Interaction of Rice Quantitative Trait Locus *gw9.1* with Three Grain Shape Genes

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ABSTRACT Grain size is one of the most important factors determining grain yield in rice breeding. In previous studies, we constructed high-density maps for two quantitative trait loci (QTL) for grain weight, *tgw2* and *gw9.1*, using progeny derived from crosses between the *japonica* cultivar Hwaseong and *Oryza grandiglumis*, and Hwaseong and *O. rufipogon* (IRGC 105491), respectively. The wild alleles contributed an increase in grain weight at these two loci. We developed an F₂ population (146 plants) by crossing two near isogenic lines (NILs) harboring *tgw2* and *gw9.1* to know how they interact in the near isogenic background. Simple sequence repeat markers tightly linked to two QTL were used to check the genotype of the F₂ population. Based on the genotype at two loci, 146 F₂ plants were classified into 9 groups with a combination of three genotypes at each two loci. Two gene interaction was not significant ($P=0.99$) in the F₂. Homozygous plants with wild alleles at two loci showed significantly higher 1,000 grain weight than plants with a single QTL in the F₂ and F₃. These results indicate that two QTLs act additively in distinct or complementary pathways in controlling GW. Gene expression analysis was also performed to know the relationship of the *gw9.1* QTL with three major grain size genes with Hwaseong and two NILs plants at the transcription level. The results from this study provide insight into grain size regulation in rice and are likely to be useful for marker aided selection for grain size.

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

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal food crop. Rice yield is closely related with grain weight. Grain weight shows a positive correlation with grain size which is measured by grain length (GL), grain width (GW), and grain thickness (GT). The identification of major quantitative trait loci (QTL) for grain shape is an important objective of rice genetic research and breeding program (Kang *et al.* 2011; Huang *et al.* 2013; Nagata *et al.* 2015). Extensive studies were carried out to identify QTL for grain size and nearly 200 QTL for GL and GW have been reported in rice (Nagata *et al.* 2015). More than ten genes to control grain size have been cloned and molecularly characterized, *GS3* (Fan *et al.* 2006), *GW2* (Song *et al.* 2007), *qGL3* (Hu *et al.* 2012), *GS5* (Li *et al.* 2011), *qSW5* (Shomura *et al.* 2008), *GW8* (Wang *et al.* 2012), *HGW* (Li *et al.* 2012), *TGW6* (Ishimaru *et al.* 2013), *GW6a* (Song *et*

al. 2015), and *GS6* (Sun *et al.* 2013). *GW2* encodes an E3 ubiquitin ligase and affects GW by restricting cell division. A significant increase of 49.8% in grain weight was observed in the near isogenic line (NIL)-*GW2* compared with the control, FAZ1-NIL (Song *et al.* 2007). *GS5* encodes a putative serine carboxypeptidase and functions as a positive factor for GW (Li *et al.* 2011). *SPL16* (*GW8*) promotes cell division leading to increase in GW and showed a 13.9% advantage in 1,000 grain weight (TGW; Wang *et al.* 2012). These studies improved our understanding of genetic control over grain size traits; however the employment of these major QTL in practical breeding program for enhancing rice yield potential is hampered. This is because the information of additive and epistatic interactions across multiple grain size genes essential for combining favorable alleles resulting in enhanced traits is not sufficient for fine-tuning grain shape in practical breeding programs (Huang *et al.* 2013). In this regard, it is

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necessary to decipher the interactions among grain size genes and combine appropriate genes/QTLs.

QTL-NILs are promising materials to detect interactions among genes and to characterize gene actions in details because the background genetic effects on the analysis can be minimized in QTL-NILs. However, a few studies reported the employment of QTL-NIL for analyzing the interaction among grain size QTL. Jin *et al.* (2011) detected an epistatic interaction between two QTL, *gw8.1* and *gw9.1*. Wang *et al.* (2012) also examined the interaction between *GS3* and *qGW8* in a near-isogenic (HJX74) background. Lu *et al.* (2013) compared the grain size among haplotypes of *GW2*, *GS3*, *qSW5*, and *GS5* in the Zhanshan97 background. However, detailed analysis of interaction among grain shape QTLs will provide more insights into the molecular mechanisms underlying seed size development in rice and is likely to be useful for gene pyramiding aimed at improving rice grain yield.

