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Full Length Research Paper

Molecular genetic analysis on percentage of grains with chalkiness in rice (*Oryza sativa* L.)

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Percentage of grains with chalkiness (PGWC) is an important factor in determining the appearance quality of milled rice. A total of 10 main-effect quantitative trait loci QTLs were identified in four environments using 71 recombinant inbred lines (RILs) derived from a cross between Asominori (*Japonica*) and IR24 (*Indica*). These QTLs were further investigated using target chromosome segmental substitution lines (CSSLs) harboring them. The PGWC of the target CSSLs, harboring *qPGWC-9*, *qPGWC-3* and *qPGWC-5* alleles, was significantly different from Asominori ($P < 0.05$), indicating these three QTLs with relatively high stability. Importantly, PGWC of CSSL64 was significantly higher than that of Asominori in all the eight environments. We constructed an F_2 secondary population derived from Asominori × CSSL64 to explore the genetics of CSSL64 with high PGWC and *qPGWC-9* was identified repeatedly in the three consecutive years (2005 to 2007). Further restriction in the interval of RM23958-RM1328 on chromosome 9, explained 19.3% of the phenotypic variance by its F_3 population. These results will be useful in fine-mapping and cloning of the *qPGWC-9* allele and marker-assisted selection in rice high quality breeding programs.

Key words: Chromosome segmental substitution line (CSSL), recombinant inbred line (RIL), percentage of grains with chalkiness (PGWC), rice.

INTRODUCTION

Low percentage of grains with chalkiness (PGWC) is an important target in breeding for appearance of quality in rice. Rice cultivars with little or no chalkiness in the rice endosperm are preferred by consumers, because PGWC is closely related to milling quality. Chalky grains have a lower density of starch granules than vitreous ones and are therefore more prone to breakage during milling (Del Rosario et al., 1968). Rice cultivars with more than 20% chalky kernels are not generally acceptable in the world's markets (ISO, 2002).

To improve the efficiency of breeding for rice quality, it is necessary to understand the genetic mechanism of some important rice quality traits. Many studies have shown that PGWC is a quantitative trait affected by genetic background and environmental conditions (especially weather) during the grain-filling period (Yamakawa et al., 2007). If marker assisted selection (MAS) strategies are to be applied for this trait, it is very important to identify the underlying major quantitative trait loci (QTLs). Over 40 QTLs for PGWC have been detected in rice 12 chromosomes (He et al., 1999; Tan et al., 2000; Yoshida et al., 2002; Zeng et al., 2002; Li et al., 2003; Septiningsih et al., 2003; Kobayashi et al., 2007; Li et al., 2004; Tabata et al., 2007; Ebitani et al., 2008). Additionally, Zhou et al. (2009) used an F_2 population from the cross C51 × 9311 to perform fine mapping of *qPGWC-7*, which was located into a 44 kb chromosome fragment containing 13 predicted genes. Meanwhile, at least six genes, including *OsPPDKB* (Kang et al., 2005), starch synthase *IIIa* (*SSIIIa*) (Fujita et al., 2007), *GIF1* (Wang et al., 2008), *GW2* (Song et al., 2007), *UGPase* (Koh et al., 1999; Woo et al., 2008) and *Pho1* (Satoh et al., 2008), showed pleiotropic effects for producing chalky

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Table 1. Characteristics of the eight environments where the RIL and CSSL populations were grown.

Environment	Population	Location
E1	RILs, CSSLs	Nanjing, China, N 31.2°, E 118.4°, May-October, 2002
E2	RILs, CSSLs	Jinhu, Jiangsu, China, N 32.7° , E 119.6°, May- October, 2002
E3	RILs, CSSLs	Donghai, Jiangsu, China, N 35.1°, E 118.4°, May- October, 2002
E4	RILs, CSSLs	Rice Breeding Base of Lingshui County, Sanya, Hainan, China, N 18.2°, E 108.9°, December, 2002-May, 2003
E5	CSSLs	Nanjing, China, N 31.2°, E 118.4°, May- October, 2001
E6	CSSLs	Jinhu, Jiangsu, China, N 32.7° , E 119.6°, May- October, 2001
E7	CSSLs	Donghai, Jiangsu, China, N 35.1°, E 118.4°, May- October, 2001
E8	CSSLs	Rice Breeding Base of Lingshui County, Sanya, Hainan, China, N 18.2°, E 108.9°, December, 2001-May, 2002

endosperm. All of these results indicated that rice starch grain development and endosperm transparency were not only controlled by multiple genetic factors, but were also affected by environmental conditions (Tashiro and Wardlaw, 1991).

Stable expression of QTLs across environments is one of the most important factors for MAS application of target genes/QTL. In this study, a set of recombinant inbred lines (RILs) derived from Asominori (a *Japonica* cultivar) × IR24 (an *Indica* cultivar) and a set of chromosome segmental substitution lines (CSSLs) created using Asominori as the recurrent parent and IR24 as the donor parent, were used to investigate the genetic basis of PGWC in rice grown under different conditions. The objectives were to: (1) detect major QTLs affecting PGWC in the two sets of mapping populations grown in multiple environments; (2) identify a stable QTL by using secondary F_2 and F_3 populations derived from a cross between the target CSSL64 and the recurrent parent Asominori; (3) locate stable QTL to a smaller chromosomal segment; (4) obtain markers for MAS breeding in rice.

MATERIALS AND METHODS

Plant materials

Four populations, RILs, CSSLs and F_2 and F_3 secondary populations, were used in this study. 71 RILs were derived from Asominori × IR24 by single-seed descent (Tsunematsu et al., 1996). To produce a series of CSSLs in a largely Asominori background, 19 selected RILs were crossed and then backcrossed with Asominori, without selection, until the BC_3F_1 generation when 66 individuals were selected on the basis of a whole genome survey (116 RFLP loci). These plants, denoted as CSSL1-CSSL66 had characteristics representative of the whole IR24 genome (Kubo et al., 1999; Wan et al., 2004). One CSSL and CSSL64, with significantly higher PGWC than the background parent Asominori in all the eight environments was again backcrossed to Asominori and the progeny were used to produce secondary F_2 and F_3 populations.

Phenotypic data collection

Asominori, IR24 and their 71 RILs were grown in four environments (E1-E4) and the parental varieties and their 66 CSSLs in eight environments (E1-E8) (Table 1). Each experimental plot consisted of two replicates represented by 10 rows each of 10 plants grown in a randomized block design. At maturity, each plot was harvested in bulk. After drying, the milled rice thus obtained was used for determining PGWC.

Using a spacing pattern of 25 cm (between rows) × 13.3 cm (within rows), 526, 4,000 and 96 F_2 individuals were planted in Nanjing in 2005, 2006 and 2007, respectively. Meanwhile, 224 F_3 individuals were planted at Nanjing in 2008. After drying (to about 13.5% moisture content), the grain was milled for determining PGWC. To separate chalky from vitreous grains, we assessed 100 grains per entry on a chalkiness visualizer constructed at the China National Rice Research Institute (NSPRC, 1999) and calculated PGWC. The quality traits of CSSL64 and Asominori (2008) grain length (GL), grain width (GW) and 100 grain weight (HGW) were measured following the method of Tan et al. (2000). Plant height (PH), spikelet per panicle (SP) and seed setting rate (SS) were determined after heading.

