain yield.

In the previous studies, two QTL-NILs related to grain weight, *tgw2*-NIL (Yoon *et al.* 2006) and *gw8.1*-NIL (Xie *et al.* 2006), were developed by introgressing the chromosomal segments of two different wild species, *O. grandiglumis* (IRGC 101154) and *O. rufipogon* (IRGC 105491), into the Korean elite *japonica* cultivar Hwaseong. The present study was carried out to elucidate the interactions between the two QTLs, *gw8.1* and *tgw2*.

MATERIALS AND METHODS

Plant materials

Molecular marker-assisted backcrossing was used to

pyramid 2 QTLs, *gw8.1* and *tgw2*. A total of 186 F_2 plants were produced from a cross between the 2 QTL-NILs and genotyped using SSR markers tightly linked to the two QTLs. Four F_2 plants, including 1 of each of the following 4 types of QTL-NILs, were selected and advanced to F_3 lines: NIL-1 (Hwaseong homozygous at both loci), NIL-2 (*O. grandiglumis* homozygous at *tgw2* and Hwaseong homozygous at *gw8.1*), NIL-3 (Hwaseong homozygous at *tgw2* and *O. rufipogon* homozygous at *gw8.1*), and NIL-4 (*O. grandiglumis* and *O. rufipogon* homozygous at *tgw2* and *gw8.1*).

Field trials and trait evaluation

During the summer of 2012, 186 F_2 plants were grown at the experimental farm of Chungnam National University, Daejeon, Korea, at a density of 15 cm $\times$ 30 cm (line $\times$ row). The seeds were sown on April 25, and transplanted to a field on May 29. To minimize border effects, the seedlings of the F_2 plants were planted in the middle, excluding the first five and last five lines in each row. The length, width, and thickness of the grain, and 1000-grain weight was measured in brown rice of the F_2 plants. Fully-filled seeds were re-dried in an oven at 30°C for 24h. The 1,000-grain weight (TGW) was evaluated by measuring the weight of 100 randomly-selected brown rice grains. This procedure was performed in triplicate, and the values were averaged to yield a single mean. The four F_3 lines selected from the F_2 population were grown in the field during the summer of 2013. The planting density was the same as in 2012. The 4 F_3 lines were evaluated for 8 traits (days to heading, plant height, panicle length, panicle number). Each line was represented by a row of 30-day-old seedlings, planted with 15 cm between plants and 30 cm between rows in a completely randomized block design with two replications. Days to heading (DTH) was estimated as the number of days from sowing in the field until 50% panicle in the plant headed. Plant height (PH) was measured in centimeters from the surface of the soil to the tip of tallest panicle. Panicle length (PL) was measured in centimeters from the panicle neck to the tip of the panicle. Panicle number (PN) was calculated as the number of panicles per plant. The middle 10 plants from each line were chosen for evaluation, and the average of the measurements was used as the

phenotype of each line for the 1,000-grain weight (TGW). Grains with hulls were allowed to dry naturally after harvesting, and partial or unfilled seeds were removed by soaking the grains in water. Fully filled seeds were re-dried in an oven at 30°C for 24 h. The TGW was evaluated by measuring the weight of 250 randomly selected brown rice grains per plant (10 plants per line). Grain length (GL), grain width (GWD), and grain thickness (GTH) were also measured, for 15 grains (brown rice) per plant (10 plants per line) using a 150-mm Vernier caliper (Mitutoyo Corp., Japan). The 1,000-grain weight was corrected for 12% grain moisture content.

Means of the two replications were averaged for each trait and used in data analysis.