In our previous studies, two QTL-NILs, *tgw2*-NIL (Yoon *et al.* 2006) and *gw9.1*-NIL (Xie *et al.* 2008) related with grain size were developed by introgressing chromosomal segments of two wild species, *O. grandiglumis* (IRGC 101154) and *O. rufipogon* (IRGC 105491) into the Korean elite *japonica* cultivar Hwaseong, respectively. This study was carried out to elucidate the interaction of two QTL, *tgw2* and *gw9.1*. Also, to know the relationship of the *gw9.1* QTL with three major grain size genes *GW2*, *GS3*, and *HGW* at the transcription level, gene expression analysis was performed with three lines, Hwaseong and two NILs.

MATERIALS AND METHODS

Plant materials

Molecular marker-assisted backcrossing was used to

Fig. 1. Graphical genotypes of 4 representative groups. The two circles on the chromosomes represent regions for 2 quantitative trait loci. Open bars and solid bars in black show segments of the chromosomes derived from Hwaseong and *Oryza grandiglumis* (*O. gra*) or *O. rufipogon* (*O. rufi*). Each number on top of the bars are chromosome number. (A) Hwaseong (Hwa). (B) *tgw2*-NIL. (C) *gw9.1*-NIL. (D) *tgw2*+*gw9.1*-NIL. Chr.: chromosome, NIL: near isogenic line.

pyramid two QTL, *tgw2* and *gw9.1*. 146 F₂ plants were produced from a cross between two QTL-NILs and genotyped using simple sequence repeat (SSR) markers tightly linked to the two QTL. Four F₂ plants with 1 of 4 types of QTL-NILs: Hwaseong (Hwaseong homozygous at both loci), *tgw2*-NIL (*O. grandiglumis* homozygous at *tgw2* and Hwaseong homozygous at *gw9.1*), *gw9.1*-NIL (Hwaseong homozygous at *tgw2* and *O. rufipogon* homozygous at *gw9.1*), and *tgw2+gw9.1*-NIL (*O. grandiglumis* and *O. rufipogon* homozygous at *tgw2* and *gw9.1*) were selected and advanced to F₃ lines (Fig. 1).

Field trials and trait evaluation

During the summer of 2013, 146 F₂ plants were grown in the experimental farm, Chungnam National University, Daejeon, Korea at a density of 15×30 cm (line×row). Seeds were sown on April 25 and transplanted to the field on May 29. To minimize border effect, seedlings of F₂ plants were planted in the middle, excluding the first five and last five lines in each row. 146 F₂ plants were measured for GL, width, thickness and TGW in brown rice. Fully-filled seeds were re-dried in an oven at 30°C for 24 hours. TGW was evaluated by measuring the weight of 250 randomly-selected brown rice; this method was performed in triplicates and the values were averaged to yield a single mean. Four F₃ lines selected from the F₂ population were grown in the field during the summer of 2014. The planting density was the same as 2013. Four F₃ lines were evaluated for 11 traits (culm length [CL], panicle length [PL], panicle number [PN], spikelets per panicle [SPP], fertility [FER]). Each line was represented by a row of 30-day-old seedlings planted with 15 cm between plant and 30 cm between rows in a completely randomized block design with two replications. CL was measured in centimeters from the surface of soil to panicle neck. PL was measured in centimeters from panicle neck to the tip of panicle. PN was calculated as the number of panicles per plant. For SPP, all seeds on panicle were counted including unfilled seeds. For FER, filled seeds were counted and divided with SPP (%). The middle 10 plants from each line were chosen for the evaluation, and the average of the measurements was used for the phenotype of each line for the TGW. Grains with hulls were allowed to dry naturally after harvesting, seeds

were re-dried in an oven at 30°C for 24 hours. The TGW was evaluated by measuring the weight of 250 randomly selected brown rice per plant (10 plants per line). GL, GW, and GT were measured. GL, GW, and GT were measured for 15 grains (brown rice) per plant (10 plants per line) using a 150-mm Vernier caliper (Mitutoyo Corp., Kawasaki, Japan). TGW was corrected for the 15% grain moisture content. Amylose content (%) and grain chalkiness were checked using a category of 0 (no chalky in the center and belly) to 9 (the grain opaque area >75%) (Juliano 1985). Means for two replications were evaluated for each trait and used in data analysis.

