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ORIGINAL PAPER

Mapping and validation of quantitative trait loci for spikelets per panicle and 1,000-grain weight in rice (*Oryza sativa* L.)

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Abstract This study identified four and five quantitative trait loci (QTLs) for 1,000-grain weight (TGW) and spikelets per panicle (SPP), respectively, using rice recombinant inbred lines. QTLs for the two traits (*SPP3a* and *TGW3a*, *TGW3b* and *SPP3b*) were simultaneously identified in the two intervals between RM3400 and RM3646 and RM3436 and RM5995 on chromosome 3. To validate QTLs in the interval between RM3436 and RM5995, a BC_3F_2 population was obtained, in which *TGW3b* and *SPP3b* were simultaneously mapped to a 2.6-cM interval between RM15885 and W3D16. *TGW3b* explained 50.4% of the phenotypic variance with an additive effect of 1.81 g. *SPP3b* explained 29.1% of the phenotypic variance with an additive effect of 11.89 spikelets. The interval had no effect on grain yield because it increased SPP but decreased TGW and vice versa. Grain shape was strongly associated with TGW and was used for QTL analysis in the BC_3F_2 population. Grain length, grain width, and grain thickness were also largely controlled by *TGW3b*. At present, it is not clear whether one pleiotropic QTL or two linked QTLs were located in the interval. However, the conclusion could be made ultimately by isolation of *TGW3b*. The strategy for *TGW3b* isolation is discussed.

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Introduction

An important component of yield in rice is 1,000-grain weight (TGW), which is determined by grain length (GL), grain width (GW), and grain thickness (GT) (Evans 1972). GL is regarded as the strongest determinant of TGW (Xie et al. 2006). GL mutants have been observed and were used to isolate genes controlling GL in rice. *Mi* and *mik*, conferring minute grain, were identified from mutants (Takamure and Kinoshita 1994; Takeda and Saito 1977). The long-kernel genes *lk1*, *Lk2*, *Lkf*, *lki1*, and *lki2* were also identified in rice mutants (Takamure et al. 1995; Takeda and Kato 1992; Takeda and Saito 1980). All these genes regulate grain weight by changing GL, and knowledge of these genes enabled researchers to understand the genetic bases of TGW and GL. Due to the complex inheritance of TGW, however, natural mutants alone are not sufficient to systematically examine the genetic nature of TGW in rice.

Since the advent of molecular marker genetic linkage maps and quantitative trait locus (QTL) analysis approaches, many QTLs for TGW and grain shape have been identified in populations from crosses between diverse rice cultivars or cultivars and wild rice (Li et al. 1998; Lin et al. 1996; Qiao et al. 2007; Rahman et al. 2007; Xing et al. 2002). By developing near isogenic lines (NILs), which can eliminate the genetic background noise and visualize a QTL as a Mendelian factor, several QTLs for TGW have been fine-mapped or cloned. *Gw8.1*, a QTL controlling TGW and grain shape, was mapped in a 306.4-kb region on chromosome 8 (Xie et al. 2006). *Gw3.1,* a major QTL for GL and TGW, was fine-mapped in a 93.8-kb region (Li et al. 2004). Fan et al. (2006) further delimited it (as *GS3*) to a 7.9-kb region, and the candidate gene was predicted to encode a putative transmembrane protein. *GW2*, a major QTL for rice GW and TGW, was narrowed down to the

interval between W236 and W239 on rice chromosome 2 by using a BC_2F_2 population from WY3/Fengaizhan-1. It encodes a previously unknown RING-type E3 ubiquitin ligase (Song et al. 2007). *tgw6*, a QTL increasing TGW, was identified using an NIL population, and function analysis showed that it improves the carbohydrate storage capacity and consequently increases yield potential (Ishimaru 2003).

The number of spikelets per panicle (SPP) is another important rice yield component that is quantitatively inherited. Previous studies showed that epistasis and genotype × environment (G × E) interaction also frequently had considerable effects on SPP although their contribution to SPP was less than that of the genetic main effects (Li et al. 1997; Xing et al. 2002). The case makes it challenging to precisely evaluate SPP QTLs. Because of its important role in grain yield, many studies have investigated QTLs for SPP in rice (http://www.gramene.org/db/qtl/qtl_display?trait_category=Yield). Moreover, QTLs for SPP expressed the characteristics of a single Mendelian factor in QTL-based NILs (Xing et al. 2008; Zhang et al. 2006, 2009) and were fine-mapped (Cheng et al. 2007; Tian et al. 2006). The first cloned QTL for SPP, *Gn1a*, encodes a cytokinin oxidase and negatively regulates SPP in rice by breaking down the cytokinin (Ashikari et al. 2005). QTLs for SPP were also identified from rice mutants. For instance, *LAX PANICLE* (*LAX*) and *SMALL PANICLE* (*SPA*), which encode a basic helix–loop–helix transcription factor, regulate the axillary meristem formation and consequently change the grain number (Komatsu et al. 2003). In addition, some genes controlling flowering time also change SPP. Overexpression of *RCN1* and *RCN2*, which encode *TERMINAL FLOWER 1* (*TFL1*)/*CENTRORADIALIS* (*CEN*)-like genes, delays the transition to the reproductive phase and alters panicle size in rice (Nakagawa et al. 2002). *Ghd7*, a major QTL for SPP and heading date, was fine-mapped to a 0.2-cM region on chromosome 7 and finally cloned (Xing et al. 2008; Xue et al. 2008).