DNA preparation and PCR protocol

DNA was extracted from fresh leaves of F_2 and F_3 individuals using the method described by Dellaporta et al. (1983). Suitable DNA samples were diluted with double distilled water and stored at 4°C for polymerase chain reaction (PCR). PCR was performed using the procedure of Chen et al. (1997) with minor modifications. PCR products were separated on 8% non-denaturing polyacrylamide gels and detected using silver staining (Sanguinetti et al., 1994).

QTL analysis

The linkage map of the Asominori × IR24 RIL population constructed by Tsunematsu et al. (1996), including 375 RFLP markers, was used in this QTL analysis. Tests of QTL main effects and QTL × environment interactions (QEI) were carried out using the computer program QTL Mapper 1.0 (Wang et al., 1999). In this study, the likelihood ratio (LR) value corresponding to P= 0.005 (equivalent to LOD= 4.03) was used as the threshold for claiming the presence of main-effect QTLs. Relative contributions of QTL were calculated as the phenotypic variation explained (PVE).

Gene action of the main-effect QTLs 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 QTL allele derived from the IR24 donor (Wan et al., 2004).

The linkage map of the CSSL64 × Asominori F_2 population was

Table 2. Phenotypic variation of PGWC among Asominori, IR24 and RIL population in four environments.

Trait	Location	Parent		RIL population		
		Asominori	IR24	Range	Mean	CV[a] (%)
PGWC (%)	Nanjing	29.0±2.1	21.5±2.1	0.5 - 100.0	41.1±33.3	81.2
	Jinhu	28.3±2.5	18.3±1.8	0.0 - 100.0	40.6±35.0	86.2
	Donghai	28.5±0.7	14.8±2.5	0.0 - 100.0	40.4±34.2	84.5
	Hainan	25.0±2.8	16.0±2.8	1.5 - 100.0	40.7±32.8	80.7

[a]Coefficient of variation

constructed by Mapmaker 3.0 including 34 SSR markers. The main-effect QTL analysis was carried out using the composite interval mapping (CIM) method and by the computer program QTLCART 2.5 (http://statgen.ncsu. edu/qtlcart/WQTLCart.htm).

Estimation of the expression stability of main-effect QTLs for PGWC

A total of 526, 284 and 96 F_2 plants grown in Nanjing in 2005, 2006 and 2007, respectively, were genotyped using 34 SSR markers to construct a small-scale linkage map. PGWC phenotypic values of the F_2 plants were measured and genetic mapping was performed using the Mapmaker/Exp 3.0 program to combine the genotypic and molecular marker data. By means of QTLCART2.5 software, we analyzed the main QTLs controlling PGWC in different environments adopting a LOD value 2.5 (P= 0.05). In addition, 224 F_3 individuals were used to focus on the main and stable *qPGWC-9* using the same method.

RESULTS

Main-effect QTLs for PGWC and their interactions with environments in the RIL population

The phenotypic variation of the RILs and their parents in the four environments is shown in Table 2. Significant differences were observed for PGWC between the parental cultivars. Phenotypic values of PGWC in the RIL population showed a continuous distribution with bidirectional transgressive segregation. This was observed repeatedly in the four environments, suggesting that PGWC was a quantitative trait controlled by multiple genes

10 main-effect QTLs for PGWC were identified in the four environments and mapped to nine rice chromosomes (Figure 1), with LOD values ranging from 5.29 to 31.24 (Table 3). Among them, *qPGWC-2* was consistently detected in all four environments and mapped to the interval C132-XNpb223 on chromosome 2, with an average PVE of 27.1%. The IR24 allele at *qPGWC-2* increased PGWC by an average of 27.1%. *qPGWC-3* and *qPGWC-8* were mapped to the intervals C393B-XNpb164 on chromosome 3 and XNpb41-C259G on chromosome 8, respectively, under E1, E2 and E4 and accounted for 7.4 and 18.6% of PVE. *qPGWC*-9 and *qPGWC-11a* were detected in two environments and mapped to the intervals XNpb36-XNpb103 on chromosome 9 and G1465-C6 on chromosome 11, with average PVE of 10.5 and 6.5%. Four QTLs (*qPGWC-1*, *qPGWC-4*, *qPGWC-10* and *qPGWC-11b*) were each identified in only one environment, with PVEs ranging from 4.9 to 16.1%, showing significant QEI effects except *qPGWC-1*.

Validation of putative QTLs in the CSSL population

The RFLP genotype of each CSSL was used to identify a set of lines that carried IR24-derived segments in regions where the RIL analysis indicated the presence of a putative QTL. There were 11 CSSLs fitting this criterion (such as, CSSLs 10, 20, 21, 26, 28, 29, 32, 52, 53, 62 and 64). One of these lines (CSSL10) harbored the chromosome segment (C132-XNpb223) from IR24 (Figure 2). There were significant differences in PGWC between Asominori and CSSL10 in seven environments. Similarly, CSSL52, CSSL53 and CSSL64 harboring the IR24 allele of *qPGWC-9* had higher PGWC than Asominori in all the eight environments (Figure 2). These two IR24 alleles appeared to generate an increase of PGWC in the background of Asominori irrespective of the growing environments and thus, were considered to be stable QTLs. In contrast, two CSSLs (CSSL20 and CSSL21) harboring the IR24 allele of *qPGWC-3* and three CSSLs (CSSL28, CSSL29 and CSSL32) harboring the IR24 allele of *qPGWC-5* showed lower PGWC than Asominori in the seventh and eighth environments, respectively (Figure 2). QTLs *qPGWC-8* and *qPGWC-11a* showed lower stability. For example, CSSL26 carrying the IR24 allele of *qPGWC-8* showed no significant difference in PGWC from Asominori in any of the eight environments (Figure 2). Therefore, particular attention was given to *qPGWC-9* because of its expression stability under the eight environments.

Phenotypic variation of PGWC in the F_2 populations

The plant morphology of CSSL64 was similar to that of Asominori; while PGWC of CSSL64 was about 3.3 fold