DNA extraction and SSR analysis

DNA was extracted from the fresh leaf tissue of each of the 186 F₂ plants. DNA extraction was performed as described in Causse *et al.* (1994). Simple sequence repeat (SSR) analysis of the F₂ population was performed according to the method described by Panaud *et al.* (1996). SSR markers with tight linkage to the two QTLs (RM12813 for *tgw2* and RM23204, RM23208, RM30000 and RM30002 for *gw8.1*, Xie *et al.* 2006), were used for genotype analysis of the 186 F₂ plants. The protocols for marker amplification using polymerase chain reaction (PCR), for size separation using polyacrylamide gel electrophoresis (PAGE), and for marker detection using the silver staining procedure were as described in Xie *et al.* (2006). The silver staining kits were purchased from Bioneer Co., Korea (www.bioneer.co.kr).

Statistical analysis

To analyze the effect of digenic interaction on grain weight, nine genotypes were identified from the 186 F₂ plants based on the SSR genotypes tightly linked to each target QTL. In the F₂ generation, a QTL was declared if the phenotype was associated with a marker locus at $P < 0.05$ using one-way ANOVA. Two-way ANOVA was used for analysis of the epistatic interaction between the two QTLs. The proportion of observed phenotypic variation attributable to a particular QTL was estimated by the coefficient of determination (R^2). The mean values of the four QTL-NILs in the F₃ generation were compared using Duncan's multiple range test, performed in SAS9.2.

RESULTS

Confirmation of QTLs

The existence of the two QTLs was confirmed by QTL analysis of the F₂ population (Table 1). The *O. grandiglumis* and *O. rufipogon* alleles contributed to positive effects on grain weight in the Hwaseong background. The results were consistent with those in earlier reports (Yoon *et al.* 2006; Xie *et al.* 2006). The distribution in the F₂ plants, based on genotypes at the two loci, is shown in Fig. 1. Wide variation was observed in the F₂ population. The grain weights of the two NILs with *tgw2* and *gw8.1* were 26.4g and 24.1g, respectively, which were significantly higher than that of Hwaseong-NIL (21.9g) ($P < 0.05$). At the *tgw2* locus, the mean TGW of the homozygous *O. grandiglumis* class was significantly higher than those of the heterozygous

Table 1. QTLs detected for grain weight based on one-way ANOVA in the F₂ population.

Cross	QTL	Chr.	SSR marker	P value	R ^{2z}	Mean ^{y)}		
						HH	HG/HR	GG/RR
<i>tgw2/gw8.1</i>	<i>tgw2</i>	2	RM 12813	<0.0001	49.7	21.6a ^{x)}	23.8b	26.4c
	<i>gw8.1</i>	8	RM 23204	0.02	9.9	21.6a	22.8ab	23.6b

^{z)}Percentage of phenotypic variance explained.

^{y)}HH, GG, RR, HG, and HR: homozygous for Hwaseong, *O. grandiglumis*, and *O. rufipogon*, and heterozygous for Hwaseong/*O. grandiglumis*, Hwaseong/*O. rufipogon*, respectively.

^{x)}Numbers followed by the same letter in a row are not significantly different at $P < 0.05$ based on Duncan's multiple range test.

and Hwaseong homozygous class. A significant difference was also observed between the heterozygotes and the Hwaseong homozygote (Table 1). This result indicated that the *O. grandiglumis* allele increased TGW in the Hwaseong background in an additive fashion.

Similarly, the TGW of the homozygous *O. rufipogon* class at *gw8.1* was significantly higher than that of the Hwaseong homozygous class. However, no significant difference was detected between the heterozygotes and the

Hwaseong homozygote.

The existence of the two QTLs was also confirmed by QTL analysis. *tgw2* and *gw8.1* were found to explain 49.7% and 9.9% of the phenotypic variance of TGW in the F_2 population, respectively (Table 1).

Pyramiding of two grain weight QTLs

Based on the genotypes at two loci, RM12813 for *tgw2* and RM23204 for *gw8.1*, the 186 F_2 plants were classified

Fig. 1. Frequency distributions of 1,000 grain weight in 3 genotype classes (homozygous for Hwaseong and *O. grandiglumis* /*O. rufipogon*, and heterozygous for *tgw2* and *gw8.1*, respectively) of individual QTLs in the F_2 population. Arrows indicate mean 1,000 grain weight for Hwaseong and each of the QTL-NILs.