A negative correlation at the significance level of $P < 0.01$ between SPP and TGW has been reported with correlation coefficients of −0.19 (Kato and Takeda 1996) and −0.71 (Samonte et al. 1998). In this study, to dissect the genetic bases of the two negatively correlated traits, a recombinant inbred line (RIL) population derived from two *indica* rice varieties, Teqing and Minghui 63, with striking differences in SPP and TGW, was used for QTL mapping. Furthermore, a BC_3F_2 population segregating in the small region between RM3436 and RM5995 on chromosome 3 was developed. The population harbors two QTLs, *TGW3b* and *SPP3b*, controlling TGW and SPP, respectively, and was used to validate the identity of the QTLs and to discriminate their locations.

Materials and methods

Field experiment of RILs for QTL analysis

An RIL population was used to identify QTLs controlling SPP and TGW. This population consisted of 190 RILs derived by single-seed descent from a cross between two parents, Minghui 63, with large TGW but small SPP, and Teqing, with small TGW but large SPP. For the field test, the seeds were sown in a seedling bed in May 2005 and 2006. The RILs (F_7 and F_8) and two parents were transplanted to a bird net-equipped field in the experimental farm of Huazhong Agricultural University in Wuhan, China, in the 2005 and 2006 rice-growing seasons. Field experiments were carried out following a randomized complete block design with two replicates. Fourteen seedlings (approximately 25 days old) for each family line were transplanted into a two-row plot, with a distance of 16.5 cm between plants within a row and 26.4 cm between rows. The 10 plants in the middle of the two rows of each plot were harvested individually to score SPP and TGW.

Development of an NIL-F_2 population

Two QTLs, *TGW3b* and *SPP3b*, were identified in the interval between RM3436 and RM5995 in the RIL population. To develop NILs targeted for the interval, a cross was made between Minghui 63 and Teqing. The resulting F_1 plants were used as the male parent in a backcross with Minghui 63. The BC_1F_1 plant with a heterozygous target region was then crossed with Minghui 63. Of the 133 polymorphic simple sequence repeat (SSR) markers located in the linkage map in RILs, 85 evenly distributed on all 12 chromosomes were selected to investigate the genetic makeup of 12 BC_2F_1 plants. The BC_2F_1 plant with a heterozygous target region and the least genetic background noise was selected to cross with Minghui 63. Accordingly, 12 BC_3F_1 plants were investigated. Finally, plant BC_3F_1-11, which was only heterozygous at two markers besides the target region (RM566 on chromosome 9 and RM511 on chromosome 12), was selected to produce BC_3F_2.

BC_3F_2 population experiment and its progeny test

A BC_3F_2 population of 288 plants derived from BC_3F_1-11 was planted in Hainan, China, in January 2007. Twelve seedlings (approximately 25 days old) per row were transplanted in the field with a distance of 16.5 cm between plants within a row and 26.4 cm between rows, in 24 rows. In total 215 plants, except those growing along the boundary, were individually harvested for trait measurement at the ripening stage. In May 2007, BC_3F_3 families of 215 BC_3F_2 plants

were sown in a seedling bed. Twenty seedlings (approximately 20 days old) of each family were transplanted into a two-row plot in the experimental farm of Huazhong Agricultural University, with a distance of 16.5 cm between plants within a row and 26.4 cm between rows. Sixteen plants in the middle of each plot were individually harvested for trait measurement at the ripening stage. Only 193 families were used to conduct the progeny test due to the poor germination of 22 families. Each plant was harvested individually to score the traits of TGW and SPP.

Trait measurement

SPP was calculated as the total number of spikelets from the whole plant divided by the number of tillers; TGW was calculated as the grain weight per plant divided by the number of grains multiplied by 1,000. The seed-setting ratio was calculated as the grain number per plant divided by the number of spikelets per plant. Ten randomly chosen grains from each plant were placed lengthwise along a ruler to measure 10-GL, and GL (mm) was calculated as 10-GL divided by 10. GW and GT (mm) were determined for each grain individually using a Vernier caliper. The mean values of the 10 measured grains were used as the measurements of each plant. In RILs, trait mean values of each line across two replicates in each year and trait values of individuals in BC_3F_2 population were used for corresponding data analysis.