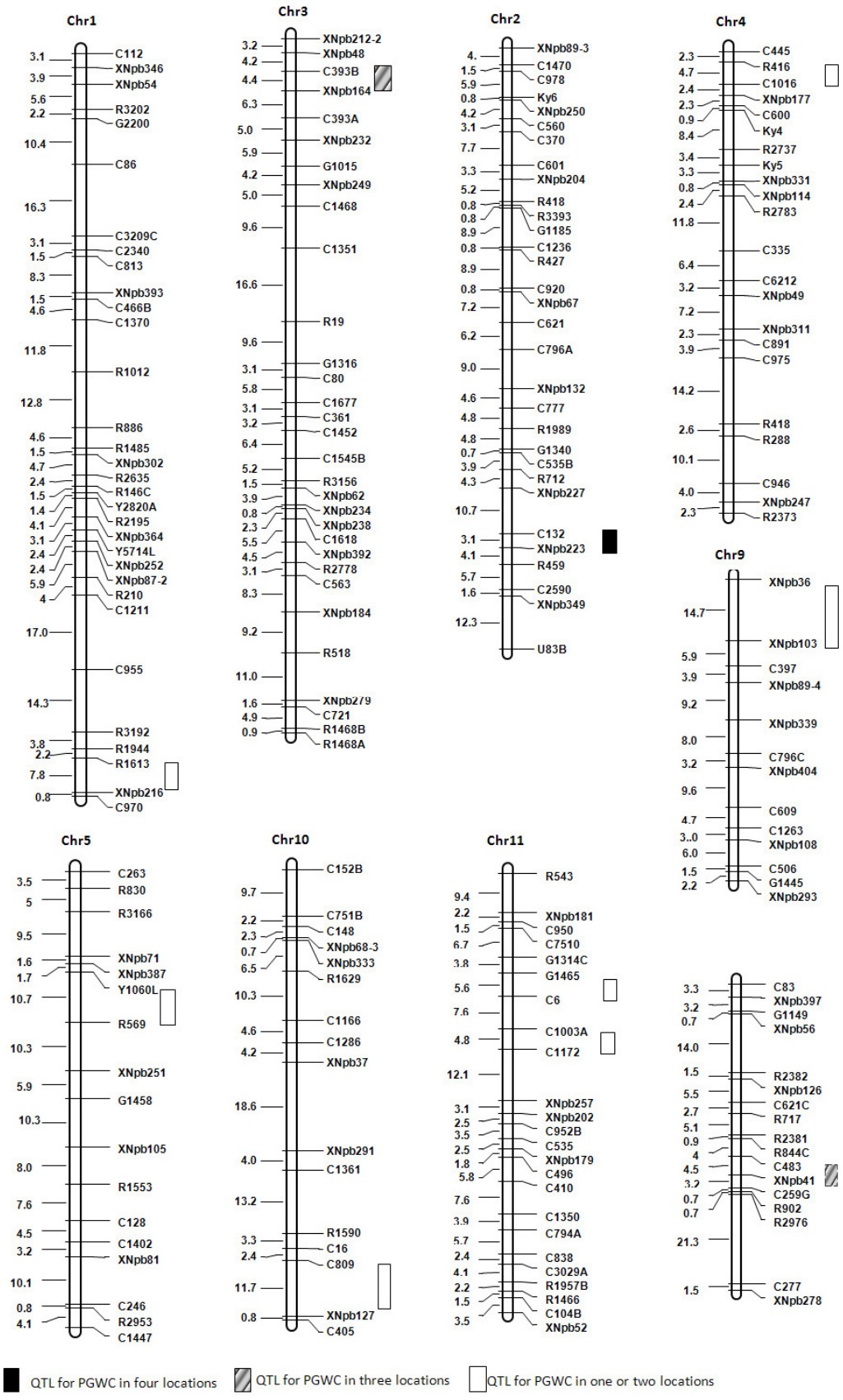


Figure 1. Map locations of identified QTLs affecting PGWC of Asominori × IR24 RIL population in the four environments.

Table 3. Putative main-effect QTLs and their environmental interactions for PGWC of milled rice detected using the Asominori/ IR24 RIL population.

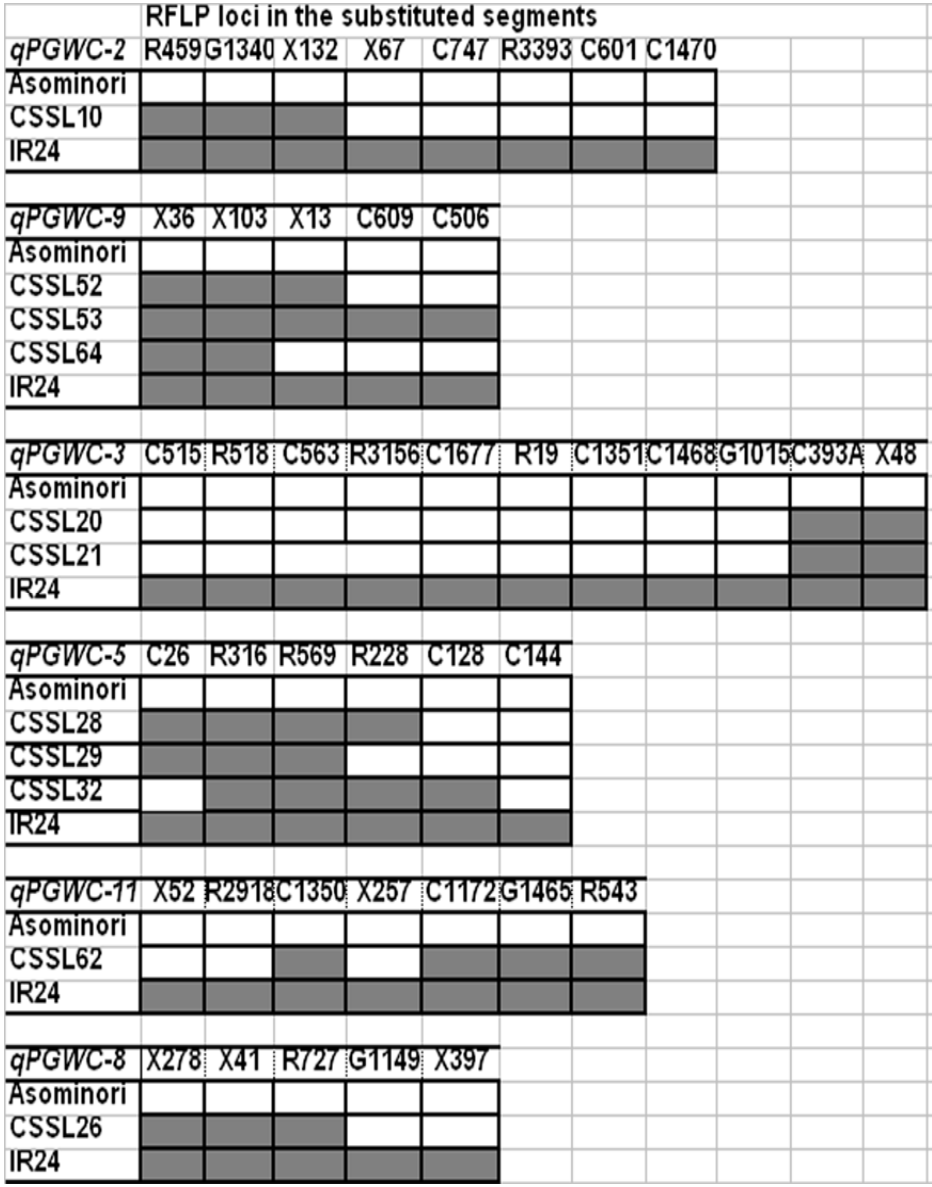
QTL	Chromosome	marker interval	Environment	LOD	PVE[a] (%)	Effect	Source of positive allele	QEI[b]
qPGWC-2	2	C132-XNpb223	E1	7.47	6.3	-8.41	[c] I	8.34**
			E2	21.18	45.4	-28.17	I	-11.36**
			E3	31.24	32.8	-15.67	I	[d] ns
			E4	12.14	23.9	-14.85	I	ns
qPGWC-3	3	C393B-XNpb164	E1	15.21	13.0	12.04	A	5.44**
			E2	4.81	3.6	7.97	A	ns
			E4	4.45	5.6	7.16	A	ns
qPGWC-8	8	XNpb41-C259G	E1	22.16	18.8	-14.47	I	10.89**
			E2	17.97	19.6	-12.96	I	-7.74**
			E4	20.09	17.3	-11.38	I	-4.12*
qPGWC-5	5	Y1060L-R569	E1	13.18	7.9	9.42	A	ns
			E3	6.46	13.6	10.10	A	ns
qPGWC-9	9	XNpb36-XNpb103	E1	12.71	12.9	-11.99	I	-7.84**
			E2	7.57	7.2	-11.25	I	ns
qPGWC-11a	11	G1465-C6	E1	9.18	5.4	-7.76	I	3.94*
			E2	6.95	7.3	-11.27	I	-4.83**
qPGWC-1	1	R1613-XNpb216	E1	5.68	16.1	-13.41	I	ns
qPGWC-4	4	R416-C1016	E4	5.67	7.7	8.40	A	5.00**
qPGWC-10	10	C809-XNpb127	E2	5.29	4.9	9.21	A	5.56**
qPGWC-11b	11	C1003A-C1172	E2	5.92	7.5	11.46	A	5.89**

