DOI: 10.1534/genetics.108.089862

Quantitative Trait Loci (QTL) Analysis For Rice Grain Width and Fine Mapping of an Identified QTL Allele *gw-5* in a Recombination Hotspot Region on Chromosome 5

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Manuscript received April 2, 2008
Accepted for publication May 20, 2008

ABSTRACT

Rice grain width and shape play a crucial role in determining grain quality and yield. The genetic basis of rice grain width was dissected into six additive quantitative trait loci (QTL) and 11 pairs of epistatic QTL using an F_7 recombinant inbred line (RIL) population derived from a single cross between Asominori (*japonica*) and IR24 (*indica*). QTL by environment interactions were evaluated in four environments. Chromosome segment substitution lines (CSSLs) harboring the six additive effect QTL were used to evaluate gene action across eight environments. A major, stable QTL, *qGW-5*, consistently decreased rice grain width in both the Asominori/IR24 RIL and CSSL populations with the genetic background Asominori. By investigating the distorted segregation of phenotypic values of rice grain width and genotypes of molecular markers in BC_4F_2 and BC_4F_3 populations, *qGW-5* was dissected into a single recessive gene, *gw-5*, which controlled both grain width and length–width ratio. *gw-5* was narrowed down to a 49.7-kb genomic region with high recombination frequencies on chromosome 5 using 6781 BC_4F_2 individuals and 10 newly developed simple sequence repeat markers. Our results provide a basis for map-based cloning of the *gw-5* gene and for marker-aided gene/QTL pyramiding in rice quality breeding.

RICE (*Oryza sativa* L.) is the world's most important cereal crop and is considered a model cereal crop due to its relatively small genome size, vast germplasm collection, enormous repertoire of molecular genetic resources, and efficient transformation system (Paterson *et al.* 2005). Rice grain quality consists of several components: cooking texture, palatability, flavor, grain appearance, milling efficiency, and nutritional quality. Among these, the cooking, eating, and appearance qualities constitute important economic concerns that influence rice production in many rice-producing areas of the world. Rice grain appearance is mainly specified by grain shape as defined by grain length, width and the length–width ratio (LWR), and chalkiness of the endosperm (Zhang 2007). Preference for rice grain shape, however, varies among consumer groups. For instance, long and slender grain varieties are preferred by consumers in the United States and Western Europe and in most Asian countries or areas, including China, India, Pakistan, and Thailand; in contrast, consumers in Japan, South Korea, and Sri Lanka prefer short and bold grain cultivars (Unnevehr *et al.* 1992; Juliano and Villareal 1993). Therefore, breeding for the appropriate grain shape needs to be considered in the context of market preference. Additionally, rice grain width and shape can greatly affect other important rice quality traits, such as endosperm chalkiness, milling efficiency, eating, and cooking properties (McKenzie and Rutger 1983; Takita 1989; Chauhan *et al.* 1995; Tan *et al.* 2000; Xu *et al.* 2004). Similarly, rice grain width and shape also play important roles in determining grain yield (Lin and Wu 2003; Yoon *et al.* 2006). Notably, grain shape is also an important indicator of the evolution of crops due to the continuous selection for large seeds during early domestication. However, small seeds are usually favored by natural selection, as small seed size is frequently associated with more seeds per plant, early maturity, and wider geographic distribution (Harlan 1992). Therefore, the great economic importance associated with grain width and shape necessitates in-depth study of their genetic basis and development mechanism(s) to better understand biological development processes and to facilitate breeding in rice.

Several independent studies have identified a number of QTL for rice grain width in primary mapping

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populations (Lin *et al.* 1995; Huang *et al.* 1997; Redoña and Mackill 1998; Tan *et al.* 2000; Xu *et al.* 2002; Aluko *et al.* 2004; Li *et al.* 2004b). In these studies, grain width was evaluated in a single environment, so the stability of the resultant QTL remained unknown. However, this stability information is critical in determining the usefulness of the QTL in marker-aided selection (MAS) breeding. Additionally, among the 31 grain-width QTL detected in these studies, one was consistently identified in the same genomic region of rice chromosome 5, with an average percentage of phenotypic variation explained (PVE) of 35.9%. Whether this QTL corresponds to only one gene or to a cluster of genes, each with relatively small contribution to the total genetic effects, remains unknown. Thus, it is necessary to establish whether this QTL is a single Mendelian factor and to further narrow down its location on genetic and physical maps.

Primary mapping populations such as $F_{2:3}$, backcross (BC), doubled haploids (DH), and recombinant inbred lines (RILs) are not appropriate for fine mapping of an identified QTL, as they simultaneously segregate at the whole parental chromosome segments (Yamamoto *et al.* 2000). On the other hand, chromosome segment substitution lines (CSSLs) or near-isogenic lines can simplify or eliminate the effect of genetic background on the expression of QTL and therefore facilitate fine mapping and isolation of the alleles of target QTL (Frary *et al.* 2000; Fridman *et al.* 2004; He *et al.* 2006; Konishi *et al.* 2006; Wan *et al.* 2006). A recent study reported that QTL *GW2* for rice grain width and weight on chromosome 2 encodes a RING-type E3 ubiquitin ligase that negatively regulates cell division by targeting its substrate(s) to proteasomes for proteolysis. Loss of *GW2* results in increasing cell numbers and wider spikelet hull, thus enhancing rice grain width, weight, and yield (Song *et al.* 2007). Furthermore, a major grain-width QTL *qGW-5* on chromosome 5 was consistently detected in all eight environments including two cropping seasons (2001 and 2002) and four locations (Nanjing, Jinhu, Donghai, and Hainan in China), and the donor IR24 allele of *qGW-5* had a significant and stable effect on decreasing rice grain width in the isogenic background of Asominori (Wan *et al.* 2005).

The objectives of this study were the following: (1) to obtain information on rice grain-width QTL including additive QTL, epistatic QTL, QTL by environment interactions, and gene action of identified additive QTL; (2) to dissect the major QTL *qGW-5* into a single gene; and (3) to localize the *gw-5* gene to a narrow genomic region.

MATERIALS AND METHODS

Plant materials: Four different populations (RILs, CSSLs, BC_4F_2, and BC_4F_3) were used to conduct QTL analysis and fine mapping in this study. Seventy-one RILs were derived from the single cross between Asominori and IR24 by single-seed descent (Tsunematsu *et al.* 1996). A total of 66 CSSLs with a genetic background of mostly Asominori, denoted as CSSL1-66, were produced by crossing and backcrossing 19 selected RILs with Asominori (without selection) until the BC_3F_1 generation, as described in supplemental Figure S1 (Kubo *et al.* 1999; Wan *et al.* 2004a). Of two narrow-grain lines (CSSL28 and 29) harboring the *gw-5* gene, CSSL28 was used to develop BC_4F_2 and BC_4F_3 populations by backcrossing to Asominori with subsequent self-pollination.

Field experiment design and phenotypic evaluation: This study was carried out in 12 environments, E1–E12, including five locations and eight cropping seasons (supplemental Table 1S). Asominori, IR24, and their 71 RILs were grown in 4 environments (E1–E4), and the parental varieties and their 66 CSSLs were planted in 8 environments (E1–E8). Each experimental plot consisted of two replicates, with the design of 10 rows × 10 plants for each line. At maturity, each plot was harvested in bulk. After drying, grains were milled using the method of Wan *et al.* (2004a), and grain width was determined on the basis of the method of Wan *et al.* (2005).

Two thousand one hundred seventy-one, 1248, 2465, and 897 CSSL28/Asominori F_2 (BC_4F_2) plants were grown in E9, E10, E11, and E12, respectively. One hundred BC_4F_3 families (16 plants per family) were planted in E11. At maturity, seeds were collected from primary panicles. Paddy and brown rice were used to evaluate grain length and grain width using the methods of Wan *et al.* (2005, 2006), and the LWR, which represents the grain shape, was calculated as the ratio of grain length/width. Of the 6781 BC_4F_2 plants, 805 homozygous individuals with wide grain were used to precisely map the *gw-5* gene.