DNA extraction and molecular marker development

DNA was extracted from fresh leaves of BC_3F_2 population at the seedling stage by using the CTAB method (Murray and Thompson 1980). SSR markers were identified from the Gramene database (http://www.gramene.org/), and the SSR primers were designed according to this public database (International Rice Genome Sequencing Project 2005; McCouch et al. 2002; Temnykh et al. 2000). One new indel marker (W3D16) was developed based on the publicly available rice genome sequences (http://www.rgp.dna.affrc.go.jp; http://www.rise.genomics.org.cn/) with a forward primer sequence of CCCGCTTGAAAACTAACAC and a reverse primer sequence of ACACGCCTAAGCAAA TGTA. Five SSR markers (RM3436, RM15774, RM15855, RM5475, and RM5995) plus one indel marker, W3D16, around the target region were used to genotype the BC_3F_2 population. The SSR marker assay was conducted as described by Wu and Tanksley (1993).

Data analysis

The molecular linkage map was constructed using Mapmaker 3.0 at the logarithm of odds (LOD) value of 3.0 (Lincoln et al. 1992). The Kosambi function was used to calculate the genetic distance. Composite interval mapping was performed for QTL analysis with RILs using WinQTL Cartographer v2.0 (Wang and Zeng 2003). QTL analysis was based on the statistical model, which is defined as:

$$Y = xb + zd + XB + E,$$

where Y is a vector of trait values, b and d are the additive and dominance effects of the putative QTL being tested, x and z are indicator variable vectors specifying the probabilities of an individual being in different genotypes for the putative QTL constructed by flanking markers, B is the vector of effects of other selected markers fitted in the model, X is the marker information matrix for those selected markers, and E is the error vector. To select the marker as cofactor to control the genetic background, we selected a model that is defined as model 6 in the WinQTL Cartographer documentation, which required specification of two additional parameters including the number of markers to control for the genetic background and a window size (Basten et al. 2003). When using model 6, forward stepwise regression and backward elimination regression methods were used to choose significant markers for each trait and the most important five markers of the significant markers were selected as cofactors to control genetic background; window size was set at 10 cM. Thresholds for the LOD score were estimated by permutation tests with 1,000 (Churchill and Doerge 1994). In the RIL population, the threshold values of LOD for SPP and TGW at genome-wide significance level of 0.05 were 2.5 and 2.6, respectively. Interval QTL mapping was performed with both BC_3F_2 and BC_3F_3 data using the program Mapmaker/QTL 1.1 (Lincoln et al. 1993). In the progeny test, the mean data of each BC_3F_3 family were used for QTL analysis combining with BC_3F_2 genotypic data.

Trait correlation analysis was executed by STATISTICA software. Heritability for the traits SPP and TGW was estimated in RILs using the formula:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + n^{-1}\sigma_{ge}^2 + (nr)^{-1}\sigma_e^2},$$

where σ_g^2, σ_{ge}^2, and σ_e^2 were the estimates of genetic (G), G × E, and error variances derived from the mean square expectations of the analysis of variance, with $n = 2$ being the number of environments and $r = 2$ being the number of replicates.

Results

Trait variations in RILs and BC_3F_2 population

The two parents, Minghui 63 and Teqing, showed highly significant differences in the traits of SPP and TGW

Table 1 Descriptive statistics of the traits for parents and the RIL population

Traits	Heritability (%)	Years	RIL population		Parents		
			Range	Mean ± SD	Teqing	Minghui 63	*t* value
SPP	87.3	2005	95.7–237.8	160.5 ± 28.4	210.5	138.8	10.7**
		2006	100.4–228.0	158.1 ± 26.3	196.7	136.0	9.8**
TGW	93.6	2005	16.3–33.6	24.4 ± 2.73	22.6	25.9	9.7**
		2006	18.3–29.7	23.6 ± 2.25	22.0	28.2	14.2**

** Significance level at $P = 0.001$

(Table 1). Minghui 63 exhibited a small panicle of about 130 spikelets but large TGW of about 28 g, and Teqing had a large panicle with about 200 spikelets but only 22 g of TGW (Table 1). Large variations in RILs were observed for SPP and TGW in 2005 and 2006, and their phenotypic values showed a normal frequency distribution. In the RIL population, TGW and SPP showed high heritability of 93.6 and 87.3%, respectively (Table 1). Transgressive segregation was observed for TGW in RILs. However, no transgressive segregation was observed for SPP and TGW in the BC_3F_2 population; the SPP distribution was skewed toward the small-value parent, Minghui 63, and the TGW distribution was skewed toward the large-value parent, Minghui 63 (Fig. 1). In the BC_3F_2 population, both the seed setting ratio and tillering number showed a normal frequency distribution from 70.2 to 91.2% and 4–14 tillers, respectively.