DNA extraction and SSR analysis

DNA was extracted from the fresh leaf tissue of each of 146 F₂ plants. DNA extraction was performed as described in Causse *et al.* (1994). SSR analysis of the F₂ population was performed according to the method described by Panaud *et al.* (1996). SSR markers tightly linked to two QTLs, RM12813 for *tgw2* and ex9 for *gw9.1* (Xie *et al.* 2008) were used for genotyping 146 F₂ plants. Protocols for marker amplification using the polymerase chain reaction (PCR) and for size separation using polyacrylamide gel electrophoresis and marker detection using the silver staining procedure were as described in Xie *et al.* (2008). The silver staining kits were purchased from Bioneer Co., Daejeon, Korea (www.bioneer.co.kr).

RNA extraction and gene expression analysis

Total RNA was extracted from panicles, less than 1 cm long from plants around 23 days before heading; Hwaseong, *tgw2*-NILs, and *gw9.1*-NILs using Nucleospin RNA plant kit (MACHEREY-NAGEL, Düren, Germany). Sampling time and procedure followed those described in previous studies (Song *et al.* 2007; Mao *et al.* 2010; Li *et al.* 2012). The first-strand cDNA was synthesized using extracted RNA using a PrimeScript RT-PCR Kit (TaKaRa Biotechnology Co. Ltd, Tokyo, Japan). Gene expression was performed by quantitative Real-Time PCR (CFX Connect Real-Time System; Bio-Rad, Hercules, CA, USA) with IQTM SYBR[®] Green Supermix (BIO-RAD). Quantitative RT-PCR was done by using 3 steps protocol; 95°C for 1 minute for denaturation, 40 cycles of 95°C for 15

seconds (denaturation), 55°C for 30 seconds (annealing) and 72°C for 30 seconds (extension), followed by final extension at 72°C for 7 minutes, and melting curve, temperature increased from 72°C to 95°C by 0.2°C for 5 seconds. For qRT-PCR primers, *OsUBQ5* (*Oryza sativa* Ubiquitin 5) was used as control and three primers for qRT-*GW2* (Yan *et al.* 2011; Lu *et al.* 2013), qRT-*GS3* (Yan *et al.* 2011), and *HGW* were designed (Table 1). Gene expression was performed in three replicates and the average values of three replicates were used in data analysis.

Statistical analysis

To analyze the interaction effect for grain weight, nine genotypes were identified from 146 F₂ plants based on SSR genotypes tightly linked to each target QTL. In the F₂ generation, 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 to analyze the epistatic interaction between two QTL. The proportion of observed phenotypic variation attributable to a particular QTL was estimated by the coefficient of determination

(R^2). The mean values of four QTL-NILs in the F₃ were compared using Duncan's multiple range test using SAS 9.3 program (SAS Institute, Cary, NC, USA).

RESULTS

QTL Confirmation

The existence of two QTL was confirmed by QTL analysis of the F₂ population (Table 2, Fig. 2). The *O. grandiglumis* and *O. rufipogon* alleles increased grain weight in the Hwaseong background which is consistent with those in earlier reports (Yoon *et al.* 2006; Xie *et al.* 2008). The genotype-based distribution of the grain weight in the F₂ at the two loci is shown in Fig. 2. Grain weight of two NILs for *tgw2* and *gw9.1* was 29.6 g and 24.7 g, respectively, and significantly higher than that of Hwaseong-NIL (23.0 g) ($P < 0.05$). At the *tgw2* locus, the mean TGW of the homozygous *O. grandiglumis* class was significantly higher than those of the heterozygous and Hwaseong homozygous class. There was also a significant difference between heterozygote plants and Hwaseong

Table 1. Primers for quantitative RT-polymerase chain reaction.

Primer ²⁾		Sequences (5'→3') ^{y)}	Predicted size (bp)
<i>OsUBQ5</i>	F	GAAGTAAGGAAGGAGGAGGA	100
	R	AAGGTGTTTCAGTTCCAAGG	
qRT- <i>GW2</i>	F	CTGCAGCAGGGAAGTGGT	96
	R	ATGTCTGAGCCCGCGTAG	
qRT- <i>GS3</i>	F	CATCGGAGAAGCGAAGTCA	109
	R	CAGCAGCAGATCCAGGAGA	
<i>HGW</i>	F	AGAACCCCAACTGGAGCAAG	118
	R	ACTGGATCCCCTGGAGTAGC	

²⁾F: forward, R: reverse.

^{y)}All sequences are based on cDNA sequences.