DNA preparation, PCR protocol, and DNA marker analysis: DNA was extracted from fresh rice leaves of BC_4F_2 plants as described by Dellaporta *et al.* (1983). PCR was performed using the procedure of Chen *et al.* (1997). PCR products were separated on an 8% nondenaturing polyacrylamide gel and detected using the silver staining method of Sanguinetti *et al.* (1994). The required density of markers in the genomic region harboring the *gw-5* gene was achieved using previously published simple sequence repeat (SSR) and expressed sequence tag (EST) markers on rice chromosome 5 (McCouch *et al.* 2002; Wu *et al.* 2002) as well as the SSR markers newly developed in this study. New SSR markers were designed using the method of Wan *et al.* (2006). Primer sequences, map positions, and amplified lengths of 10 new SSR markers are listed in supplemental Table 2S.

QTL analysis and gene action of identified additive QTL: The linkage map of the Asominori/IR24 RIL population including 375 markers (Tsunematsu *et al.* 1996) was used for QTL analysis. Tests of QTL additive effects, epistatic interactions and QTL × environment interactions (QEI) were performed using the QTL Mapper 1.0 program (Wang *et al.* 1999). The LOD = 4.03 (up to $P = 0.005$) was used as the threshold for determining the presence of additive or epistatic QTL. The relative contribution was calculated as the PVE by the QTL. Additionally, gene action of identified additive QTL was evaluated by a *t*-test to show the presence of significant differences between the phenotypic values of the recurrent parent Asominori and those of CSSLs harboring the target QTL alleles derived from the donor parent IR24.

Fine mapping of the *gw-5* gene: For fine mapping of the *gw-5* gene, the bulked-extreme and recessive-class approach as described in Zhang *et al.* (1994) was used to calculate recombination frequencies between the *gw-5* gene and molecular markers in the 805 homozygous BC_4F_2 plants with wide grain. Thus, the recombination frequency = $(N_1 + N_2/2)/N$,

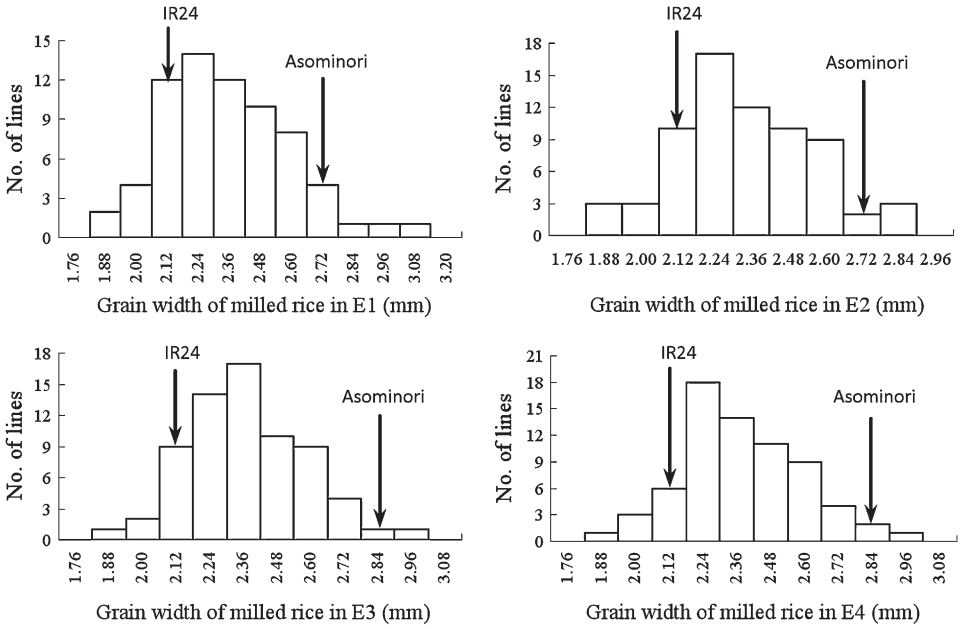


FIGURE 1.—Phenotypic distributions of rice grain width in the Asominori/IR24 recombinant inbred lines in four environments (E1–E4).

where N is the total number of wide-grain plants surveyed, N_1 is the number of wide-grain plants with the band pattern of the narrow-grain parent, and N_2 is the number of wide-grain plants with heterozygous band patterns.

RESULTS

Stability of QTL for rice grain width and gene action of identified QTL: The phenotypic distributions of rice grain width in the RIL populations grown in the E1–E4 environments (supplemental Table 1S) are shown in Figure 1. Variance among genotypes (G) was highly significant for grain width, but was not significant among environments (E). The significant G × E interaction explained 3.5% of the total phenotypic variation. Six additive effect QTL for rice grain width were identified and mapped to six chromosomes, with LOD values ranging from 7.68 to 23.77 (Table 1). Among the QTL, *qGW-5* was consistently located in the Y1060L–R569 interval on chromosome 5 in populations grown across all four environments, and the average PVE by *qGW-5* was 24.3%. The IR24 allele at the *qGW-5* locus was found to reduce grain width by an average of 0.115 mm. Moreover, stability of *qGW-5* was relatively high, as its QEI was not significant. Additionally, 11 pairs of epistatic QTL for rice grain width were detected and

TABLE 1

The putative additive QTLs and their environmental interactions for grain width of milled rice

Loci	Chromosome	Marker interval	Environment	LOD score	PVE[a] (%)	Additive effect (mm)	Positive allele	QEI effect
qGW-5	5	Y1060L–R569	E1	21.66	24.4	0.11	A[b]	NS[c]
			E2	23.77	22.2	0.11	A	NS
			E3	17.50	25.9	0.12	A	NS
			E4	16.45	24.7	0.12	A	NS
qGW-9	9	G1445–XNpb293	E1	10.06	12.9	−0.08	I[b]	NS
			E2	10.20	11.7	−0.08	I	NS
			E3	9.08	11.5	−0.08	I	NS
			E4	7.68	11.0	−0.08	I	NS
qGW-10	10	C16–XNpb127	E1	10.09	12.9	0.08	A	NS
			E2	10.03	9.0	0.07	A	0.02*
qGW-12	12	R1869–R367	E3	10.45	14.6	−0.09	I	−0.02*
			E4	8.88	11.0	−0.08	I	−0.03**
qGW-1	1	R1613–XNpb216	E2	11.37	14.8	−0.09	I	−0.04**
qGW-4	4	C335–C6212	E2	8.29	6.6	−0.06	I	−0.03**

*$P < 0.05$; **$P < 0.01$.

[a] Percentage of phenotypic variation explained.

[b] Positive effects of QTL contributed by Asominori (A) and IR24 (I) alleles.

[c] Nonsignificant effect of the QEI.

TABLE 2

The putative epistatic QTLs and their environmental interactions for grain width of the milled rice

Chr.	Interval *i*	A-QTL[a]	Chr.	Interval *j*	A-QTL	Environment	LOD score	PVE[b] (%)	AA*ij*[c]	E-AA*ij*[d]
1	R1613–XNpb216	*qGW-1*	7	XNpb50–C105		E1	12.48	9.49	0.09	0.030**
						E2	6.04	5.51	0.06	NS
1	XNpb252–XNpb87-2		2	C560–C601		E2	6.22	3.83	−0.05	NS
						E3	6.85	5.21	−0.06	NS
1	C112–XNpb346		3	G1316–C80		E1	9.31	4.22	0.06	0.030*
						E4	6.17	6.91	−0.07	−0.021*
6	XNpb27–XNpb16∼1		9	XNpb103–C39		E3	8.24	7.1	0.07	NS
						E4	7.50	9.03	0.08	0.026*
2	XNpb349–V83B		11	C410–C1350		E3	9.27	9.27	−0.08	−0.029**
						E4	8.92	11.43	−0.09	−0.023**
2	XNpb227–C132		4	C335–C1612	*qGW-4*	E2	14.83	7.5	−0.08	−0.032**
2	G1185–C1236		4	Ky4–R2737		E3	7.06	9.27	−0.08	−0.027**
3	C80–C1677		7	C1008–R2394		E1	8.35	4.22	0.06	0.021*
3	XNpb392–R2778		11	C6–C1003A		E1	8.41	5.74	−0.07	NS
3	C1351–R19		12	R1869–R367	*qGW-12*	E2	7.92	3.83	0.05	−0.022*
8	R2976–C277		11	C6–C1003A		E1	10.90	11.72	0.10	0.040**

Chr., chromosome. *$P < 0.05$; **$P < 0.01$.
[a] Additive effect QTL.
[b] Percentage of phenotypic variation explained.
[c] Additive by additive epistatic effects of QTL.
[d] Interaction effect of epistatic QTL by environment.

mapped on 10 chromosomes. Of these, three digenic interactions occurred between an additive effect QTL (*qGW-1*, *qGW-4*, or *qGW-12*) and a modifying factor (Table 2).