Correlation between SPP and TGW

A significant negative correlation was observed between SPP and TGW in both RILs ($r = -0.330$ and -0.270 in 2005 and 2006, respectively) and the BC_3F_2 population ($r = -0.467$). The correlation between SPP and grain shape was also analyzed in the BC_3F_2 population. TGW showed a significant positive correlation with GL, GW, and GT, although TGW showed a significant negative correlation with the ratio of GL/GW (Table 2). SPP was significantly negatively correlated with all investigated grain shape traits. GL was significantly correlated with GW and GT, but no significant correlation was observed between GW and GT (Table 2).

QTLs identified in RILs

A total of 133 polymorphic SSR markers evenly distributed on the 12 chromosomes were used for construction of the genetic linkage map with the RIL population. The genetic map spanned 1,371.4 cM in length, with an average distance of 10.3 cM between adjacent markers. The longest interval was 31.7 cM between RM493 and RM1349 in this genetic map.

Five and three SPP QTLs were identified in 2005 and 2006, respectively (Table 3). Three SPP QTLs (*SPP1*, *SPP3b*, and *SPP8*) were detected in both years, whereas the

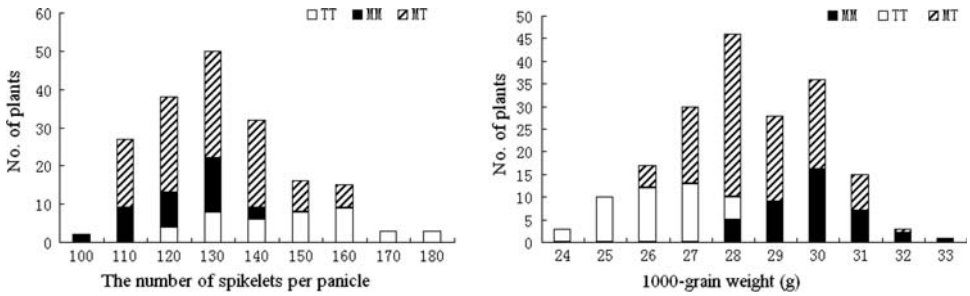

Fig. 1 Frequency distribution of number of spikelets per panicle and TGW in the BC_3F_2 population. The three genotypes of homozygous Teqing (*TT*), homozygous Minghui 63 (*MM*), and heterozygote (*MT*) were identified based on a progeny test

Table 2 Correlation among spikelets per panicle, 1,000-grain weight, and grain shape in the BC_3F_2 population

Traits	Spikelets per panicle	1,000-grain weight	Grain length	Grain width	Grain thickness
1,000-grain weight	–0.467**				
Grain length (GL)	–0.203**	0.380**			
Grain width (GW)	–0.373**	0.491**	0.184**		
Grain thickness	–0.216**	0.520**	0.250**	0.104	
Ratio of GL/GW	0.274**	–0.203**	0.498**	–0.761**	0.115

** Significant correlation at $P < 0.01$

Table 3 QTLs identified for spikelets per panicle (SPP) and 1,000-grain weight (TGW) from Teqing/Minghui 63 RIL population in 2005 and 2006

Traits	QTL	Chr.	Interval	2005			2006		
				LOD	ADD[a]	Var (%)[b]	LOD	ADD[a]	Var (%)[b]
SPP	*SPP1*	1	RM1195–RM490	2.9	8.6	10.2	2.5	8.3	9.8
	SPP3a	3	RM3400–RM3646	3.9	8.5	9.7			
	SPP3b	3	RM3436–RM5995	8.9	12.7	22.4	6.6	11.2	18.1
	SPP7	7	RM18–RM248	4.0	13.2	24.3			
	SPP8	8	RM126–RM483	2.9	7.8	8.3	3.4	8.5	10.3
TGW	*TGW3a*	3	RM3400–RM3646	10.3	–1.3	24.0	10.1	–1.1	24.8
	TGW3b	3	RM3436–RM5995	8.2	–1.2	19.6	4.8	–0.8	12.3
	TGW5	5	RM574A–RM3381	2.6	0.9	10.7	3.3	0.8	13.2
	TGW9	9	RM215–RM1013				2.8	0.6	7.8

[a] Additive effect, positive value means the Teqing allele increases the trait

[b] Percentage of total phenotypic variance explained by the QTL

other two SPP QTLs (*SPP3a* and *SPP7*) were detected only in one year (Fig. 2).