Table 2. QTLs detected for grain weight based on one-way ANOVA in the F₂ population.

Cross	QTL ²⁾	Chr.	Marker	P-value	R ²	Mean		
						HH	GH/HR	GG/RR
<i>tgw2/gw9.1</i>	<i>tgw2</i>	2	RM12813	< 0.0001	0.73	22.3b ^{y)}	24.3b	26.8a
	<i>gw9.1</i>	9	ex9	0.01	0.39	22.3b	22.8ab	23.9a

²⁾QTL: quantitative trait loci, Chr.: chromosome, HH: homozygous for Hwaseong, GH/HR: heterozygous for *Oryza grandiglumis* or *O. rufipogon*, GG/RR: homozygous for *O. grandiglumis* or *O. rufipogon*.

^{y)}Numbers followed by the same letter in each row are not significantly different at $P < 0.05$.

Fig. 2. Frequency distribution of 1,000 grain weight in 3 genotype classes in an F₂ population. NIL: near isogenic line, *O.*: *Oryza*.

homozygote plants (Table 2). This result indicated that the *O. grandiglumis* allele increased TGW in the Hwaseong background in an additive fashion.

TGW of the homozygous *O. rufipogon* class at *gw9.1* was significantly higher than the Hwaseong homozygous class. However, no significant difference between TGW of the homozygous *O. rufipogon* class and the heterozygous class at *gw9.1* was detected. The finding that *tgw2* and *gw9.1* explained 73.5% and 38.8% of the phenotypic variance for TGW in the F₂, respectively confirms the existence of the two QTLs (Table 2).

Pyramiding two grain weight QTL

Based on the genotypes at the two loci, RM12813 for *tgw2* and ex9 for *gw9.1*, 146 F₂ plants were classified 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 QTL, the average grain weight of nine genotype classes in the F₂ population was compared. Digenic interaction was not detected based on two-way ANOVA ($P=0.99$) (Fig. 3). The TGW increasing effect of the *O. grandiglumis* allele was observed in three genotype classes at the *tgw2* locus. Also, the *O. rufipogon* allele increased TGW in three genotype classes at the *gw9.1* locus. The highest grain weight was observed in genotype class homozygous for *O. grandiglumis* and *O. rufipogon* at

Fig. 3. Differences in 1,000 grain weight for different genotype classes between *tgw2* and *gw9.1* in the F₂ population from the cross between two quantitative trait loci (QTL)-near isogenic lines (NILs) *tgw2*-NIL and *gw9.1*-NIL. HH: homozygous for Hwaseong, GH/HR: heterozygous for *Oryza grandiglumis* or *O. rufipogon*, GG/RR: homozygous for *O. grandiglumis* or *O. rufipogon*.

both loci *tgw2* and *gw9.1* indicating no epistatic interaction between *tgw2* and *gw9.1*. To further confirm this finding that no interaction exists between *tgw2* and *gw9.1*, grain weight of four QTL-NILs was measured in the F₃ (Table 3). Four F₃ lines were selected from the F₂ population based on the genotypes at two loci (Fig. 1). The effect of the *O.*

Table 3. Comparison of 11 traits among 4 different groups at F₃ generation.

Line	Trait										
	CL ²⁾	PL	PN	SPP	FER	GL	GW	GT	TGW	CH	AC
Hwaseong	92a	19.3a	13a	102a	92.0a	5.29c	2.87c	2.17b	23.0d	0	20.1b
<i>tgw2</i> -NIL	90a	17.8b	11b	100a	84.3b	5.37b	3.18b	2.35a	29.6b	4	24.1a
<i>gw9.1</i> -NIL	90a	20.2a	13a	105a	92.9a	5.36b	3.10b	2.20b	24.7c	1	20.9b
<i>tgw2</i> + <i>gw9.1</i> -NIL	91a	19.3a	11b	98a	85.4b	5.42a	3.31a	2.40a	31.8a	4	24.2a

Numbers followed by the same letter in each column are not significantly different at $P < 0.05$.

²⁾CL: culm length, PL: panicle length, PN: panicle number, SPP: spikelets per panicle, FER: fertility, GL: grain length, GW: grain width, GT: grain thickness, TGW: 1,000 grain weight, CH: chalkiness, AC: amylose content, NIL: near isogenic line.