Eighteen lines were selected from the total 66 CSSLs and used to analyze gene action of the six additive effect QTL. The donor IR24 segments harboring *qGW-5* and *qGW-9* were transferred into 2 (CSSL28 and 29) and 4 CSSLs (CSSL53–55 and 57), respectively (Kubo *et al.* 1999). Significant difference in grain width of milled rice was observed between Asominori and each of CSSL28 and CSSL29 across all eight environments, indicating that the gene action of *qGW-5* was significant and stable in the Asominori genetic background (Figure 2). With *qGW-9*, significant difference in grain width between Asominori and each of the four target CSSLs was found only in a few environments. The direction of the effect of *qGW-9* in CSSL53 and CSSL55 was consistent with that in the RIL population (Table 1), but opposite to that in CSSL54 (Figure 2). Thus, the gene action of *qGW-9* was sensitive to both the genetic background and environmental conditions. Similar results were also observed for *qGW-1*, *qGW-4*, *qGW-10*, and *qGW-12* (supplemental Figure S2). Therefore, the observed stability and gene action of these QTL indicates that *qGW-5* is the most important genetic factor that controls rice grain-width difference between the Asominori and IR24 parental lines.

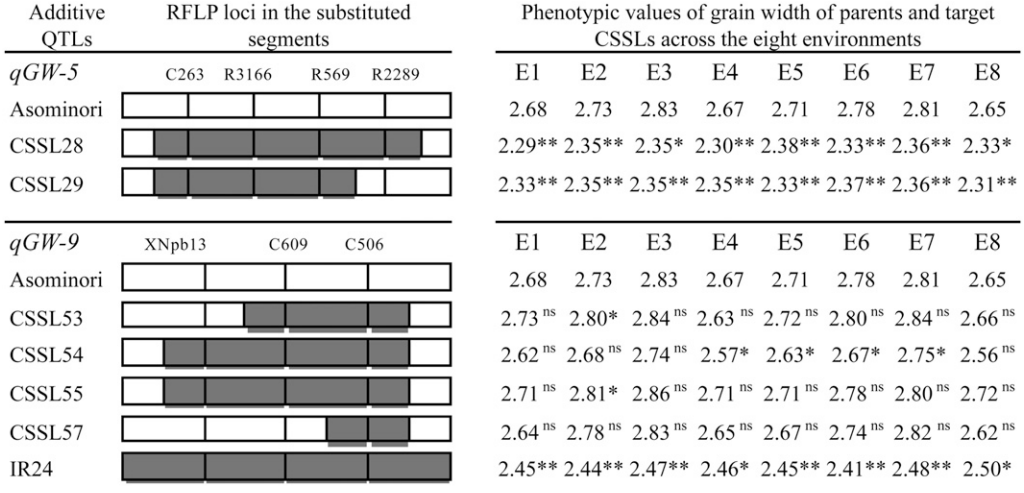


Figure 2.—Grain width of the milled rice in Asominori and target CSSLs carrying *qGW-5* or *qGW-9* alleles in eight environments (E1–E8). * and ** indicate significance levels of 5 and 1%, respectively; ns, nonsignificant difference. The open and shaded boxes represent the chromosomal segments from Asominori and IR24, respectively. The test was conducted between Asominori and IR24 or one target CSSL.

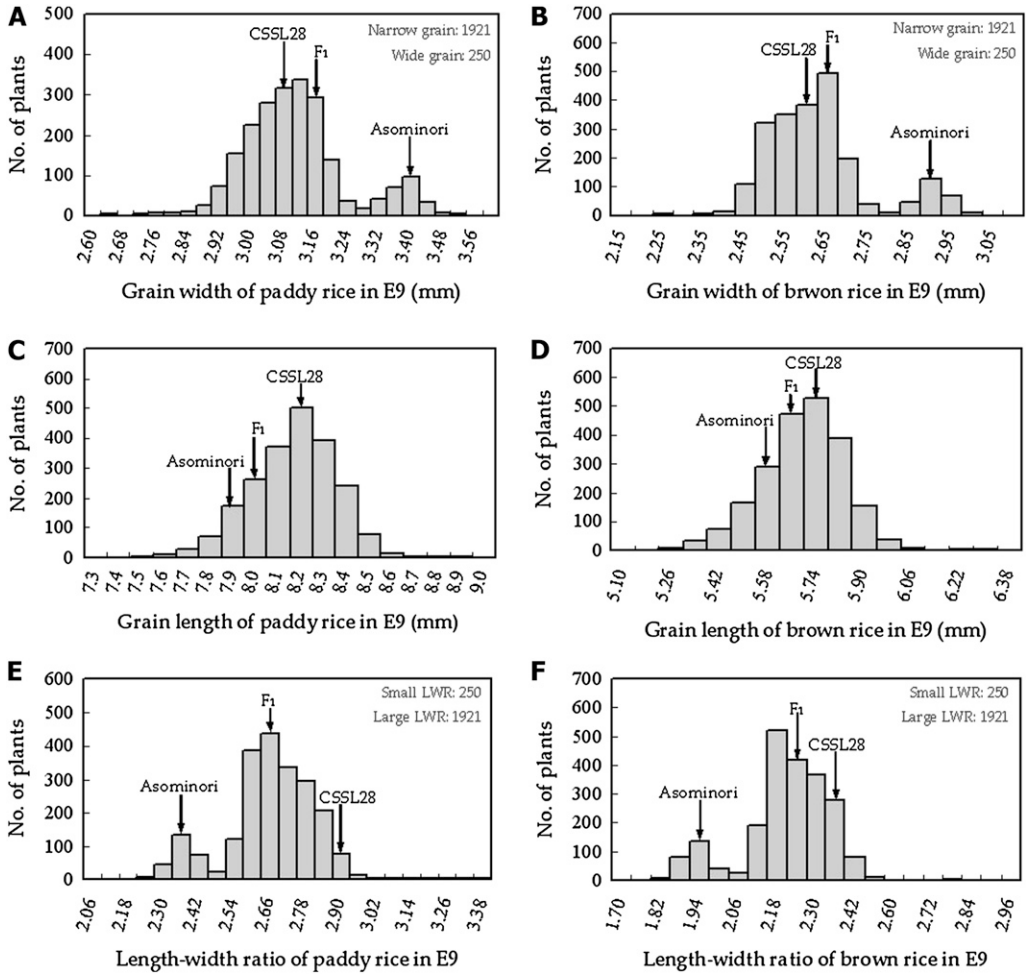


FIGURE 3.—Frequency distributions of grain width, grain length, and length–width ratio in the CSSL28/Asominori BC_4F_2 population (2171 individuals) in E9. (A) Grain width of paddy rice; (B) grain width of brown rice; (C) grain length of paddy rice; (D) grain length of brown rice; (E) length–width ratio of paddy rice; (F) length–width ratio of brown rice.