Three and four TGW QTLs were identified in 2005 and 2006, respectively (Table 3). Three TGW QTLs (*TGW3a*, *TGW3b*, and *TGW5*) were detected in both years, whereas *TGW9* was only detected in one year on chromosome 9 (Fig. 2). Two pairs of QTLs, *SPP3a*/*TGW3a* and *SPP3b*/*TGW3b*, were identified with opposite effect for SPP and TGW in the same intervals between RM3400 and RM3646 and RM3436 and RM5995, respectively.

Validation of *SPP3b* and *TGW3b* in the BC_3F_2 population

The BC_3F_1-11 plant was heterozygous at only five markers among the 85 investigated. Three were located in the target QTL region. The other two, RM566 and RM511, were located on chromosomes 9 and 12, respectively. No QTL was identified near these two markers in RILs. An *F* test showed the two markers were not associated with SPP and TGW in BC_3F_2 (data not shown).

Five SSR markers plus one indel marker around the target region were used to genotype the 215 BC_3F_2 individuals produced by BC_3F_1-11 selfing, and then a local linkage group covering 11.8 cM was constructed (Fig. 3). One TGW QTL, *TGW3b*, and one SPP QTL, *SPP3b*, were simultaneously detected in a 2.6-cM region between the markers RM15855 and W3D16 in the BC_3F_2 population. The LOD peak position for *TGW3b* was 1.6 cM from RM15855, and it explained 50.4% of the TGW variance with an additive effect of 1.81 g. The LOD peak position for *SPP3b* was located exactly at the locus of RM15855,

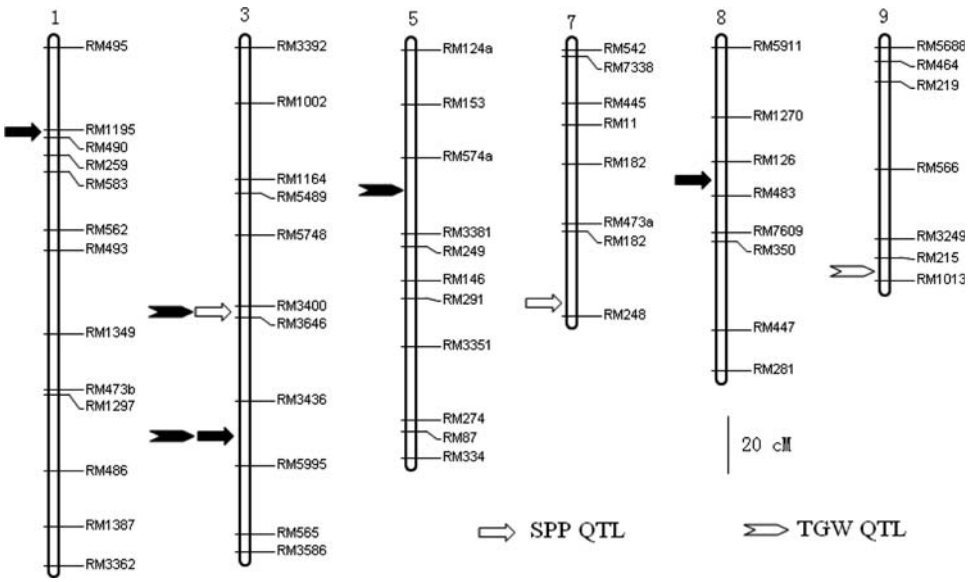

Fig. 2 This molecular linkage map showing the position of spikelets per panicle (SPP) and 1,000-grain weight (TGW) QTLs identified in an RIL population. The black and white arrows indicate that the QTLs were detected in both environments and a single environment, respectively

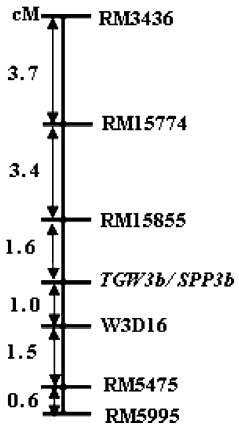

Fig. 3 The local linkage map of *TGW3b* and *SPP3b* region on chromosome 3 constructed with the BC_3F_2 population. The positions of QTLs were identified by a progeny test

and it explained 29.1% of the SPP variance with an additive effect of 11.89 spikelets (Table 4). In the BC_3F_3 progeny test, *TGW3b* and *SPP3b* were also detected in the same region between RM15855 and W3D16. They explained 34.5 and 20.2% of the corresponding trait variation, respectively (Table 4). No QTL for seed setting and tillers per plant was identified in the BC_3F_2 population.