Fig. 4. Comparison of the grain shape traits among 4 groups. (A) Grain length. (B) Grain width. (C) Grain thickness. a: Hwaseong, b: *tgw2*-near isogenic line (NIL), c: *gw9.1*-NIL, d: *tgw2*+*gw9.1*-NIL. White bar on picture indicates 10 mm.

rufipogon allele at *gw9.1* on TGW was observed based on comparison of grain weight between Hwaseong and *gw9.1*-NIL. The effect of the *O. grandiglumis* allele at *tgw2* on TGW was observed based on comparison of grain weight between Hwaseong and *tgw2*-NIL plants. Also, the additive effect of the *O. grandiglumis* allele at *tgw2* and the *O. rufipogon* allele at *gw9.1* on TGW was observed based on comparison of grain weight between *tgw2*-NIL and *tgw2*+*gw9.1*-NIL. TGW difference between *tgw2*-NIL and *tgw2*+*gw9.1*-NIL caused by the genotype of *gw9.1* was observed. The finding indicates that there is no interaction between *tgw2* and *gw9.1*. To clarify which grain shape trait was associated with the increase in TGW, NILs were evaluated for GL, GW, and GT (Table 3, Fig. 4). GL and width of *tgw2*+*gw9.1*-NIL plants were significantly higher than those of *tgw2*-NIL and *gw9.1*-NIL plants. A phenotypic difference between *tgw2*-NIL and *tgw2*+*gw9.1*-NIL caused

by the *O. rufipogon* allele at *gw9.1* was observed. Similar results were observed for GT: grains of the *tgw2*-NIL and *tgw2*+*gw9.1*-NIL plants were significantly thicker than *gw9.1*-NIL grains. These results indicate that the variation in grain weight in *gw9.1*-NIL is associated with GL and width whereas three grain shape traits are responsible for the variation in GW in *tgw2*-NIL.

Impact of QTL pyramiding on agronomic traits

To know the effect of the chromosomal segment harboring *tgw2* and *gw9.1* from *O. grandiglumis* and *O. rufipogon* on agronomic traits, NILs were evaluated for 11 agronomic traits (Table 3). Results showed that no significant difference was observed for CL and SPP. PL was significantly higher in *gw9.1*-NIL than the other NILs and Hwaseong. However, *tgw2*-NIL and *tgw2*+*gw9.1*-NIL plants had significantly lower number of panicles per plant

than Hwaseong and this result seems to imply that QTL associated with panicle number is located in the *O. grandiglumis* introgression on chromosome 2 and is consistent with the previous study (Yoon *et al.* 2006). Also, *tgw2*-NIL and *tgw2+gw9.1*-NIL plants displayed higher ratio of grain chalkiness and amylose content than Hwaseong.

Gene expression

To investigate whether *gw9.1* interacts with other three grain size genes, *GW2*, *GS3*, and *HGW*, we performed the

Fig. 5. Gene expression level of 3 different grain shape genes in Hwaseong (HS), *tgw2*-near isogenic line (NIL), and *gw9.1*-NIL. (A) Panicle samples for RNA extraction. White bar indicates 10 mm. Samples sizes are less than 1 cm. (B) *GW2*, (C) *GS3*, and (D) *HGW*. *OsUBQ5* was used as normalization control for the RNA level. Error bars indicate standard deviation of three replicates. Each expression was repeated three times. One way ANOVA and Duncan's multiple range test were done with each stage in *GW2*, *GS3*, and *HGW* at the significant level of $P=0.05$. Numbers followed by the same letter above the error bar are not significantly different at the $P=0.05$ based on Duncan's multiple range test. a: HS, b: *tgw2*-NIL, c: *gw9.1*-NIL.

quantitative RT-PCR with three groups: Hwaseong, *tgw2*-NIL and *gw9.1*-NIL (Fig. 5). For *GW2* expression (Fig. 5B), three groups did not show any significant difference supporting the results from phenotypic data that *tgw2* and *gw9.1* act in an additive manner. A significant difference was detected in *GS3* expression level between Hwaseong and *gw9.1*-NIL, and *tgw2*-NIL and *gw9.1*-NIL (Fig. 5C). The difference between Hwaseong and *tgw2*-NIL in the expression level of *GS3* was observed ($P=0.189$) although it did not reach the significance level ($P<0.05$). The expression of *GS3* in *gw9.1*-NIL was remarkably reduced compared to other two groups. This result might imply that the expression of *GS3* may be negatively regulated by *gw9.1*. However additional experiments are needed to confirm the effect of *gw9.1* on the expression of *GS3* because the molecular function of *gw9.1* is not known and *gw9.1*-NIL harbors several *O. rufipogon* genes in the introgressed segment (Xie *et al.* 2008). It is speculated that *GS3* expression was affected by other *O. rufipogon* genes in the introgression in *gw9.1*-NIL. To analyze the interaction between *gw9.1* and *GS3*, cloning of the *gw9.1* gene is necessary. No significant difference among three groups was observed in *HGW* expression (Fig. 5D).