QTL *qGW-5* controlled both rice grain width and length–width ratio in the BC_4F_2 population: In E9, the phenotypic distributions of grain width of paddy and brown rice in the CSSL28/Asominori F_2 population appeared to be bimodal with boundaries of 3.28 and 2.80 mm, respectively (Figure 3, A and B). The ratio of narrow- (1921) to wide-grain individuals (250) was 7.68:1, which does not fit with the expected segregation ratio of 3:1 for the inheritance of a single gene. Similarly, the ratio of small to large LWR individuals was 1:7.68 (Figure 3, E and F). Interestingly, all 250 small LWR individuals showed wide grains, and all 1921 large LWR plants presented narrow grains. Moreover, significantly negative correlations ($r = -0.94^{**}$ and -0.94^{**}) were observed between grain width and LWR of paddy rice as well as brown rice in the total 2171 BC_4F_2 individuals. Furthermore, although LWR is a complex trait reflecting grain length and width, QTL for grain length had relatively small effects on the phenotypic variation of LWR in the BC_4F_2 population, as shown by Figure 3, C and D, which clearly indicated that minor factor(s) conferred the phenotypic variations of grain length. These results suggest that *qGW-5* mainly affected both rice grain width and LWR in the BC_4F_2 population. This is further supported by the fact that the ratios of narrow- to wide-grain individuals were 7.05:1, 7.36:1, and 7.54:1 in the CSSL28/Asominori F_2 populations that included 1248 plants in E10, 2465 in E11, and 897 in E12 (Figure 4, A–C); their corresponding ratios of small to large LWR individuals were 1:7.05, 1:7.36, and 1:7.54, respectively.

Dissecting QTL *qGW-5* into a single gene, *gw-5*: To dissect *qGW-5* into a single gene, we analyzed four genetic models that could possibly lead to the observed distortion of segregation in the BC_4F_2 population. Wide rice grain could be controlled by one of the following models: (1) two recessive linked genes at the *qGW-5* locus, (2) a recessive gene linked with gamete sterility gene(s), (3) a recessive gene linked with gene(s) for weak germination or seedling viability, or (4) a recessive gene linked with hybrid partial sterility gene(s). If case 1 is correct, genotypes of the markers near *qGW-5* including *Asominori(A)/A*, *A/CSSL28(L)*, and *L/L* should show the normal 1:2:1 ratio in the BC_4F_2 population. However, the ratios of 8 SSR markers in the interval 20.6–39.2cM near *qGW-5* did not exhibit the predicted 1:2:1 ratio (Figure 5, A1–D1). If case 2 is the correct model, the above ratios of 7.68:1, 7.05:1, 7.36:1, and 7.54:1 should correspond to 1:3.9:3.8, 1:3.7:3.4, 1:3.7:

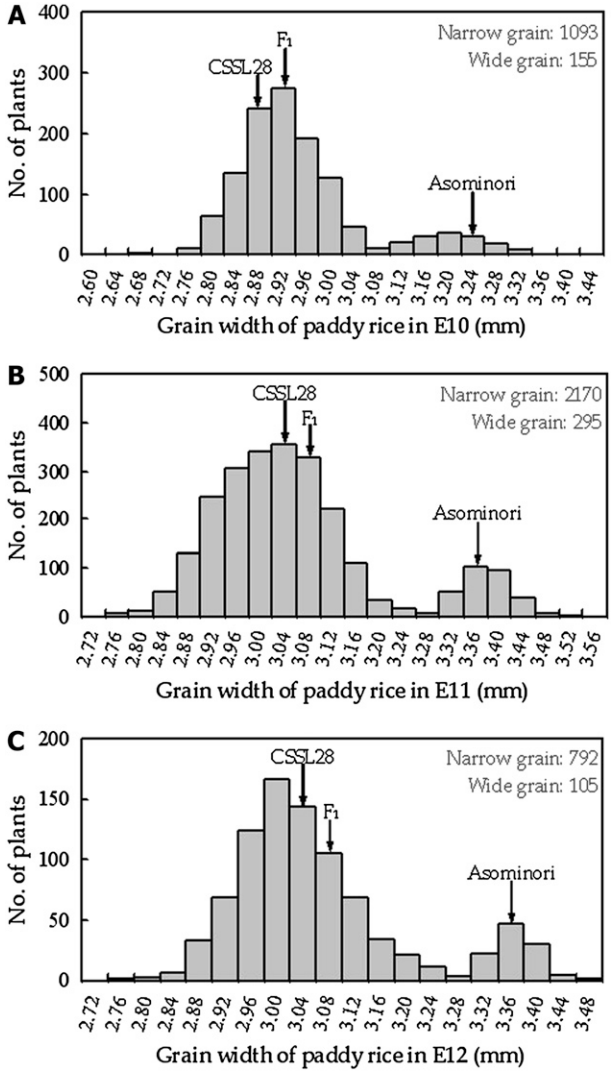


FIGURE 4.—Frequency distributions of grain width of paddy rice in the CSSL28/Asominori F_2 populations grown in E10, E11, and E12. (A) Grain width of paddy rice in E10 (1248 individuals); (B) grain width of paddy rice in E11 (2465 individuals); (C) grain width of paddy rice in E12 (897 individuals).

3.6, and 1:3.9:3.8 for *A/A*:*A/L*:*L/L*, respectively. But the observed actual ratios listed in Figure 5, A1–D1, clearly exclude case 2. Additionally, rice seeds with the genotype of *A/A*, *A/L*, or *L/L* had similar germination ability and seedling viability (supplemental Table 3), ruling out the model presented in case 3.

Several observations support the fourth case. First, complete fertility was observed for Asominori, IR24, and CSSL28, whereas partial sterility occurred in Asominori/CSSL28 F_1 plants, which evidently resulted from hybrid partial sterility gene(s). Second, an *indica–japonica* hybrid partial sterility gene *S31(t)* mapped to 20.9–24.7 cM on chromosome 5 in our laboratory (ZHAO *et al.* 2006) was linked with *qGW-5* located at 36.4–44.9 cM (Table 1). Third, eight SSR markers in the 20.6–39.2cM region showed regularly progressive segregation distortions in the three genotypes, with ratios of *LL* + *AL*/*AA* from 7.33:1 at 39.2 cM to 14.79:1 at 20.6 cM (Figure 5, A1), indicating that the closer the distance from the SSR markers to the *S31(t)* gene, the stronger the degree of segregation distortion. Thus, the *S31(t)* gene played a crucial role in inducing the distortion of the ratios. In contrast, four SSR markers (RM163, RM430, RM506, and RM408) far from the *qGW-5* locus showed a nearly normal ratio of 3:1 for *LL* + *AL*/*AA* (Figure 5, A1). Similar results were also observed in E10–E12 (Figure 5, B1–D1). Additionally, when 300 randomly selected BC_4F_2 plants were divided into three groups on the basis of the three genotypes of the RMw513 marker, 35, 37, 42, and 36 plants with the genotype *A*/*A* in E9–E12, respectively, showed wide rice grains (Figure 5, A2–D2), whereas all other narrow-grain plants presented the genotype of *A/L* or *L/L*, indicating that the distorted segregation of marker genotypes was consistent with that of grain-width phenotype. Finally, all individuals in 35 BC_4F_3 families with the genotype *A/A* at the RMw513 locus showed wide grains without segregation. Of 2408 BC_4F_3 plants derived from 152 BC_4F_2 plants with the genotype *A/L*, the ratio of narrow- (2124) to wide-grain plants (284) was 7.48:1, consistent with that in the BC_4F_2 population (Figures 3 and 4). These findings clearly indicate that *qGW-5* behaves like a single gene in the CSSL28/Asominori F_2 population and that both wide rice grains and small length–width ratio are mainly controlled by a recessive *qGW-5* allele, named *gw-5*.

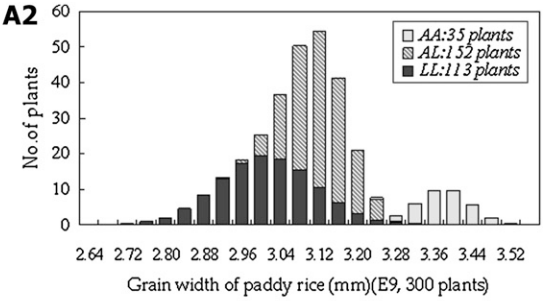
Environmental impact on the effect of the *gw-5* gene: Since wide rice grain was controlled by the *gw-5* gene in the BC_4F_2 population, the environmental impact on the effect of the *gw-5* gene could be evaluated by comparing the average grain width of BC_4F_2 individuals with the genotype of *gw-5/gw-5* among the E9–E12 environments. The largest effect of the *gw-5* gene occurred in E9 and E11, with average grain widths of 3.36 ± 0.05 and 3.36 ± 0.04 mm, and 2.88 ± 0.04 and 2.88 ± 0.05 mm for paddy rice and brown rice, respectively; *gw-5* had the smallest effect in E10, in which paddy rice and brown rice had average grain widths of 3.20 ± 0.05 and 2.66 ± 0.04 mm, respectively. Moreover, significant differences of grain width were observed between E9/E11 and E12, as well as E12 and E10 (Table 3), thus showing that the environment had a significant impact on the effect of the *gw-5* gene. Likewise, a similar environmental impact was also found on the effect of the *GW-5* gene (supplemental Table 4).