[a]Percentage of phenotypic variation explained; [b]QTL by environment interaction; [c]the positive effects of QTL alleles contributed by Asominori and IR24, respectively; [d]ns, non-significant effects of the QEI.

that of Asominori (Table 3 and Figure 3). The heading date of CSSL64 and Asominori was 90 and 99 days, respectively. The weight of brown rice of CSSL64 was lower than that of Asominori, while there was no significant difference in plant height, spikelet per panicle, seed setting rate, rice grain width and length between them (Table 4). This line (CSSL64) was thus used as the parent for constructing a secondary F_2 population to perform fine mapping of *qPGWC-9* in Nanjing during 2005, 2006 and 2007. The genetic background of CSSL64 is shown in Figure 4a. In 2005, 2006 and 2007, the frequencies of PGWC among the 526, 284 and 96 F2 individuals appeared to be continuously distributed without boundaries (Figure 4b to d). These results suggested that PGWC in the CSSL64 × Asominori F2 secondary population was controlled by multiple QTLs.

QTL analysis of PGWC using F_2 secondary populations at Nanjing in 2005, 2006 and 2007

In this study, seven main-effect QTLs for PGWC in the CSSL64 × Asominori secondary F_2 population tested in three environments were mapped to 5 chromosomes with LOD values between 2.7 and 8.57 (Table 5, Figure 5 and Supplementary Table 1). Among them, *qPGWC-9*, *qPGWC-6* and *qPGWC-1a* were consistently detected in two years. *qPGWC-9* was mapped to the interval RM444-RM1328 overlapping with the interval XNpb36-XNpb103. *qPGWC-6* and *qPGWC-1a* were mapped to the intervals RM253-RM5531 and RM486-RM1003, respectively. Positive effects of PGWC at *qPGWC-9*, *qPGWC-6* and *qPGWC-1* were provided by IR24, Asominori and IR24 alleles, respectively. The other four QTLs, including



	RFLP loci in the substituted segments								Phenotypic values of PGWC of parents and target CSSLs across the eight							
qPGWC-2	R459	G1340	X132	X67	C747	R3393	C601	C1470	E1	E2	E3	E4	E5	E6	E7	E8
Asominori									24.5±2.5	26.5±0.5	29.0±3.0	27.0±1.0	29.0±1.5	28.3±1.75	28.5±0.5	25.0±2.0
CSSL10									45.4±7.9	50±2.0**	48.5±1.5*	61.5±1.5**	64.2±10.8*	60±10.0*	54.8±6.3*	67.8±6.3*
IR24									20.0±0.0	15.0±2.0*	18.0±3.0	16.0±3.0*	21.5±1.5*	18.3±1.3*	14.8±1.8*	16.0±2.0*
qPGWC-9	X36	X103	X13	C609	C506				E1	E2	E3	E4	E5	E6	E7	E8
Asominori									24.5±2.5	26.5±0.5	29.0±3.0	27.0±1.0	29.0±1.5	28.3±1.75	28.5±0.5	25.0±2.0
CSSL52									77.8±2.8**	71.5±0.5**	84±2.0**	86.5±0.5**	84.1±3.4**	79.25±7.8*	87.5±3.5**	83.7±2.8**
CSSL53									76.7±1.7**	76.5±2.5**	78.5±2.5**	74.5±2.5**	77.4±0.9**	79.0±2.0**	76.8±3.3**	77.3±2.8**
CSSL64									85.9±2.4**	81.5±0.5**	89.5±1.5**	94.0±1.0**	92.4±4.1**	88.8±7.3**	92.3±2.8**	87.0±7.0**
IR24									20.0±0.0	15.0±2.0*	18.0±3.0	16.0±3.0*	21.5±1.5*	18.3±1.3*	14.8±1.8*	16.0±2.0*
qPGWC-3	C515	R518	C563	R3156	C1677	R19	C1351	C1468	G1015	C393A	X48					
Asominori												E1	E2	E3	E4	E5	E6	E7	E8
CSSL20												24.5±2.5	26.5±0.5	29.0±3.0	27.0±1.0	29.0±1.5	28.3±1.75	28.5±0.5	25.0±2.0
CSSL21												11.7±2.7*	10±3.0*	27.5±0.5	5.5±0.5**	9.9±4.4*	8.0±2.0**	21.3±6.3	8.8±3,3*
IR24												6.4±1.6**	3.5±0.5**	3.0±1.0**	8.0±1.0**	7.2±2.3**	5.8±2.3**	2.5±0.5**	6.0±2.0**
												20.0±0.0	15.0±2.0*	18.0±3.0	16.0±3.0*	21.5±1.5*	18.3±1.3*	14.8±1.8*	16.0±2.0*
qPGWC-5	C26	R316	R569	R228	C128	C144						E1	E2	E3	E4	E5	E6	E7	E8
Asominori												24.5±2.5	26.5±0.5	29.0±3.0	27.0±1.0	29.0±1.5	28.3±1.75	28.5±0.5	25.0±2.0
CSSL28												4.3±3.3**	10.0±2.0**	12.5±0.5*	0.5±0.5**	3.8±3.8*	6.5±3.5*	8.8±3.8*	0.8±0.3**
CSSL29												6.3±1.3*	9.0±2.0**	5.0±1.0**	1.0±1.0**	9.5±4.5*	9.5±0.5**	5.5±0.5**	0.5±0.5**
CSSL32												5.4±2.1**	1.5±0.5**	3.0±1.0**	5.5±0.5**	10.4±7.1	6.8±5.3*	7.0±4.0*	11.3±5.8
IR24												20.0±0.0	15.0±2.0*	18.0±3.0	16.0±3.0*	21.5±1.5*	18.3±1.3*	14.8±1.8*	16.0±2.0*
qPGWC-11	X52	R2918	C1350	X257	C1172	G1465	R543					E1	E2	E3	E4	E5	E6	E7	E8
Asominori												24.5±2.5	26.5±0.5	29.0±3.0	27.0±1.0	29.0±1.5	28.3±1.75	28.5±0.5	25.0±2.0
CSSL62												24.1±1.1	25.0±2.0	26.0±2.0	24.5±0.5	32.8±7.7	46±11.0	30.5±4.5	18.3±6.3
IR24												20.0±0.0	15.0±2.0*	18.0±3.0	16.0±3.0*	21.5±1.5*	18.3±1.3*	14.8±1.8*	16.0±2.0*
qPGWC-8	X278	X41	R727	G1149	X397							E1	E2	E3	E4	E5	E6	E7	E8
Asominori												24.5±2.5	26.5±0.5	29.0±3.0	27.0±1.0	29.0±1.5	28.3±1.75	28.5±0.5	25.0±2.0
CSSL26												23.9±1.9	27.0±2.0	35.0±5.0	15.5±1.5*	26.4±0.6	32.5±5.5	29.0±6.0	12.3±3.3*
IR24												20.0±0.0	15.0±2.0*	18.0±3.0	16.0±3.0*	21.5±1.5*	18.3±1.3*	14.8±1.8*	16.0±2.0*

Figure 2. Differences in phenotypic values of PGWC between Asominori, IR24 and the target CSSLs carrying putative QTLs in eight environments. * and ** indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) between PGWC of Asominori and IR24, and target CSSLs carrying QTL alleles, respectively. Black (IR24) and white (Asominori) bars denote the parental origin of chromosome segments.