Fig. 2. Differences in TGW for different genotype classes between *tgw2* and *gw8.1* in the F_2 population from the cross between two QTL-NILs. The *P*-value was calculated using two-way ANOVA, and indicated no interaction between the two QTLs. Numbers in parenthesis indicate the number of each genotype class. HH, HG and GG indicate Hwaseong homozygotes, Hwaseong/*O. grandiglumis* heterozygotes and *O. grandiglumis* homozygotes, respectively. HH, HR and RR indicate Hwaseong homozygotes, Hwaseong/*O. rufipogon* heterozygotes and *O. rufipogon* homozygotes, respectively.

into nine groups, with a combination of three genotypes (homozygous for Hwaseong, *O. rufipogon*/*O. grandiglumis* or heterozygous) at each locus. To test the pyramiding effect of the two QTLs, the average grain weights of the nine genotype classes in the F_2 population were compared. Digenic interaction was not detected, based on two-way ANOVA ($P=0.98$) (Fig. 2). The TGW-increasing effect of the *O. rufipogon* allele at the *tgw2* locus was observed in three genotype classes at the *tgw2* locus. In addition, presence of the *O. grandiglumis* allele at the *tgw2* locus increased the TGW in three genotype classes at the *gw8.1* locus. The highest grain weight was observed in the genotype class homozygous for *O. grandiglumis* and *O. rufipogon* at both loci, *tgw2* and *gw8.1*, indicating no interaction between *tgw2* and *gw8.1*. To further confirm the lack of interaction, the grain weights of four QTL-NILs in the F_3 generation were measured (Table 2). The four F_3 lines were selected from the F_2 population based on the genotypes at the two loci (Fig. 3). The effect of the *O. rufipogon* allele at *gw8.1* on TGW was observed based on comparison of the grain weight between NIL-1 and NIL-3. The effect of the *O. grandiglumis* allele at *tgw2* on TGW

was observed based on comparison of grain weight between NIL-1 and NIL-2 plants. Finally, the additive effect of the *O. grandiglumis* allele at *tgw2* and the *O. rufipogon* allele at *gw8.1* on TGW was observed based on comparison of the grain weight between NIL-2 and NIL-4. Difference in TGW between NIL-2 and NIL-4, caused by the genotype of *gw8.1*, was observed. The finding further supports that there is no interaction between *tgw2* and *gw8.1*. To clarify which grain shape trait was associated with the increase in GW, the grain length, width and thickness was evaluated for the 4 NILs (Table 2, Fig. 4). The grain length of the NIL-4 plants was significantly higher than in the NIL-2 and NIL-3 plants. A phenotypic difference was observed between NIL-2 and NIL-4, as a result of the *O. rufipogon* allele at *gw8.1*. There was no difference in grain width between the NIL-2 and NIL-4 plants, whereas grain widths of the NIL-2 and NIL-4 plants were significantly higher than NIL-3. Similar results were observed for grain thickness: grains of the NIL-2 and NIL-4 plants were significantly thicker than NIL-3 grains. These results indicate that the variation in GW seen in NIL-3 was associated with grain length, whereas all three

Fig. 3. Graphical genotype of the 4 NIL-QTLs in the F_3 generation. The two circles on the chromosomes represent regions of the 2 QTLs. Open bars and solid bars show chromosome segments derived from Hwaseong and *O. grandiglumis* or *O. rufipogon*, respectively.

grain shape traits were responsible for the variation in GW in NIL-2.

Impact of QTL pyramiding on agronomic traits

To determine the effects of the chromosomal segment harboring *tgw2* and *gw8.1* from *O. grandiglumis* and *O. rufipogon* on agronomic traits, four agronomic traits of the NILs were next evaluated (Table 2). The results indicated

no significant differences in DTH, PH and PL. However, the NIL-2 and NIL-4 plants had significantly lower numbers of panicles per plant than NIL-1 and NIL-3. This result seems to imply that the QTL associated with panicle number is located in the *O. grandiglumis* introgression on chromosome 2, which is consistent with the previous study (Yoon *et al.* 2006).