Fine mapping of the *gw-5* gene in a recombination hotspot region on chromosome 5: For fine mapping, 805 wide-grain homozygotes of the total 6781 BC_4F_2 individuals were used to calculate the recombination frequency between the *gw-5* gene and the surrounding molecular markers. Using previously published SSR and EST markers, we detected three SSR markers (RM3328, RM3322, and RM5874) and one EST marker (C53703)

A1

Chr.	Location	Marker	*AA*	*AL*	*LL*	*LL+AL/AA*
5	20.6 cM	RM3345	19	151	130	14.79 : 1
5	27.7 cM	RM7444	25	157	118	11.00 : 1
5	36.4 cM	RM3328	33	153	114	8.09 : 1
5	37.2 cM	RMw534	34	152	114	7.82 : 1
5	37.2 cM	RMw535	34	152	114	7.82 : 1
5	38.3 cM	RMw513	35	152	113	7.57 : 1
5	38.6 cM	RMw522	35	147	118	7.57 : 1
5	39.2 cM	RMw528	36	153	111	7.33 : 1
5	78.7 cM	RM163	69	158	73	3.35 : 1
5	78.7 cM	RM430	70	152	78	3.29 : 1
8	0 cM	RM506	73	155	72	3.11 : 1
8	0.5 cM	RM408	72	158	70	3.17 : 1

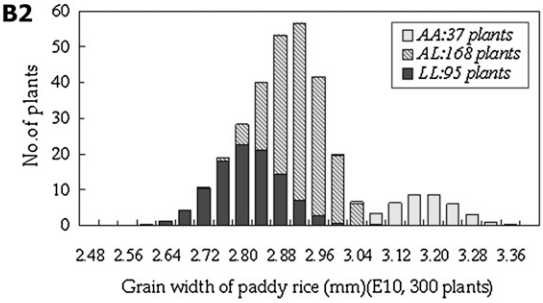
A2



B1

Chr.	Location	Marker	*AA*	*AL*	*LL*	*LL+AL/AA*
5	20.6 cM	RM3345	19	160	121	14.79 : 1
5	27.7 cM	RM7444	25	160	115	11.00 : 1
5	36.4 cM	RM3328	31	169	100	8.68 : 1
5	37.2 cM	RMw534	35	169	96	7.57 : 1
5	37.2 cM	RMw535	36	167	97	7.33 : 1
5	38.3 cM	RMw513	37	168	95	7.11 : 1
5	38.6 cM	RMw522	37	173	90	7.11 : 1
5	39.2 cM	RMw528	39	171	90	6.69 : 1
5	78.7 cM	RM163	68	151	81	3.41 : 1
5	78.7 cM	RM430	70	146	84	3.29 : 1
8	0 cM	RM506	70	149	81	3.29 : 1
8	0.5 cM	RM408	73	149	78	3.11 : 1

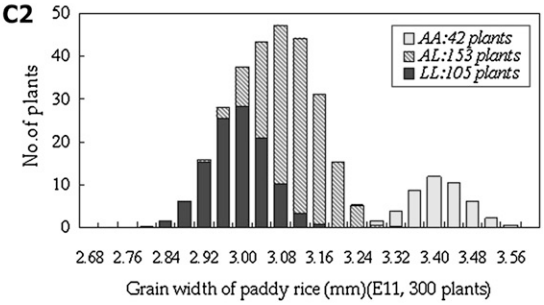
B2



C1

Chr.	Location	Marker	*AA*	*AL*	*LL*	*LL+AL/AA*
5	20.6 cM	RM3345	21	148	131	13.29 : 1
5	27.7 cM	RM7444	27	148	125	10.11 : 1
5	36.4 cM	RM3328	38	154	108	6.89 : 1
5	37.2 cM	RMw534	42	153	105	6.14 : 1
5	37.2 cM	RMw535	42	153	105	6.14 : 1
5	38.3 cM	RMw513	42	153	105	6.14 : 1
5	38.6 cM	RMw522	42	156	102	6.14 : 1
5	39.2 cM	RMw528	42	152	106	6.14 : 1
5	78.7 cM	RM163	72	158	70	3.17 : 1
5	78.7 cM	RM430	72	154	74	3.17 : 1
8	0 cM	RM506	79	139	82	2.80 : 1
8	0.5 cM	RM408	77	143	80	2.90 : 1

C2



D1

Chr.	Location	Marker	*AA*	*AL*	*LL*	*LL+AL/AA*
5	20.6 cM	RM3345	22	150	128	12.64 : 1
5	27.7 cM	RM7444	28	159	113	9.71 : 1
5	36.4 cM	RM3328	33	159	108	8.09 : 1
5	37.2 cM	RMw534	35	158	107	7.57 : 1
5	37.2 cM	RMw535	35	158	107	7.57 : 1
5	38.3 cM	RMw513	36	156	108	7.33 : 1
5	38.6 cM	RMw522	37	157	106	7.11 : 1
5	39.2 cM	RMw528	38	156	106	6.89 : 1
5	78.7 cM	RM163	70	158	72	3.29 : 1
5	78.7 cM	RM430	74	155	71	3.05 : 1
8	0 cM	RM506	70	159	71	3.29 : 1
8	0.5 cM	RM408	73	155	72	3.11 : 1

D2

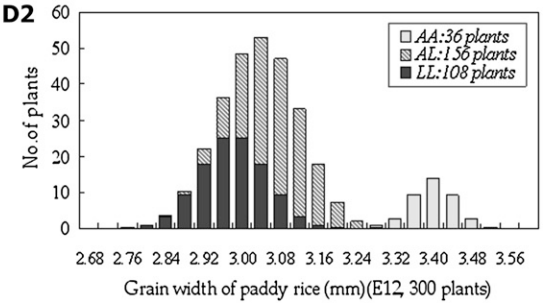


FIGURE 5.—The ratios of three genotypes of SSR markers and frequency distributions of grain width in 300 BC_4F_2 individuals across four environments (E9–E12). A1–D1 represent the ratios of three genotypes of 12 SSR markers in E9–E12, respectively; A2–D2 show the frequency distributions of grain width at the RMw513 locus in E9–E12, respectively.

on one side of the *gw-5* gene, and one SSR marker (RM5994) on the other side (Figure 6C). Ten polymorphic SSR markers were then designed using DNA sequences of seven BAC/PAC contigs in Nipponbare (supplemental Table 2S and Figure 6B). Among the markers, RMw530 and RMw513 had 23 and 6 recombinants with the *gw-5* gene in the 805 homozygotes, respectively (Figure 6C). Thus, the *gw-5* gene was located in the 1.8-cM genetic region on chromosome 5. Searching for the contigs OJ1097_A12 (harboring RMw530) and B1007D10 (carrying RMw513) in the Nipponbare genome (http://www.gramene.org), we found that RMw530 and RMw513 reside at 5,309,078 and 5,358,806 bp on chromosome 5, respectively. Thus, the *gw-5* gene was narrowed down to a 49.7-kb genomic region (Figure 6C).

The 17.1-cM genetic region (RM5874–RM5994) can be divided into 10 intervals, with ratios of physical-to-genetic distance of 226.3, 261.7, 183.9, 68.9, 27.6, 40.0, 14.2, 257.3, 267.4, and 255.4 kb/cM (Figure 7). The *gw-5* gene was located in the RMw530–RMw513 interval with the kb/cM of 27.6. In rice, the genomewide average is estimated at 244 kb/cM (CHEN *et al.* 2002), indicating that the crossover frequency between RMw530 and RMw513 was approximately nine times that of the whole genome in rice.