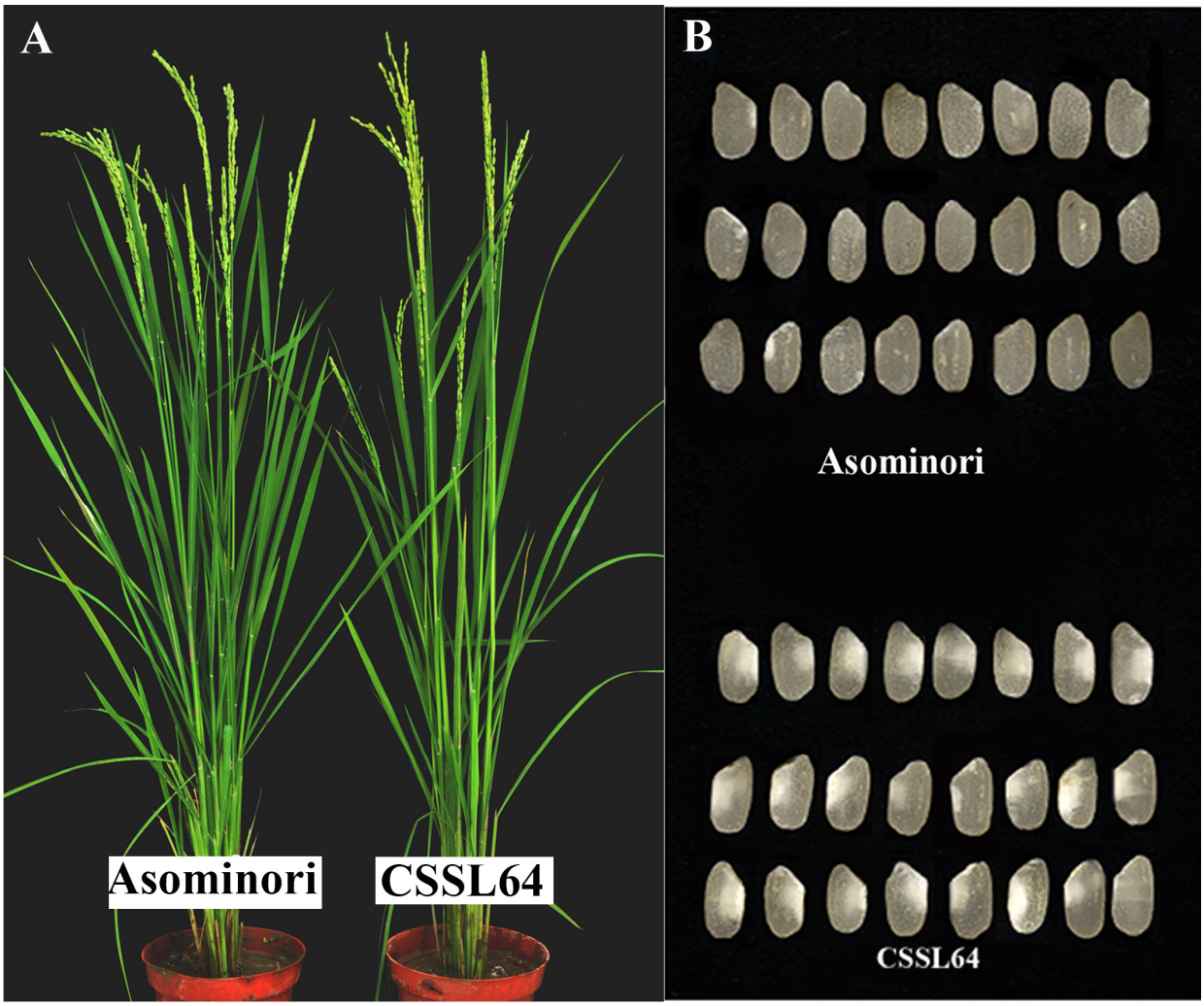


Figure 3. Plant morphology and milled rice of CSSL64 and the recurrent parent Asominori. A, Plant morphology; B, milled rice morphology.

qPGWC-1b, *qPGWC-11a, qPGWC-11b* and *qPGWC-10*, were each detected only in one environment. *qPGWC-11a* and *qPGWC-11b* were mapped to the intervals RM224-RI01666 and RM21-RM5961, respectively. Of the four QTLs, the only Asominori allele to provide a positive effect for PGWC was *qPGWC-10*.

QTL mapping of *PGWC-9* using the secondary F_3 populations in Nanjing in 2008

Among F_2 individuals grown at Nanjing in 2006, we selected two individuals heterozygous at the *qPGWC-9* locus (markers ID162, RM444, RM23904, RM23958 and RM1328), whereas other QTL loci for PGWC were homozygous. Four groups of markers [RM3403, RM11734, RM11694 and RM212 (chr 1); RM253 and RM276 (chr 6); RM333 and RM228 (chr 10); RM5961, RI01365 and RM224 (chr 11)] were used for selecting *qPGWC-1*, *qPGWC-6*, *qPGWC-10* and *qPGWC-11*, respectively (Figure 6). Composite interval mapping on data for the F_3 lines indicated that *qPGWC-9* was located in the interval RM23958-RM1328, equivalent to a genetic distance of 5 cM; the PVE was 19.3% (Figure 6).

DISCUSSION

Direct evidence for QTLs detected in the RIL and CSSL populations

In order to reduce the PGWC, the rice varieties with low PGWC generally will be chosen as parents in hybrid breeding. While due to the interaction or recombinant of genes, there were lines with high PGWC in the according progeny. Our aim was to identify these negative sites using genetic populations, such as RIL and CSSL.

Although, the 10 QTLs in the RIL population were

Table 4. Comparison of traits between CSSL64 and Asominori.

Parent and CSSL	PGWC (%)	PH (cm)	SP	SS (%)	HGW (g)	GW (mm)	GL (mm)
Asominori	46±2.6	95.07±2.7	82.6±8.8	94.6±1.1	2.24±0.05	2.862±0.17	5.558±0.22
CSSL64	94±1.0**	94.93±5.1	78.4±8.0	93.7±4.6	2.13±0.06*	2.811±0.20	5.454±0.24

PGWC, Percentage of grains with chalkiness; PH; plant height; SP spikelet per panicle; SS ,seed setting rate; HGW, 100-grain weight; GW, rice grain width; GL, rice grain length . *and **Significant difference at 5% and 1% based on *t*-test.