Based on the performance of 16 plants within a family, the 193 families were classified into three groups: (1) identical small trait values with a small standard deviation, (2) identical large trait values with a small standard deviation, or (3) varied trait values with a large standard deviation. In theory, the three groups corresponded to two homozygotes and a heterozygote (MT) at target QTLs. However, groups 1 and 2 had some overlapping values, which caused difficulty in making clear attribution of plants falling into the overlapping region (Fig. 2). Thus, the genotypes of these plants could not be identified by only trait performance. For this situation, we inferred the QTL genotypes of these plants according to the flanking markers. If both markers were genotyped with Teqing or Minghui 63 homozygotes, the genotypes of these plants at the target QTL should be Teqing or Minghui 63 homozygotes, respectively. For TGW, only four plants fell into the overlapping region; three of them were assigned to group 1 and the other to group 2. Finally, 193 BC_3F_3 families (193 BC_3F_2 plants) were assigned to the three groups corresponding to Teqing homozygote (TT), Minghui 63 homozygote (MM), and a heterozygote (MT) at *TGW3b*. There were 47 TT, 42 MM, and 104 MT plants. The TGW variations of TT, MM, and MT ranged from 23.8 to 27.9, 27.3 to 32.0, and 25.4 to 31.6 g, respectively (Table 5; Fig. 1). Accordingly, for SPP, 23 plants in the overlapping region were attributed to corresponding groups. The 193 families could also be classified into three groups, which coincidently shared the same individuals as those classified based on TGW. The SPP variation of TT, MM, and MT genotypes at *SPP3b* ranged from 116.7 to 179.6, 99.3 to 133.0, and 102.2 to 154.0 spikelets, respectively. The averaged TGW and SPP of heterozygotes were 28.0 g and 123.5 spikelets, which were skewed toward the 29.4 g and 117.7 spikelets of MM but were far away from the 25.8 g and 141.5 spikelets of TT. These results suggested that *TGW3b* and *SPP3b* were co-segregated and the Minghui 63 allele acted dominantly (Table 5; Fig. 1). Finally, *TGW3b* and *SPP3b* were mapped as a molecular marker to a locus 1.6 cM from RM15855 and 1.0 cM from W3D16 (Fig. 3).

QTL analysis for grain shape

TGW was strongly associated with grain shape, which was defined by three dimensions, GL, GW, and GT. Because the 193 investigated families could be classified into three groups according to TGW, grain shape was calculated for the three groups. GL variations of TT, MM, and MT ranged from 9.25 to 10.03, 9.77 to 10.41, and 9.57 to 10.29 mm, respectively; GW variations ranged from 2.9 to 3.0, 2.8 to 3.1, and 2.8 to 3.1 mm, respectively; and GT variations were from 1.9 to 2.0, 1.9 to 2.1, and 1.9 to 2.1 mm, respectively (Table 5).

To understand which one among the three dimensions was most affected by the target chromosome region, QTLs

Table 4 Effects of the QTL for spikelets per panicle (SPP), 1,000-grain weight (TGW), and grain shape in the BC_3F_2 population

Traits	Generation	QTL	LOD	A[a]	D[b]	D/A	Var (%)[c]
SPP	F_2	*SPP3b*	12.8	11.89	–5.94	–0.5	29.1
	F_3	*SPP3b*	8.8	7.85	–1.9 ns		20.2
TGW (g)	F_2	*TGW3b*	26.2	–1.81	0.35	–0.19	50.4
	F_3	*TGW3b*	17.0	–0.89	0.21 ns		34.5
Grain length (mm)	F_2	*GL3*	14.9	–0.17	0.035 ns		33.7
Grain width (mm)	F_2	*GW3*	6.9	–0.05	0.009 ns		18.1
Grain thickness (mm)	F_2	*GT3*	3.9	–0.02	0.008 ns		10.0

[a] Additive effect, positive value means the Teqing allele increases the trait

[b] Dominance effect, ns not significant at the level of 5%

[c] Percentage of total phenotypic variance explained by the QTL

Table 5 Trait performance of three genotypes at the targeted QTL on the basis of progeny test of BC_3F_2 population

Traits	Minghui 63 homozygotes (MM)				Heterozygotes (MT)				Teqing homozygotes (TT)			
	No. of F_2 plants	Mean	CV (%)	Range	No. of F_2 plants	Mean	CV (%)	Range	No. of F_2 plants	Mean	CV (%)	Range
SPP	42[a]	117.7	8.4	99.3–133.0	104[a]	123.5	10.0	102.2–154.0	47[a]	141.5	12.2	116.7–179.6
TGW (g)	42	29.4	4.1	27.3–32.0	104	28.0	4.6	25.4–31.6	47	25.8	4.7	23.8–27.9
GL (mm)		10.04	1.8	9.77–10.41		9.88	1.6	9.57–10.29		9.71	1.9	9.25–10.03
GW (mm)		2.99	2.3	2.84–3.12		2.95	2.4	2.78–3.1		2.90	1.7	2.76–2.97
GT (mm)		2.01	2.0	1.94–2.11		1.99	2.0	1.90–2.10		1.97	2.0	1.89–2.04