On the basis of the available sequence annotation, we found five predicted candidate genes in the 49.7-kb

TABLE 3

Comparison of grain width in the BC_4F_2 individuals with wide-grain phenotype (*gw-5/gw-5*) across four environments (E9–E12)

Environment	Number of individuals	Grain width of paddy rice			Grain width of brown rice		
		Mean (mm)	Standard variation	TSR[a]	Mean (mm)	Standard variation	TSR[a]
E9	250	3.36	0.05	A	2.88	0.04	A
E11	295	3.36	0.04	A	2.88	0.05	A
E12	105	3.33	0.03	B	2.76	0.06	B
E10	155	3.20	0.05	C	2.66	0.04	C

[a] Tukey's studentized range.

region (http://www.ncbi.nlm.nih.gov/BLAST; http://www.softberry.com). Of these genes, one had unknown function, and the other four encoded an auxin-responsive protein IAA16, a 20S proteasome β-subunit, a hexokinase 6, and the Ulp1 protease-like protein. This result will be useful in the molecular cloning and functional characterization of the *gw-5* gene.

DISCUSSION

Dissection of the QTL *qGW-5* into a recessive gene *gw-5* despite the nontraditional Mendelian segregation ratio: In this study, QTL *qGW-5* was separated as a recessive gene, *gw-5*, and narrowed down to a 49.7-kb genomic region, though the segregation ratios did not fit with the inheritance mode of a single Mendelian factor under multiple environments (Figures 3–6). Several observations support this conclusion:

1. The ratios of narrow- to wide-grain individuals were 7.68:1, 7.05:1, 7.36:1, and 7.54:1 in the CSSL28/Asominori BC_4F_2 population in four environments (E9–E12) (Figures 3 and 4), indicating that the environment likely had little effect on the distorted segregation ratios.
2. A similar distorted segregation ratio (*i.e.*, 7.48:1) was also observed in the BC_4F_3 population, excluding the possibility that the distorted segregation could be attributed to the partial false F_1 hybrid seeds of CSSL28/Asominori.

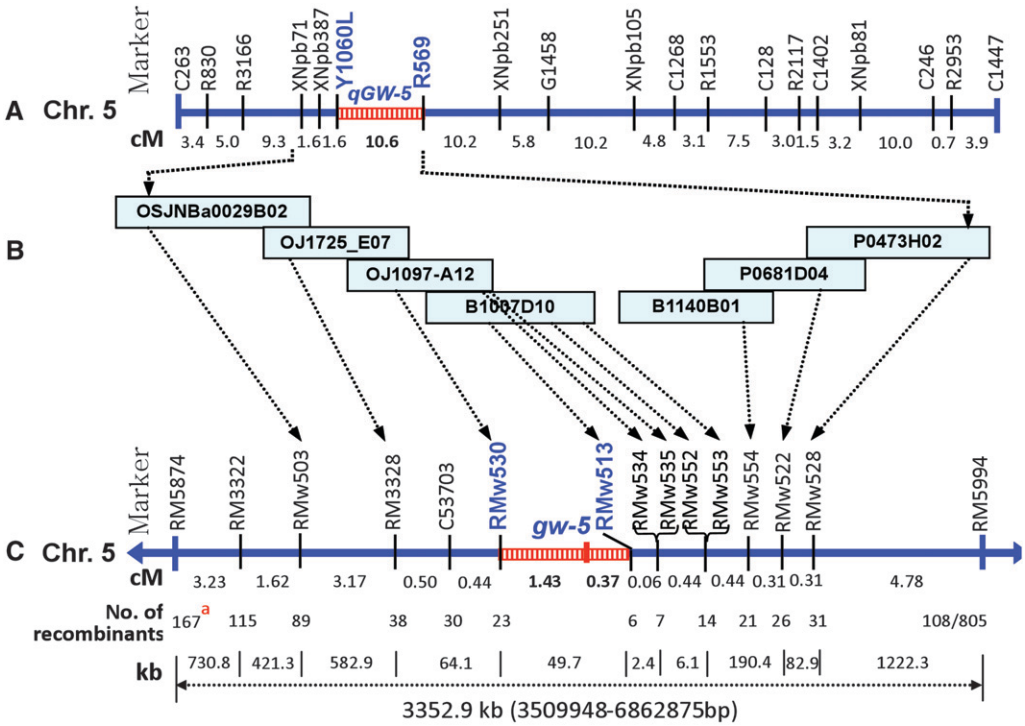


FIGURE 6.—Genetic and physical maps of the *gw-5* gene on rice chromosome 5. (A) QTL mapping of the *qGW-5* locus. (B) Six BAC/PAC contigs around the *gw-5* locus. (C) Fine mapping of *gw-5* using newly developed SSR markers. The red superscript a indicates that there are 167 recombinants of the total 805 homozygous plants between the *gw-5* gene and the marker RM5874. In fact, the 167 recombinants included 113 wide-grain plants with the heterozygous band patterns of wide- and narrow-grain parents and 27 wide-grain plants with the band pattern of the narrow-grain parent. The recombination frequency = $(N_1 + N_2/2)/N$, where N is the total number of wide-grain plants surveyed, N_1 is the number of wide-grain plants with the band pattern of the narrow-grain parent, and N_2 is the number of wide-grain plants with the heterozygous band patterns. We marked the number of recombinants between RM5874 and the *gw-5* gene as 167 to directly calculate the recombination frequency, $[(27 \times 2 + 113 = 167)/(805 \times 2)] \times 100$ (cM). The other numbers marked in the row (no. of recombinants) were also calculated on the basis of this approach.

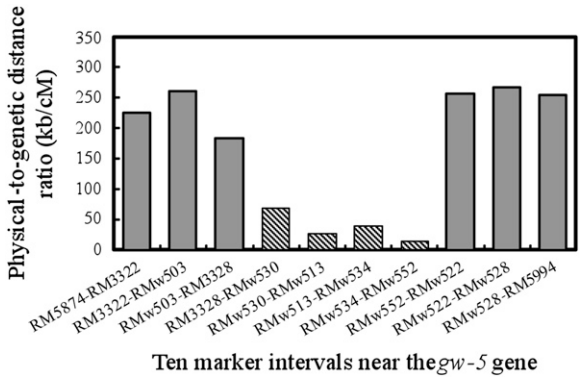


FIGURE 7.—Physical-to-genetic distance ratios in 10 marker intervals near the *gw-5* gene.

3. Eight SSR markers in the 20.6–39.2 cM region on rice chromosome 5 showed progressive distortions of segregation in three genotypes, with ratios of (*GW-5/GW-5* + *GW-5/gw-5*)/*gw-5/gw-5* from 6.14 at 39.2 cM to 14.79 at 20.6 cM (Figure 5, A1–D1), showing that the closer the SSR markers to the hybrid partial-sterility gene *S31(t)* (ZHAO *et al.* 2006), the stronger the degree of segregation distortion. However, four SSR markers located far from *qGW-5* and the *S31(t)* gene showed a nearly normal ratio of 3:1 (Figure 5, A1–D1).
4. Complete fertility was observed for Asominori, IR24 and CSSL28, whereas partial sterility occurred in Asominori/CSSL28 F_1 plants, which evidently resulted from the hybrid partial sterility. Thus, the *S31(t)* gene should play a crucial role in triggering the distorted ratios of marker genotypes near the *gw-5* gene.
5. Among 300 BC_4F_2 plants, 35, 37, 42, and 36 plants with the *gw-5/gw-5* genotype in E9–E12, respectively, showed wide rice grains (Figure 5, A2–D2), whereas all other narrow-grain plants presented the genotype of *GW5/gw-5* or *GW-5/GW-5*. This indicated that the distorted segregation of marker genotypes was consistent with that of phenotypic values of grain width.
6. Rice seeds with the genotype of *GW-5/GW-5*, *GW-5/gw-5*, or *gw-5/gw-5* had similar germination ability and seedling viability (supplemental Table 3), which ruled out the possibility that a gene(s) for weak germination or seedling viability linked to the *gw-5* gene resulted in the distorted segregation in the BC_4F_2 population.
7. We used 805 wide-grain plants and 10 SSR markers to narrow down the *gw-5* gene to a 49.7-kb genomic region on rice chromosome 5 (Figure 6), further demonstrating that *qGW-5* corresponded to the recessive *gw-5* gene. Therefore, although it did not show the inheritance mode of a single gene in the BC_4F_2 population, *qGW-5* was separated as the *gw-5* gene, and the observed distorted segregation might be caused by its linkage to the hybrid partial-sterility *S31(t)* gene located at a genetic distance of ~15.0 cM (Figure 6; ZHAO *et al.* 2006).