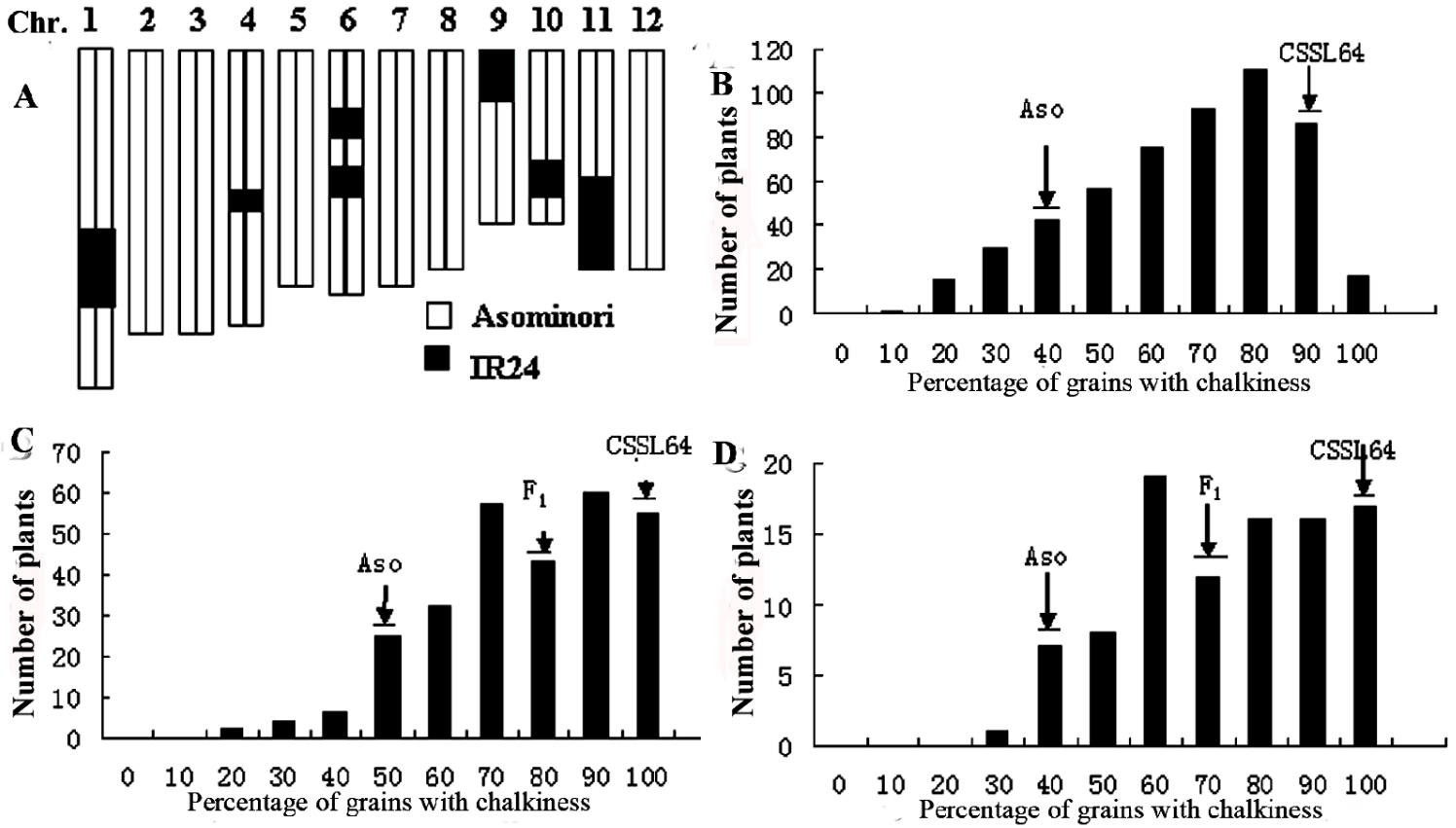


Figure 4. Graphical genotype of the CSSL64 plant (A) and frequency distribution of PGWC in the F_2 population in Nanjing 2005, 2006 and 2007 (B, C, D, respectively). F_1, CSSL64×Asominori F_1.

Table 5. Main effect QTLs of PGWC in the F_2 population grown at Nanjing in 2005, 2006, and 2007

Year	Chr.	Marker interval	LOD	PVEa (%)	Positive allele
2005	9	RM1328-RM444	20.4	9.5	bl
	11	RM224-RM5961	8.8	5.8	I
	1	RMM212-RM486	23.2	10.4	I
2006	1	RM1003-RM212	6.9	2.6	I
	1	RM543-RM11694	7	2.7	I
	6	RM253-RM276	4.5	1.5	A
	9	ID162-RM444	5.5	7.4	I
	9	RM23958-RM1328	8.4	12.2	I
	10	RI00185-RM228	10	10	A
	11	RM21-RM5961	3.1	3.7	I
2007	9	ID162-RM444	4.5	7.46	I
	9	RM23958-RM1328	3.2	9.46	I
	1	RM543-RM212	6.2	15.76	I
	6	RM253-RM276	4.6	15.96	A
	11	RM21-RM5961	3.3	1.73	I

[a] Percentage of phenotypic variation explained; [b] the positive effects of QTL alleles contributed by Asominori and IR24, respectively. Chr., chromosome.

repeatedly detected across environments, there was no direct evidence for QTL reliability. QTL effects identified in early-generation mapping have only a limited ability to distinguish between linkage and pleiotropy (Kearsey and Farquhar, 1998). However, CSSL materials provide a convenient method to analyze the inheritance of complex traits (Kubo et al., 2002) and can be used to verify the results of other mapping populations. In this study, only one of 10 QTLs affecting PGWC was identified in all of eight environments, indicating that individual QTL were sensitive to environmental conditions. We therefore used CSSL and backcross F_2 and F_3 populations to detect and validate valuable and reliable QTLs for PGWC. *qPGWC-9* proved to be a major QTL with a LOD score of more than 7.5 in the RILs and was detected repeatedly in a population of 66 CSSLs across the eight environments. Analysis of F_2 and F_3 populations obtained by introgressing the PGWC-9 target region into Asominori validated the QTL. It was also possible to narrow the QTL region and to estimate the allelic effect by using an F_2 secondary population, although further analysis with more DNA markers is necessary to detect accurate QTL and allelic effects.

qPGWC-9 was a stable QTL across populations and environments

Detailed attention was given to *qPGWC-9*. The reasons for focusing on this QTL were as follows: firstly, in CSSL52, 53 and 64, the IR24 chromosomal segment harboring *qPGWC-9* (defined by RFLP markers XNpb36 and XNpb103) was delineated in the genetic background of Asominori. There was a significant difference in PGWC between Asominori and all three CSSLs grown in all the eight environments (Figure 2). Secondly, the mean PGWC of the three lines were clearly higher than that those of Asominori. Previous studies detected QTLs for PGWC on one to all 12 chromosomes when using one single population or a single environment (He et al., 1999; Li et al., 2003; Tan et al., 2000; Yoshida et al., 2002). Li et al. (2003) detected seven QTLs for PGWC on chromosomes 1, 3, 5, 6, 8 and 12, with PVE of 6.14 to 28.15% using 98 backcross inbred lines (BILs) derived from a subspecific backcross of Nipponbare *(japonica)* × Kasalath (*indica*) × Nipponbare, showing that PGWC was controlled by polygenes. Among these QTLs, *qPGWC-6a* was detected repeatedly over two years. Tan et al. (2000) used $F_{2:3}$ and an F_{10} recombinant inbred line population from a cross between the parents of Shanyou 63, the most widely grown rice hybrid in China, to analyse the genetic basis of appearance quality of rice grains. The QTL located in the interval of RG360-C734a on chromosome 5 was the major locus for chalkiness, explaining 70.3% of the phenotypic variance. He et al. (1999) detected two QTLs located on chromosomes 8 and 12, explaining 20.9 and 10.0% of the phenotypic variance, respectively. Yoshida et al. (2002) detected two and three QTLs for percentages of occurrence of white-core (WC) and white-belly (WB), respectively, using a doubled haploid population. The QTL for WB located near the position of RM31 on chromosome 5 had the