DISCUSSION

Cloning and molecular characterization of several major QTL for grain shape traits have advanced our understanding of the grain size regulation in rice. However, the complicated additive and epistatic interactions among major grain size genes and some minor QTL are still unclear, and these impede the process of gene pyramiding program to attain higher grain yield in rice (Huang *et al.* 2013). In rice, primary mapping populations have been extensively used in QTL analyses (Yano and Sasaki 1997). It would be more difficult to determine the true genetic action of the QTL because the segregation of other QTLs often affects the genetic parameters of a given QTL in primary mapping population (Yano and Sasaki 1997). Only a limited number of interactions between QTLs were detected because primary populations cannot provide

much information about the real nature of interactions among QTLs due to background noise. For example, two studies reported different results on the interaction between QTLs for grain size. Major QTL, *GW2*, and *qSW5/GW5* for grain size were detected using an F₂ population from a cross between 'FAZ1' and 'Jizi1560' and no significant interaction was observed between three QTL, *GW2*, *GS5*, and *qSW5/GW5* (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 that *GW2* might work upstream of *qSW5* (Yan *et al.* 2011). Also, in an association study using *japonica* rice, a significant difference in GL was detected between two *GW2* genotypes in the presence of nonfunctional *GS3*-A alleles, and the result indicated that two QTL, *GW2* and *GS3* interact each other (Lu *et al.* 2013).

QTL-NIL could provide a solution to this problem because the background genetic effects on the analysis can be minimized by QTL-NIL or populations derived from crosses between QTL-NILs with a uniform genetic background. Jin *et al.* (2011) detected an epistatic interaction between *gw8.1* and *gw9.1* using four QTL-NILs with allelic combination of *gw8.1* and *gw9.1* in a near-isogenic (Hwaseong) background. Wang *et al.* (2012) also developed four contrasting allelic combinations of *GS3* and *qGW8* in a near-isogenic (HJX74) background and examined the interaction between *GS3* and *qGW8*. NIL-*GW8/gs3* plants harboring the *qgs3* allele from Basmati385 had grains heavier and longer than those from NIL-*GW8/GS3* control plants and this result implies that two QTL act in an additive manner. These results clearly indicate that utilization of QTL-NIL is a powerful strategy for analyzing the gene interaction of QTLs.

In this study, we described the pyramided effect of *gw9.1* with *tgw2* on agronomic traits. The results indicated that *tgw2* showed no interaction with another grain weight gene, *gw9.1*. Compared with TGW of Hwaseong, *tgw2* from *O. grandiglumis* increased GW by up to 28%, *gw9.1* from *O. rufipogon* by up to 7.4%, and both by up to about 38.3%. TGW of three QTL-NILs, *tgw2*-NIL, *gw9.1*-NIL and *tgw2+gw9.1*-NIL were significantly higher than that of Hwaseong. TGW of *tgw2+gw9.1*-NIL plants was signi-

ficantly higher than those of *gw9.1*-NIL and *tgw2*-NIL plants. These results indicate that two QTLs act additively in distinct or complementary pathways in controlling grain weight. Analysis of grain shape traits suggests that the increase in GL might be responsible for increased grain weight in *gw9.1*-NIL whereas the increase in GL, width and thickness is associated with increased weight in *tgw2*-NIL and *tgw2+gw9.1*-NIL. A drastic increase in grain size due to *tgw2* did not increase grain yield proportionally in *tgw2*-NIL and *tgw2+gw9.1*-NIL plants owing to decrease in grain filling and panicles per plant (data not shown) and this is a typical example of imbalance between the sink and source potentials (Takai *et al.* 2013). Also, grain quality traits (high rate of white center and white belly, higher amylose content) were observed in *tgw2*-NIL and *tgw2+gw9.1*-NIL plants compared to Hwaseong. It has to be determined whether this deleterious effect is due to a pleiotropy of the *tgw2* QTL or its linkage with other genes. This result suggests that breeders should practice caution when choosing QTL alleles for grain shape with pleiotropy or tight linkage with genes for deleterious effects.