Dissection of the genetic modes of QTL underlying complex quantitative traits: The QTL *qGW-5* allele, *gw-5*, was narrowed down to a 49.7-kb genomic region exhibiting recombination hotspots (Figures 3–7), illustrating that a QTL could be treated as a single Mendelian factor to perform research on functional genomics of a quantitative trait. Using a strategy of forward genetics, many QTL have been dissected into single genes for fine mapping and map-based cloning in recent years. The genetic modes of QTL established as single Mendelian factors can be categorized into at least six different types (Figure 8, A–F), *i.e.*, the allele of a QTL corresponding to (1) a dominant major gene for the phenotypic distribution with a discrete boundary and a ratio of 3:1 (*e.g.*, in rice, FAN *et al.* 2006; WAN *et al.* 2006; and in wheat, CUTHBERT *et al.* 2006) or (2) with a partially overlapping boundary and a ratio of 3:1 in the secondary F_2 population (*e.g.*, in rice, WAN *et al.* 2004b; YAMAMOTO *et al.* 2000); (3) an additive major gene for the phenotypic distribution with two discrete boundaries and a ratio of 1:2:1 (*e.g.*, in soybean, YAMANAKA *et al.* 2005) or (4) with two partially overlapping boundaries and a ratio of 1:2:1 (*e.g.*, in rice, YAMAMOTO *et al.* 1998); (5) an additive major gene for the continuous distribution in a secondary F_2 population and a ratio of 1:2:1 in F_3 families (*e.g.*, in rice, MONNA *et al.* 2002; TAKEUCHI *et al.* 2003; and in wheat, RÖDER *et al.* 2008) or (6) a non-Mendelian segregating major gene linked to other gene(s) for gamete sterility, hybrid partial sterility, weak germination, or seedling viability (this study). These modes not only disclose the complex genetic properties of QTL, but also provide some useful rules of how to interpret the results with non-Mendelian segregation ratios in advanced backcross progenies when establishing QTL into single genes and fine mapping QTL. Since the first use of a complete RFLP linkage map to identify QTL controlling fruit mass, concentration of soluble solids, and fruit pH in the tomato by PATERSON *et al.* (1988), >1000 QTL regulating agronomic and economic traits in crops have been identified, but only a few have been precisely mapped and isolated. One important reason may be the complex genetics of QTL as described above. Therefore, greater research contribution by the geneticists in the field of QTL mapping is required so that more QTL will be dissected into individual genes on the basis of the genetic modes of different QTL (Figure 8) and fine mapping and map-based cloning carried out. In addition, overlapping substitute lines have been used to achieve high resolution mapping of QTL in plants (PATERSON *et al.* 1990; ESHED and ZAMIR 1995). Recently, a reverse genetics approach was employed for fine mapping of QTL, in which recombinants in a target region harboring a QTL of interest were identified by comprehensive genotyp-

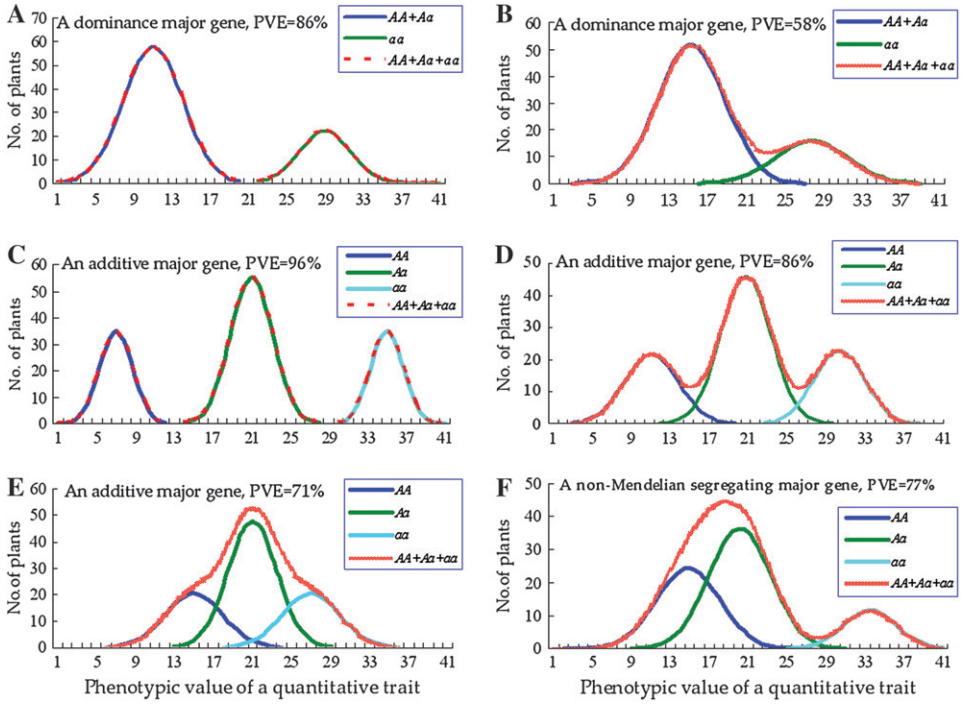


FIGURE 8.—Six models for dissecting a QTL into a single gene in a secondary F_2 population. (A) The allele of a QTL belongs to a dominance major gene leading to a ratio of 3:1 with a discrete boundary. (B) The allele of a QTL is a dominant major gene resulting in a ratio of 3:1 with an overlapping boundary. (C) The allele of a QTL belongs to an additive major gene contributing to a ratio of 1:2:1 with two discrete boundaries. (D) The allele of a QTL is an additive major gene leading to a ratio of 1:2:1 with two overlapping boundaries. (E) The allele of a QTL belongs to an additive major gene resulting in the continuous distribution in a secondary F_2 population, and a ratio of 1:2:1 can be observed by investigating F_3 families. (F) The allele of a QTL is a non-Mendelian segregating major gene linked to other gene(s) for gamete sterility, hybrid sterility, weak germination ability, and/or weak seedling viability.

ing followed by phenotyping of the resultant informative recombinants (LI *et al.* 2004a). By combining this method with tests of advanced backcross progenies, several QTL were precisely localized in narrow genomic regions (CHEN and TANKSLEY 2004; THOMSON *et al.* 2006). Furthermore, QTL isogenic recombinant analysis has been developed to speed up fine mapping of a QTL with a single population (PELEMAN *et al.* 2005). The progress on QTL fine mapping would facilitate research on the functional genomics of complex quantitative traits and provide new insights into the genetic theory of quantitative traits.