Genotypes of BC_3F_2 were determined by a progeny test. MM and TT homozygotes have identical small or large trait values with very small standard deviation within BC_3F_3 families. When the mean value within BC_3F_3 family varied within the overlapping values between small and large groups, QTL flanking marker genotypes were used to identify the genotype of target QTLs. MT heterozygotes have varied trait values within BC_3F_3 families

[a] Chi square test fit the ratio of 1:2:1 ($\chi^2 = 1.42$, $P = 0.49$)

for GL, GW, and GT were analyzed in the BC_3F_2 population. One QTL for each trait was detected in the interval flanked by RM15855 and W3D16. *GL3* explained 33.7% of phenotype variance with an additive effect of 0.17 mm, *GW3* explained 18.1% of phenotype variance with an additive effect of 0.05 mm, and *GT3* explained 10% of phenotype variance with an additive effect of 0.02 mm (Table 4).

Discussion

Either two tightly linked QTLs or a pleiotropic QTL with opposite effects on TGW and SPP

QTL locations were defined to a large interval of about 10 cM in a primary mapping population because of genetic background noise (Darvasi et al. 1993). In the present study, *TGW3b* and *SPP3b* were identified in a small region on chromosome 3 in an RIL population. Although *TGW3b* and *SPP3b* were frequently detected in many primary mapping populations, their precise positions and effects remain uncertain. However, in this study, the 1-LOD confidence intervals of *TGW3b* and *SPP3b* mostly overlapped each other, probably indicating one pleiotropic QTL. Both QTLs were validated by an advanced backcross population of BC_3F_2 in this study. According to a progeny test of the BC_3F_2, *TGW3b* and *SPP3b* were precisely mapped to a locus 1.6 cM from RM15855 and 1.0 cM from W3D16. Although *TGW3b* and *SPP3b* were mapped as a single Mendelian factor and cosegregated in this study, the sample size of 193 BC_3F_2 plants for genetic analysis was not large enough to verify their cosegregation. It is still difficult to clarify their relationship, that is, either two closely linked QTLs or a pleiotropic QTL with an opposite effect on TGW and SPP.

Recent studies on QTL fine mapping have shown that some tiny chromosome regions have pleiotropic effects on TGW and SPP. For example, *qTNSP6-1* and *qTGWT6-1*, controlling SPP and TGW, respectively, were fine-mapped to a 125-kb region on chromosome 6 and cosegregated (Cheng et al. 2007). The SPP QTL *sn9.1* and TGW QTL *gw9.1* were delimited to a 37.4-kb region on chromosome 9 (Xie et al. 2008). Moreover, pleiotropy of recently cloned QTLs has been confirmed by transformation. A major QTL controlling GW and TGW, *GW2*, was cloned; it increases TGW by 49.8% but decreases grains per main panicle by 29.9% (Song et al. 2007). *DEP1*, a major QTL conferring density and erect panicle, could significantly increase the number of grains per panicle and grain yield but decrease TGW (Huang et al. 2009). Similar phenomena were observed in tomato; *fw2.2*, controlling fruit weight, encodes a putative negative regulator of cell division (Frary et al. 2000). Transcripts from *fw2.2* in a large-fruited plant strongly decreased in developing flower bud and fructification number due to changes in carbohydrate partitioning (Baldet et al. 2006). Mutants can also provide evidence for genetic pleiotropic effects on SPP and TGW. *lk1* (*lk1.1* or *lkna*) and *Lk2* (*Lknb*), the long kernel and heavy grain weight genes, were identified from the mutants Nagayama 77402a (N179) and Nagayama 77402b (N182), respectively. Both of the *lk1* and *Lk2* mutants had heavy TGW and small SPP (Takamure et al. 1995). The above examples show that it is not haphazard which QTLs have pleiotropic effects on related traits in rice. Thus, even though the population size of BC_3F_2 was not large enough to resolve the question of pleiotropy versus close linkage, cosegregation indicated that *TGW3b* and *SPP3b* were more likely the same QTL with opposite effects on TGW and SPP.

No matter what closely linked QTLs or one pleiotropic QTL lay in the small region, the target region contains genes that could function to balance its allocation between

SPP and TGW when a rice plant partitioned carbohydrate. For instance, if a rice plant were to produce heavy grains, as compensation the number of SPP would be reduced. That is, TGW negatively correlates with SPP. Kato (1993) suggested that the negative correlation between TGW and SPP arises because most spikelets show a low rate of filling when assimilation is limited. In this study, the TT plant has a smaller size for grains, which reduces sink size per grain, but the plant has more SPP. In contrast, the MM plant has a larger size for grains but fewer SPP. As a result, total sink size per panicle could remain constant between two homozygous genotypes. The pleiotropic region will probably control the assimilation partition capacity constant by balancing the relationship between SPP and TGW.