Fig. 4. Comparison of grain morphology traits among the 4 QTL-NILs with *O. grandiglumis* and *O. rufipogon*. Five brown rice grains are arranged for comparison (Scale bar in boxes). A: *O. grandiglumis*, B: *O. rufipogon*, C: NIL-1, D: NIL-2, E: NIL-3, F: NIL-4. NIL-1 (Hwaseong homozygous at both loci), NIL-2 (*O. grandiglumis* homozygous at *tgw2* and Hwaseong homozygous at *gw8.1*), NIL-3 (Hwaseong homozygous at *tgw2* and *O. rufipogon* homozygous at *gw8.1*), and NIL-4 (*O. grandiglumis* and *O. rufipogon* homozygous at *tgw2* and *gw8.1*).

Table 2. Comparison of 8 agronomic traits among the four F3 QTL-NILs.

Line	Trait ²⁾							
	DTH	PH	PL	PN	GL	GWD	GTH	TGW
NIL-1	102a ^{y)}	107a	20a	12a	5.00c	2.85b	2.10b	21.6d
NIL-2	101a	107a	19a	10b	5.19b	3.24a	2.30a	26.4b
NIL-3	101a	109a	20a	12a	5.10b	2.93b	2.12b	23.2c
NIL-4	101a	107a	20a	10b	5.34a	3.30a	2.32a	28.6a

²⁾DTH, days to heading; PH, plant height; PL, panicle length; PN, panicle no.; GL, grain length; GWD, grain width; GTH, grain thickness; TGW, 1,000 grain weight.

^{y)}Numbers followed by the same letter in each column are not significantly different at $P < 0.05$, based on Duncan's multiple range test. NIL-1 (Hwaseong homozygous at both loci), NIL-2 (*O. grandiglumis* homozygous at *tgw2* and Hwaseong homozygous at *gw8.1*), NIL-3 (Hwaseong homozygous at *tgw2* and *O. rufipogon* homozygous at *gw8.1*), and NIL-4 (*O. grandiglumis* and *O. rufipogon* homozygous at *tgw2* and *gw8.1*).

DISCUSSION

In order to understand the genetic architecture of complex traits, it is prerequisite to know the interactions among QTL genes. In rice, primary mapping populations have been extensively used in QTL analyses (Yano and Sasaki 1997). However, these populations are not appropriate for the precise mapping of one of multiple QTLs, because of the simultaneous segregation of many genetic factors in the whole genome in such populations. Moreover, it would be more difficult to determine the true genetic action of the QTL, because the genetic parameters of a given QTL are often affected by the segregation of other QTLs (Yano and Sasaki 1997). Only a limited number of interactions between QTLs have been detected, because primary populations cannot provide much information about the real nature of interactions among QTLs due to background noise. For example, two independent studies reported contrasting results on the interaction between QTLs related to grain size. Major QTLs for grain size, *GW2* and *qSW5/GW5*, were detected using an F_2 population from a cross between 'FAZ1' and 'Jizi1560,' with no significant interaction observed between the two (Ying *et al.* 2012). However, gene expression analysis with *GW2*-RNAi lines and CSSL of *qSW5* at the transcriptional level clearly showed that *qSW5* can be down-regulated by repression of *GW2* transcription, implying the existence of interaction between *GW2* and *qSW5* (Yan *et al.* 2011). This problem can be overcome by using QTL-NILs, because the background genetic effects can be minimized by use of QTL-NILs or populations derived from crosses between QTL-NILs with a uniform genetic background. QTLs for heading date (*hd1*, *hd2*, *hd3*, *hd4*, *hd5*, *hd6*) have been mapped as single Mendelian factors, and the interactions among them were dissected through the QTL-NIL strategy by Yamamoto *et al.* (1998). In addition, SPP in four QTL-NILs expressed bimodal or discontinuous distributions and followed the expected segregation ratio of single Mendelian factor by a progeny test (Zhang *et al.* 2009). These results clearly indicate that utilization of QTL-NILs is a powerful strategy for estimating the gene action of QTLs. However, few studies have yet examined the interactions among QTLs related to grain weight through use of NILs (Jin *et al.*

2011).