The significance of this work to the basic understanding of genetic principles of QTL across taxa in plants: Classical quantitative genetics describes the aggregate behavior of suites of genes influencing a quantitative trait. However, an understanding of quantitative inheritance at the molecular level requires detailed descriptions on genetic properties of individual genes controlling the quantitative trait, which is made possible by QTL mapping (PATERSON 1995). Basic properties of individual QTL, such as additivity, dominance, and overdominance, have been analyzed, and a wide range of different modes of gene action is evident for various QTL (PATERSON *et al.* 1991; REDOÑA and MACKILL 1996). Many basic genetic phenomena are reconciled with the results from QTL mapping, such as transgression, heterosis, the number of genes for a quantitative trait, and the importance of epistasis (PATERSON 1995; YANO and SASAKI 1997). However, several key genetic questions from most identified QTL in plants still remain unclear, *e.g.*, the number of genes at individual QTL, the kinds of phenotypic distributions controlled by individual QTL alleles, and the types of genetic effects of individual QTL alleles. It is relatively difficult to answer these questions by primary QTL mapping strategies on the basis of F_2, RIL, DH, and BC populations, while CSSLs or NILs harboring target QTL are useful for in-depth exploration of genetic modes and effects of QTL in plants. In this study, we used BC_4F_2 and BC_4F_3 populations to identify the QTL *qGW-5* corresponding to a single gene *gw-5* with a recessive genetic effect. However, the phenotypic segregation ratio controlled by the *gw-5* gene did not fit the 3:1 of a single-gene inheritance (Figures 3–6). On the basis of this finding, we summarized at least six different modes of how to dissect a QTL into a single gene, which can be applied in plants such as rice, soybean, and wheat. These modes not only enable researchers in the field of QTL mapping to dissect more QTL into individual genes, but also clearly indicate the types of genetic effects of QTL alleles on the basis of phenotypic distributions in secondary F_2 and F_3 populations (Figure 8). More importantly, this study provides a classic example of the dissection of genetic properties and modes of identified QTL and further performance of QTL fine mapping when distorted phenotypic distributions are observed in advanced backcross progenies. Therefore, this report describes significant findings for dissecting and furthering the understanding of genetic principles of QTL. Furthermore, the research strategies used in this study are applicable among other taxa in plants.

Additionally, cereal species such as rice (Oryza), maize (Zea), sorghum (Sorghum), and wheat (Triti-

cum) began to diverge from a common ancestor only ~50–70 million years ago and have undergone largely parallel selection regimes associated with domestication and improvement over the past several thousand years (Paterson *et al.* 2003). This makes it possible to perform comparative mapping among these cereal species. As a result, a close correspondence among QTL affecting complex traits such as seed size has been demonstrated in sorghum, sugarcane, maize, wheat, barley, and rice (Paterson *et al.* 1995). Thus, QTL analysis in one taxon may provide significant clues to similar findings in other taxa, and correspondence of QTL across diverse taxa also provides strong empirical support for the use of model systems in research of complex phenotypes in plants (Paterson 1995). On the other hand, the rice genome sequence provides a platform for comparing and integrating genomic information about diverse cereals and together with genetic maps and sequence samples from other cereals yields new insights into both the shared and independent dimensions of cereal evolution (Paterson *et al.* 2003). Therefore, the results of genetics and QTL fine mapping of rice seed dimensions obtained here are applicable toward the molecular dissection of evolution and domestication among cereal species.

A potential application of the *gw-5* gene for improving grain quality of rice on the basis of molecular design breeding strategy: Biological data (including QTL/gene mapping) continue to dramatically accumulate with the rapid development of biotechnology and genomics. Nevertheless, the lack of appropriate tools and methods of simulation breeding renders it difficult to integrate this information into traditional crop breeding. Strategies such as molecular design breeding (MDB) can resolve, at least partly, this conflict and facilitate crop breeding. Before going to the field, breeders design a blueprint for obtaining particular breeding objectives, by which the crossing and selection process can be simulated and optimized, thus greatly enhancing breeding efficiency (Wang *et al.* 2007a,b). Until now, most of the successful research on MDB or MAS breeding has been conducted on resistance to diseases or insects conferred by major genes, but few efforts have addressed the improvement of complex quantitative traits, possibly due to the lack of information on QTL epistasis, QEI effects, gene action of QTL, and markers closely linked to target QTL (Li 2001). On the other hand, the continuous phenotypic distribution in progeny and measurement of rice grain width and shape after the reproductive stage make it difficult for breeders to efficiently improve grain appearance using conventional selection methods. In addition, rice grain width and shape can greatly affect other important quality traits such as endosperm chalkiness, milling efficiency, cooking texture, and palatability of cooked rice, and play an important role in determining rice grain yield. Therefore, grain width and shape are good candidate traits for MDB in rice breeding programs.

In the meantime, *qGW-5* can be recommended as a desirable QTL for improving rice grain quality due to the following observations:

1. *qGW-5* has been shown to control rice grain shape not only in Indica/Japonica (Xu *et al.* 2002; Tan *et al.* 2005; Wan *et al.* 2005, 2006) and Indica/Indica (Lin *et al.* 1995; Tan *et al.* 2000; Lin and Wu 2003) populations, but also in a cultivated rice germplasm collection based on the whole-genome association mapping strategy (Iwata *et al.* 2007). The rice germplasm set consists of 281 landraces and 51 modern cultivars originating from 23 rice-producing countries and is considered extremely useful for rice improvement in breeding experiments (Kojima *et al.* 2005). Therefore, *qGW-5* should be applicable and important in the improvement of grain appearance quality in traditional and molecular rice breeding programs.
2. The expression stability of *qGW-5* is relatively high both at the level of multiple QTL (Tables 1 and 2) and an individual QTL allele across multiple environments (Figure 2).
3. *qGW-5* accounts for a large proportion of the overall phenotypic variation (Table 1; Wan *et al.* 2005).
4. Dissecting *qGW-5* into the *gw-5* gene as well as its fine mapping make it a desirable candidate for MDB, as the deleterious gene(s) near the *gw-5* allele can be easily removed by MAS, and 11 flanking markers within a 2.0-cM distance (Figure 6C) can be readily used by breeders for MAS of the *gw-5* into elite cultivars.

Additionally, other major QTL for grain length (Wan *et al.* 2006), endosperm chalkiness (Wan *et al.* 2004b), starch viscosity (rapid viscosity analyzer profile characteristics), amylose content, and protein content (Wan *et al.* 2004a; Weng *et al.* 2006; our unpublished data) in rice have also been characterized in detail, dissected into single genes, and precisely mapped. The MDB methodology and computer programs have recently been developed and employed by our laboratory (Wang *et al.* 2004, 2007a,b; Li *et al.* 2007). Thus, QTL/gene pyramiding on the basis of MDB and MAS strategies should be feasible for simultaneously improving multiple rice quality traits. In this regard, the field experiment work is ongoing.

Localization of the *gw-5* gene in the recombination hotspot region: Recombination at the meiotic stage plays an important role in genome evolution, crop crossbreeding, and map-based cloning of genes/QTL. In this study, four recombination hotspot regions (Figure 7) corresponded to three BAC/PAC contigs (OJ1725_E07, OJ1097_A12, and B1007D10) in Nipponbare (Figure 6B). We found that the G + C contents of OJ1725_E07, OJ1097_A12, and B1007D10 contigs were 40.3, 41.4,

and 42.5%, the densities of the predicted genes were 13.5, 11.3, and 16.3 (genes/100 kb), and the percentages of repetitive sequences were 9.8, 10.2, and 8.4%, respectively. Thus, the relatively high densities of the predicted genes and low repetitive sequences were found to be comparable to those in the hotspot regions of rice chromosome 1 (Wu *et al.* 2003) and in the genomes of Arabidopsis and other cereals (Arabidopsis Genome Initiative 2000; Ware and Stein 2003). The G + C content in these hotspot regions was almost the same as in the hotspot regions of rice chromosome 1 (Wu *et al.* 2003), but relatively lower than that in hotspot regions of Arabidopsis, yeast, and human (Jeffreys *et al.* 1998; Gerton *et al.* 2000), indicating that a specific mechanism may exist for activating recombination in the hotspots of rice chromosomes 1 and 5. This novel finding creates an opportunity, as well as poses a challenge, for molecular geneticists in the further exploration of the relationship between fine structure and crossover frequency of rice chromosomes by dissecting the mechanism of high recombination frequencies in the four regions surrounding the *gw-5* gene between the two parents (Asominori and CSSL28). Similarly, linkage mapping strategies have been used to investigate the recombination hotspots on chromosomes of a wide variety of organisms, *e.g.*, wheat (Faris *et al.* 2000), neurospora (Yeadon *et al.* 2004), mouse (Kelmenson *et al.* 2005), and human (Minin *et al.* 2007).

We greatly thank A. Yoshimura, Kyushu University, Japan, for kindly providing us with the RIL and CSSL populations and genotype data. We also thank San Francisco Edit (http://www.sfedit.net) for kindly proofreading, editing, and refining the English of this manuscript. This research was supported by a grant from the National High Technology Research and Development Program of China (2003AA222131) and the project 111 program.

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Communicating editor: A. H. Paterson