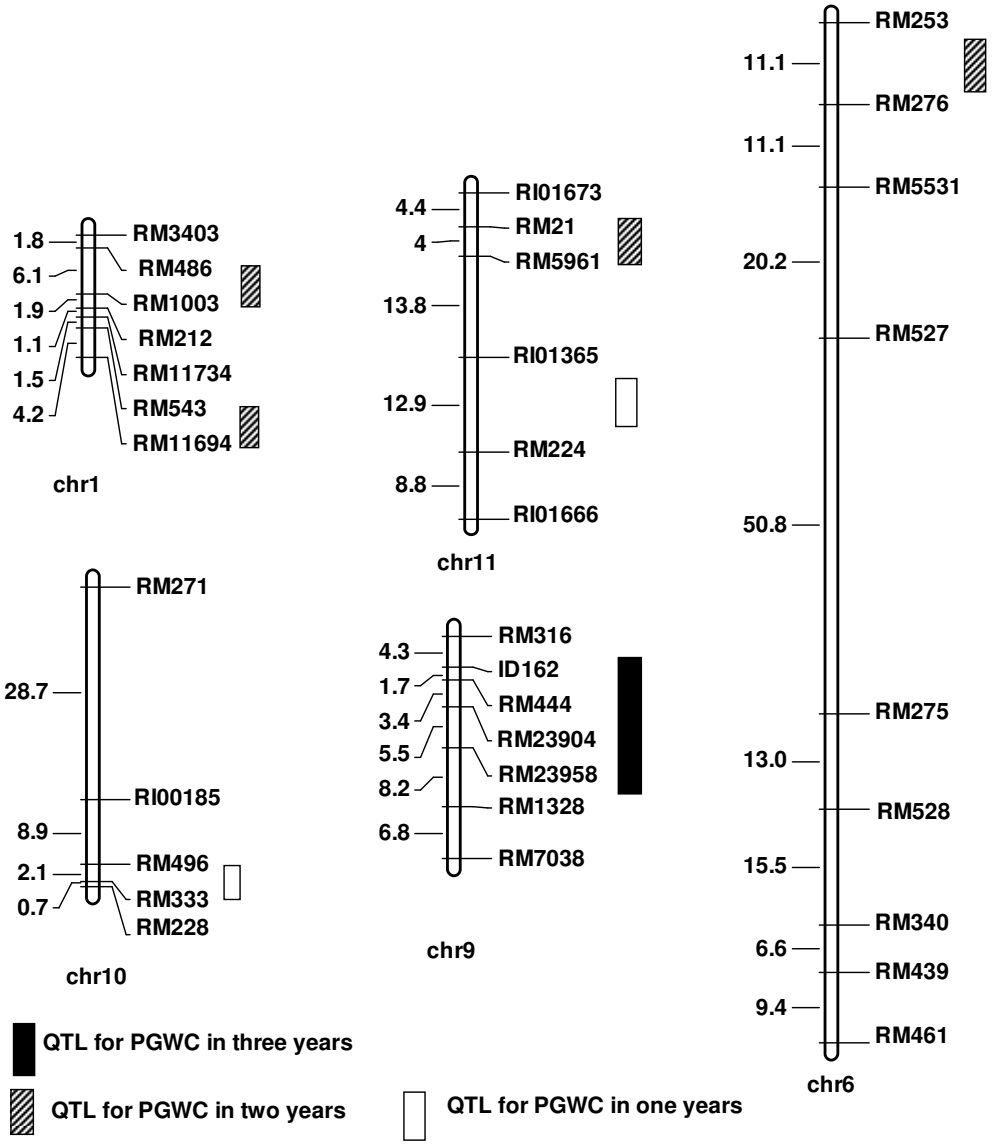


Figure 5. Genetic map of the segments derived from IR24.

highest PVE (20.7%).

qPGWC-9 was a major and stable QTL controlling PGWC in RILs, CSSLs and F_2 and F_3 secondary populations across the 12 environ-ments. Firstly, it was identified to be a main effect QTL in the RIL population across E1 and E2. The LOD and PVE were 7.57 to 12.71 and 7.2 to 11.29%, respectively. Secondly, the data for the CSSL population showed that *qPGWC-9* was relatively stable across the eight environments (E1-E8). The data for lines CSSL52,53 and 64 showed that *qPGWC-9* was stable over breeding gene-rations. Thirdly, we assessed the main-effect QTLs in an F_2 population from CSSL64 × Asominori across three environments. The results showed that *qPGWC-9* was a single stable QTL. Finally, *qPGWC-9* was dissected as a single locus (*qPGWC-9*) in two F_3 populations. Most importantly, these results illustrate a useful strategy for improving the appearance quality of rice.