We performed the quantitative RT-PCR with three groups, Hwaseong, *tgw2*-NIL, and *gw9.1*-NIL to investigate the interactions among grain size genes, *GW2*, *GS3*, and *HGW*. As for *GW2* expression, no significant difference among three groups was detected supporting the result of the phenotype data. For *GS3* expression, the difference between Hwaseong and *tgw2*-NIL was observed ($P=0.189$) although it did not reach the significance level ($P<0.05$). This result is not consistent with the results from the study by Yan *et al.* (2011) reported that *GS3* was positively regulated by *GW2* based on the gene expression level of *GW2* in both RNAi-*GS3* and RNAi-*GW2*. Additional experiments are needed to analyze their interactions using QTL-NILs in more details at the phenotypic level. A significant difference between Hwaseong and *gw9.1*-NIL, and *tgw2*-NIL and *gw9.1*-NIL were observed and these results suggest a possibility that *gw9.1* might affect the expression of *GS3*. For *HGW*, there was no significant difference among three groups. Li *et al.* (2012) analyzed the *GW2* expression level in *HGW* mutants and found that the *GW2* expression level in *HGW* mutants were significantly reduced compared to wild type

(Zhouhuan 11). The finding from this study that no significant difference between Hwaseong and *tgw2*-NIL in *HGW* expression might support the finding that *HGW* might be an upstream regulator of *GW2* (Li *et al.* 2012).

To elucidate the interaction of two QTLs, *gw9.1* and *tgw2*, an F₂ population was developed by crossing two NILs harboring *tgw2* and *gw9.1*. SSR markers tightly linked to two QTLs were used to check the genotype of the F₂ population and to select four F₃ QTL-NILs with a combination of two QTLs. Two-way ANOVA revealed no interaction between 2 QTLs in the F₂ population. TGW of homozygous plants with wild alleles at two loci was significantly higher than that of plants with a single QTL in the F₂ and F₃. These results indicate that two QTLs act additively in distinct or complementary pathways in controlling grain weight. Although the double-QTLs line gave further advantage in increasing TGW in the Hwaseong background, an increase in grain weight did not result in an increase in grain yield. However, it should be cautioned that grain filling and grain quality traits of the double-QTLs plants were decreased compared to Hwaseong indicating that *tgw2* may have some effect on plant morphology. It has to be determined whether this deleterious effect is due to a pleiotropy of the *tgw2* QTL or linkage of *tgw2* with other genes.

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REFERENCES

- Causse MA, Fulton TM, Cho YG, Ahn SN, Chunwongse J, Wu K, *et al.* 1994. Saturated molecular map of the rice genome based on an interspecific backcross population. *Genetics* 138: 1251-1274.
- Fan C, Xing Y, Mao H, Lu T, Han B, Xu C, *et al.* 2006. GS3, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. *Theor. Appl. Genet.* 112: 1164-1171.
- Hu Z, He H, Zhang S, Sun F, Xin X, Wang W, *et al.* 2012. A Kelch motif-containing serine/threonine protein phosphatase determines the large grain QTL trait in rice. *J. Integr. Plant Biol.* 54: 979-990.
- Huang R, Jiang L, Zheng J, Wang T, Wang H, Huang Y, *et al.* 2013. Genetic bases of rice grain shape: so many genes, so little known. *Trends Plant Sci.* 18: 218-226.
- Ishimaru K, Hirotsu N, Madoka Y, Murakami N, Hara N, Onodera H, *et al.* 2013. Loss of function of the IAA-glucose hydrolase gene *TGW6* enhances rice grain weight and increases yield. *Nat. Genet.* 45: 707-711.
- Jin FX, Ji SD, Xie XB, Kang JW, Ju HG, Ahn SN. 2011. Detection of epistatic interaction of two QTLs, *gw8.1* and *gw9.1*, underlying grain weight using nearly isogenic lines in rice. *Jpn. J. Breed.* 61: 69-75.
- Juliano BO. 1985. *Rice : chemistry and technology*. 2nd ed. St. Paul, Minn.: American association of cereal chemists.
- Kang JW, Yang P, Yun YT, Ahn SN. 2011. Mapping grain weight QTL Using near isogenic lines from an interspecific cross. *Korean J. Breed. Sci.* 43: 225-231.
- Li J, Chu H, Zhang Y, Mou T, Wu C, Zhang Q, *et al.* 2012. The rice *HGW* gene encodes a ubiquitin-associated (UBA) domain protein that regulates heading date and grain weight. *PLoS One* 7: e34231.
- Li Y, Fan C, Xing Y, Jiang Y, Luo L, Sun L, *et al.* 2011. Natural variation in *GS5* plays an important role in regulating grain size and yield in rice. *Nat. Genet.* 43: 1266-1269.
- Lu L, Shao D, Qiu X, Sun L, Yan W, Zhou X, *et al.* 2013. Natural variation and artificial selection in four genes determine grain shape in rice. *New Phytol.* 200: 1269-1280.
- Mao H, Sun S, Yao J, Wang C, Yu S, Xu C, *et al.* 2010. Linking differential domain functions of the *GS3* protein to natural variation of grain size in rice. *Proc. Natl. Acad. Sci. U.S.A.* 107: 19579-19584.
- Nagata K, Ando T, Nonoue Y, Mizubayashi T, Kitazawa N, Shomura A, *et al.* 2015. Advanced backcross QTL analysis reveals complicated genetic control of rice grain shape in a japonica x indica cross. *Breed. Sci.* 65:

- 308-318.
- Panaud O, Chen X, McCouch SR. 1996. Development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). Mol. Gen. Genet. 252: 597-607.
- Shomura A, Izawa T, Ebana K, Ebitani T, Kanegae H, Konishi S, *et al.* 2008. Deletion in a gene associated with grain size increased yields during rice domestication. Nat. Genet. 40: 1023-1028.
- Song XJ, Huang W, Shi M, Zhu MZ, Lin HX. 2007. A QTL for rice grain width and weight encodes a previously unknown RING-type E3 ubiquitin ligase. Nat. Genet. 39: 623-630.
- Song XJ, Kuroha T, Ayano M, Furuta T, Nagai K, Komeda N, *et al.* 2015. Rare allele of a previously unidentified histone H4 acetyltransferase enhances grain weight, yield, and plant biomass in rice. Proc. Natl. Acad. Sci. U.S.A. 112: 76-81.
- Sun L, Li X, Fu Y, Zhu Z, Tan L, Liu F, *et al.* 2013. GS6, a member of the GRAS gene family, negatively regulates grain size in rice. J. Integr. Plant Biol. 55: 938-949.
- Takai T, Adachi S, Taguchi-Shiobara F, Sanoh-Arai Y, Iwasawa N, Yoshinaga S, *et al.* 2013. A natural variant of NAL1, selected in high-yield rice breeding programs, pleiotropically increases photosynthesis rate. Sci. Rep. 3: 2149.
- Wang S, Wu K, Yuan Q, Liu X, Liu Z, Lin X, *et al.* 2012. Control of grain size, shape and quality by OsSPL16 in rice. Nat. Genet. 44: 950-954.
- Xie X, Jin F, Song MH, Suh JP, Hwang HG, Kim YG, *et al.* 2008. Fine mapping of a yield-enhancing QTL cluster associated with transgressive variation in an *Oryza sativa* x *O. rufipogon* cross. Theor. Appl. Genet. 116: 613-622.
- Yan S, Zou G, Li S, Wang H, Liu H, Zhai G, *et al.* 2011. Seed size is determined by the combinations of the genes controlling different seed characteristics in rice. Theor. Appl. Genet. 123: 1173-1181.
- Yano M, Sasaki T. 1997. Genetic and molecular dissection of quantitative traits in rice. Plant Mol. Biol. 35: 145-153.
- Ying JZ, Gao JP, Shan JX, Zhu MZ, Shi M, Lin HX. 2012. Dissecting the genetic basis of extremely large grain shape in rice cultivar 'JZ1560'. J. Genet. Genom. 39: 325-333.
- Yoon DB, Kang KH, Kim HJ, Ju HG, Kwon SJ, Suh JP, *et al.* 2006. Mapping quantitative trait loci for yield components and morphological traits in an advanced backcross population between *Oryza grandiglumis* and the *O. sativa japonica* cultivar Hwaseongbyeon. Theor. Appl. Genet. 112: 1052-1062.