QTLs for rice breeding

Pursuing high grain yield is one of the most important objectives in rice production. The genetic bases of SPP and TGW have received much attention because of their importance in rice yield. In this study, five SPP QTLs and four TGW QTLs were identified in an RIL population. All those QTLs can be repeatedly identified across multiple populations. For example, the minor QTL, *SPP1,* was mapped into the similar interval as *SPP1* and *Gn1a* (Liu et al. 2009; Ashikari et al. 2005). But *SPP1* and *Gn1a* are major QTLs with large additive effects of about 20 spikelets. The minor QTL, *SPP8*, shared the similar interval with *QSpp8*, which explained about 80% of SPP variation in NILs (Zhang et al. 2006). *SPP3* (Zhang et al. 2009) shared an overlapping region with *SPP3b,* which was detected in this study, and both had a relatively large QTL effect. Interestingly, all the four TGW QTLs, *TGW3a*, *TGW3b*, *TGW5*, and *TGW9*, and two SPP QTLs *SPP1* and *SPP3a* were identified in the mapping population of RILs, which shared the common parent, Minghui 63, with the RIL mapping population used in this study (Xing et al. 2002). In addition, *GS3* (Fan et al. 2006), and *gw9.1* (Xie et al. 2008) shared an overlapping region with *TGW3a*, and *TGW9* detected in this study. Thus, these QTLs appear to be reliable.

Mono-effect QTL is easily used for rice improvement, but the application of a pleiotropic QTL with opposite effects on different traits is complicated. If there were two tightly linked QTLs, complementary combination between them could be obtained with the aid of molecular markers. Namely, the genotype pyramiding the Minghui 63 allele at *TGW3b* and the Teqing allele at *SPP3b* would produce higher yields as compared to two parental genotypes. However, it is more likely that *TGW3b* and *SPP3b* are the same QTL with opposite effects on TGW and SPP, which creates a challenge for its application in rice improvement. The Minghui 63 homozygote increased TGW but decreased SPP, whereas the Teqing homozygote decreased TGW but increased SPP at the target QTL. This caused a counteractive effect on yield, resulting in no significant difference in grain yield per plant between Teqing homozygotes and Minghui 63 homozygotes (data not shown). Thus, the use of pleiotropic QTLs for yield improvement would not lead to a difference, no matter which allele was manipulated. At this time, more attention should be paid to rice grain appearance quality, which is mainly evaluated by the ratio of GL/GW. But the GL/GW ratios of Teqing and Minghui 63 homozygotes were also very similar. That is, both parental alleles have similar effects on grain yield and grain appearance quality. Hence, the best strategy for its application is to discover more valuable alleles hidden in wild rice germplasm after the QTL is cloned. For example, if one allele has a large positive effect on TGW and a small negative effect on SPP, an enhanced yield results. Thus, the allele would be recommended for rice breeding.

Reasonable strategy for isolation of *TGW3b*

One of the most important uses of high-quality QTL-based NILs is for target QTL isolation. In this study, trait variations did not show a bimodal distribution, and three genotypes at the target QTL could not be easily distinguished in the BC_3F_2 population. However, QTL cloning based on a large BC_3F_2 is feasible. Among the investigated traits, TGW values of TT genotype overlapped with heterozygotes (MT) to some extent and with MM genotype at a very low frequency (Fig. 1; Table 5). Trait value overlapping between both homozygotes makes fine mapping the target gene awkward because the families of two genotypes do not segregate and cannot be distinguished by a progeny test. For example, TGW variations of the TT genotype overlapped from 27.3 to 27.9 g with the MM genotype. The progeny of two homozygous plants whose TGW fell within the overlapping range expressed a similar trait value with a very small standard deviation, which obscures the identity of the genotype.

In such a case, it would seem to be impossible to isolate *TGW3b*. However, when following a map-based cloning strategy, not all the plants in the population are included. Frequently, only some plants showing extreme values are taken into consideration from recessive homozygotes. In this study, TGW is more suitable for isolation of *TGW3b* because TGW had more recessive plants, which could be distinguished from the other two genotypes by scoring phenotype. If we set TGW $\leq$26 g as a stringent standard to choose plants for *TGW3b* fine mapping, then 30 plants are screened in the small population. Of them, 25 plants belong to the TT genotype (Fig. 1). That is, about 17% of plants selected for fine mapping would be regarded as recessive ones by mistake. However, all of these plants should be

heterozygotes, whose progenies express a large variation. They could be discriminated from recessive homozygotes by a progeny test. Hence, isolation of *TGW3b* would be feasible. In addition, isolation of *TGW3b* will provide clear evidence to determine the definitive relationship between *TGW3b* and *SPP3b*.

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