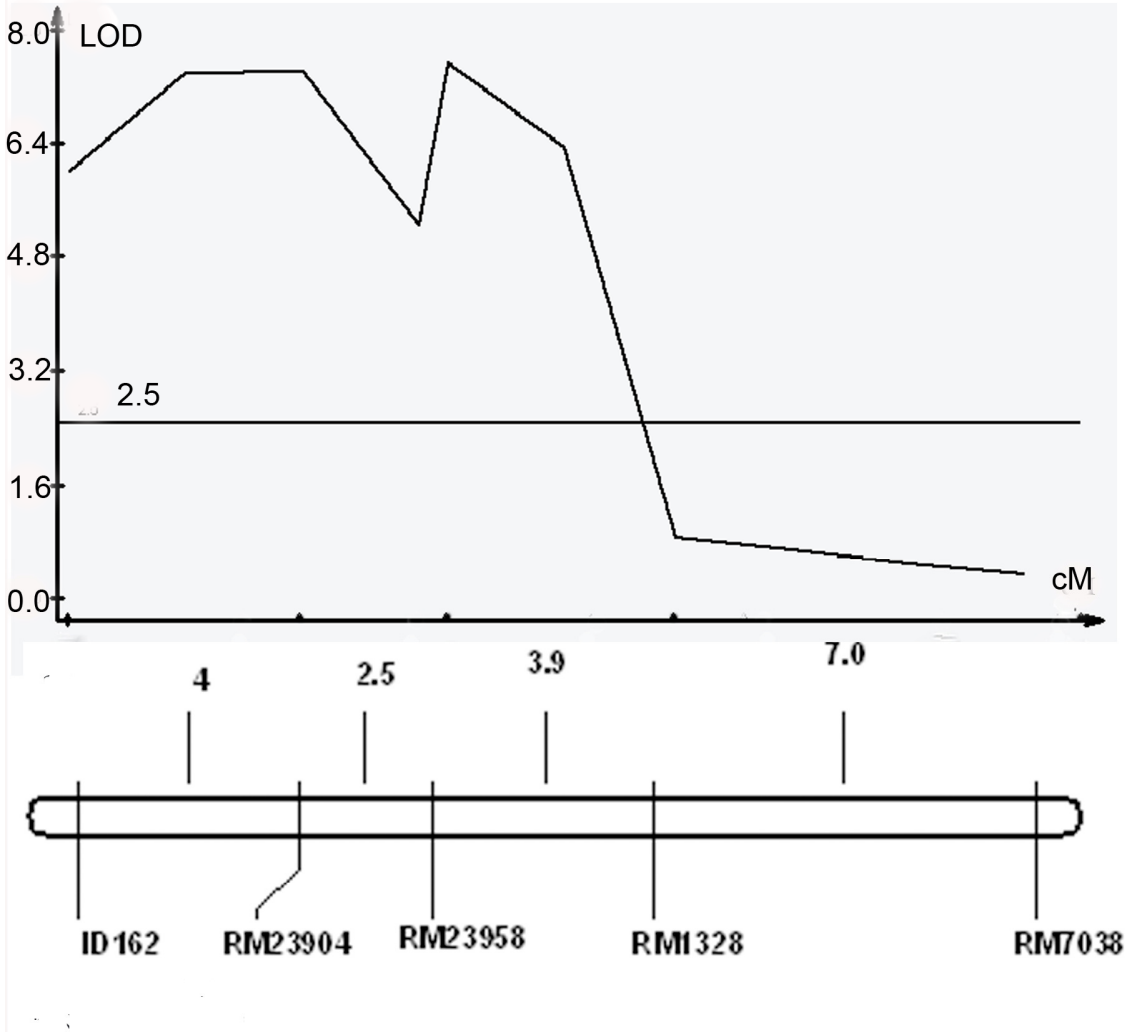
Genetic complexity of QTLs for PGWC due to minor QTLs

Minor QTLs can be responsible for a large proportion of trait variation. In this study, six minor QTLs were detected in the F_2 population (Table 5). Their gene actions were significantly environment-specific and in some instances the genetic effects on phenotype were in opposite directions. During fine-mapping, the resolution of target QTL at two or more linked loci can bring positional cloning

A

Marker	Chromosome 9				Chromosome 1				Chromosome 6		Chromosome 10		Chromosome 11		
	ID162	RM23904	RM23958	RM1328	RM3403	RM11734	RM11694	RM212	RM253	RM276	RM228	RM333	RM5961	RI01365	RM224
Line 1	H	H	H	H	A	A	A	A	I	I	I	I	A	A	A
Line 2	H	H	H	H	A	A	A	A	I	I	I	I	A	A	A

B



Chromosome	Marker interval	LOD	PVE (%)	Additive effect	Positive allele
9	RM23958-RM1328	7.6	19.4	12.0	I

Figure 6. QTLs identified for *qPGWC-9* based on composite interval mapping in F_3 populations. A, Genotypes of two secondary F_3 lines. A, I and H denote the Asominori allele, IR24 allele and heterozygote, respectively; B, QTL mapping results for PGWC in the F_3 population. Five SSR and indel markers were mapped in the interval on chromosome 9; PVE, percentage of phenotypic variance explained.

Supplementary Table 1. Primer sequences used for the linkage map of F_2 and F_3 populations.

Primer pair	Product size (bp)	Forward sequence (5'-3')	Reverse sequence (5'-3')
RM3403	120	aacgactgctccctcttcag	agcttgcaaggcattagctc
RM486	100-112	ccccctctctctctctctc	tagccacatcaacagcttgc
RM1003	128	gattcttcctcccttcgtg	ttcctgtcagaacagggagc
RM212	112-134	ccactttcagctactaccag	cacccatttgtctctcattatg
RM11734	165	caggagaccaccatttagtttgtagg	ggcaggttgaaggcaatagacc
RM543	96-104	ctgctgcagactctactgcg	aaatattacccatccccccc
RM11694	194	gcgtctatgcgtatcttcatcttacc	caactctgctagtgtgcctctgc
RI01673	255	acaaaactacctggggctca	ccaggggtttcttgttctcc
RM21	132-170	acagtattccgtaggcacgg	gctccatgagggtggtagag
RM5961	129	gtatgctcctcctcacctgc	acatgcgacgtgatgtgaac
RI01365	236	ggattcttgtcacccagcat	ccatttaagctatttcctccaagt
RM224	124-158	atcgatcgatcttcacgagg	tgctataaaaggcattcggg
RI01666	304	tacgacatctccgatgacca	gagatgtggacgacgagctt
RM253	125-143	tccttcaagagtgcaaaacc	gcattgtcatgtcgaagcc
RM276	85-153	ctcaacgttgacacctcgtg	tcctccatcgagcagtatca
5M5531	158	tttgtgttggtaagttgcttc	ttaaggagagtgttttcttttctc
RM527	221-239	ggctcgatctagaaaatccg	ttgcacaggttgcgatagag
RM275	110-114	gcattgatgtgccaatcg	cattgcaacatcttcaacatcc
RM528	232-260	ggcatccaattttacccctc	aaatggagcatggaggtcac
RM340	119-189	ggtaaatggacaatcctatggc	gacaaatataagggcagtgtgc
RM439	248-281	tcataacagtccactccccc	tggtactccatcatcccatg
RM461	191-195	gagaccggagagacaactgc	tgatgcggtttgactgctac
RM271	92-105	tcagatctacaattccatcc	tcggtgagacctagagagcc
RI00185	285	ggggagatgcatgtgcttag	gccatctactcaccctcgac
RM496	263-291	gacatgcgaacaacgacatc	gctgcggcgctgttatac
RM333	164-215	gtacgactacgagtgtcaccaa	gtcttcgcgatcactcgc
RM228	108-154	ctggccattagtccttgg	gcttgcggctctgcttac
RM316	192-212	ctagttgggcatacgatggc	acgcttatatgttacgtcaac
ID162	253	ctatgctagggttctgcc	tttccgtttgcctgttg
RM444	162-226	gctccacctgcttaagcatc	tgaagaccatgttctgcagg
RM23904	165	ctcaccggagcaccactaacc	gagagcaagactgtgaagtgtgaacc
RM23958	89	ctaccactgtttcattgtgtctcg	gaattgaaggagaagcaggaagc
RM1328	204	gaatgggattagacgatttg	ccatgagtgacatcaaaagg
RM7038	152	aggtggtgagggtgaacttg	tgggattagagctttggtgg

projects to an end when the proportion of phenotypic variability explained by each QTL is too small to be revealed with a realistically manageable number of replications. A widely adopted strategy to more accurately estimate the position and effect of a coarsely mapped QTL is to create a new experimental population by crossing near-isogenic lines (NILs) that differ only in allelic constitution in the short chromosomal segment harboring the QTL (QTL-NILs). Because of the absence of other segregating QTLs in such populations, the target QTL becomes the major source of genetic variation and the phenotypic means of the QTL genotypic classes can be statistically differentiated and genotypes recognized accordingly. Appropriate replication and/or progeny testing can generally be implemented based on the heritability of the trait. Under such conditions, the QTL is considered a Mendelized genetic factor and genetic distances between a QTL and nearby molecular markers can be estimated more precisely.

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