GW is one of the most important components of sink size in rice. The pyramiding experiment used in this study revealed that *tgw2* showed no interaction with another grain weight gene, *gw8.1*. Compared with the TGW of NIL-1, *tgw2* from *O. grandiglumis* increased the GW by up to 22.2%, *gw8.1* from *O. rufipogon* by up to 7.4%, and both by up to about 32.4%. The TGWs of three QTL-NILs, NIL-2, NIL-3 and NIL-4, were significantly higher than that of NIL-1. In addition, the TGW of NIL-4 plants was significantly higher than both NIL-3 and NIL-2 plants. These results indicate that the two QTLs act additively, in distinct or complementary pathways, to control grain weight. Analysis of the grain shape traits suggested that the increase in grain length may be responsible for the increased grain weight in NIL-3, whereas increases in the grain length, width and thickness were associated with the increased weight in NIL-2 and NIL-4. Li *et al.* (2004) also reported grain length as the main cause of the variation of TGW, based on the highly significant correlation between TGW and grain length at *gw3.1*. In some QTL studies, the genetic basis of yield and its components have been explained. For example, Xing *et al.* (2002) detected that a number of epistatic QTLs had much larger effects than the main-effect QTLs on the four traits, yield per plant, tillers per plant, grains per panicle and 1,000-grain weight, while most of the epistatic QTLs did not have main effects at the single-locus level. In contrast, a large proportion of the main-effect QTLs was involved in the epistatic interactions. This indicates that epistasis, in the form of additive by additive interactions, plays a very important role in controlling the expression of yield and yield-component traits. Similarly, Yu *et al.* (1997) detected large numbers of interactions for yield and yield components in $F_{2:3}$ families derived from the cross between Zhensan 97 and Minghui63, the parents of Sanyou63, an elite rice hybrid, while additive by additive interactions were the predominant forms of interactions. Li *et al.* (1997) also found epistasis to be an important genetic basis of three grain yield components, with digenic interactions due primarily to the additive epistatic gene action. These results indicate that epistasis plays a very important role of in controlling the expression of yield and its component traits.

The seed size is a complex quantitative trait in rice. Genetic improvement of this factor through gene pyramiding would be difficult, mainly due to the complicated interactions of seed size genes. Our analysis indicated that more comprehensive research on epistasis among QTLs is necessary to provide enough data to facilitate the accumulation of desirable QTLs in breeding lines, and to better understand the genetic mechanisms controlling grain weight. The results suggested that it is desirable to pyramid two QTLs into a single line, since the double-QTL line provided further advantages for increasing TGW in the Hwaseong background.

CONCLUSION

To elucidate the interaction of two QTLs, *gw8.1* and *tgw2*, an F₂ population was developed by crossing two near isogenic lines (NILs) harboring *tgw2* and *gw8.1*. SSR markers tightly linked to the two QTLs were used to check the genotype of the F₂ population, and to select four F₃ QTL-NILs with different combinations of the two QTLs. Two-way ANOVA revealed no interaction between the 2 QTLs in the F₂ population. The TGW of homozygous plants with wild alleles at the two loci was significantly higher than that of plants with a single QTL in the F₂ and F₃ lines. These results indicate that the two QTLs act additively in distinct or complementary pathways to control grain weight. Based on these results, it would be desirable to pyramid two QTLs into a single line, since the double-QTL line gave further advantages for increasing TGW in the Hwaseong background.

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