Research Paper

Advanced backcross QTL analysis reveals complicated genetic control of rice grain shape in a *japonica × indica* cross

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Grain shape is an important trait for improving rice yield. A number of quantitative trait loci (QTLs) for this trait have been identified by using primary F_2 mapping populations and recombinant inbred lines, in which QTLs with a small effect are harder to detect than they would be in advanced generations. In this study, we developed two advanced mapping populations (chromosome segment substitution lines [CSSLs] and BC_4F_2 lines consisting of more than 2000 individuals) in the genetic backgrounds of two improved cultivars: a *japonica* cultivar (Koshihikari) with short, round grains, and an *indica* cultivar (IR64) with long, slender grains. We compared the ability of these materials to reveal QTLs for grain shape with that of an F_2 population. Only 8 QTLs for grain length or grain width were detected in the F_2 population, versus 47 in the CSSL population and 65 in the BC_4F_2 population. These results strongly suggest that advanced mapping populations can reveal QTLs for agronomic traits under complicated genetic control, and that DNA markers linked with the QTLs are useful for choosing superior allelic combinations to enhance grain shape in the Koshihikari and IR64 genetic backgrounds.

Key Words: chromosome segment substitution lines (CSSLs), advanced mapping population, quantitative trait loci (QTL), grain shape, *Oryza sativa* L.

Introduction

The agronomic traits of rice are generally controlled by multiple quantitative trait loci (QTLs; Yamamoto *et al.* 2009, Yano and Sasaki 1997). Identification of QTLs that control grain production and resistance to biotic and abiotic stresses is a primary step in efforts to enhance rice cultivars by incorporating beneficial QTL alleles into superior genetic backgrounds. Grain shape in rice is an important trait, and QTL alleles that increase grain size and to control shape are a target in allele mining studies with the goal of increasing yield (Huang *et al.* 2013). Grain shape in Asian cultivated rice is diverse, and extensive research has been conducted to identify QTLs controlling it. More than 100 papers have reported nearly 200 QTLs for grain length and grain width

(reviewed by Huang *et al.* 2013), of which 15 have been mapped on a fine scale (Bai *et al.* 2010, Guo *et al.* 2009, Li *et al.* 2004, Qiu *et al.* 2012, Shao *et al.* 2012, Wan *et al.* 2006) and 16 have been cloned including *GS3* (Fan *et al.* 2006, Mao *et al.* 2010), *GW2* (Song *et al.* 2007), *GW5/ qSW5* (Shomura *et al.* 2008, Weng *et al.* 2008), *GS5* (Li *et al.* 2011), *TGW6* (Ishimaru *et al.* 2013), and *GW6a* (Song *et al.* 2015). Although this progress has encouraged marker-assisted breeding to target these QTLs and genes in rice, those studies focused mainly on QTLs with large effects, and the information available on QTL alleles is not currently sufficient for fine-tuning grain shape in practical breeding programs.

A drastic increase in grain size usually does not increase grain productivity proportionally, owing to decreases in both grain filling and grain quality as result of imbalances between the sink and source potentials (Peng *et al.* 2008, Takai *et al.* 2013, Takita 1983). Therefore, grain shape should be improved by using appropriate QTL alleles to

maintain an appropriate balance between sinks and sources and thus to allow an increase in grain yield. Thus, alleles that govern various effects on grain size are needed for efficient improvement of rice, since this would provide a "toolbox" of QTL alleles that provide an appropriate grain shape in genotypes with diverse source capacities.

Most QTL mapping studies in rice use primary mapping populations such as F_2 populations, recombinant inbred lines (RILs), or backcross inbred lines (BILs), and thus might not detect QTLs with small effects owing to the lower detection power of using such early generations (Yamamoto *et al.* 2009). In contrast, using advanced mapping populations can overcome this problem because their more uniform genetic background increases the ability to detect even QTLs with small effects (Tanksley and Nelson 1996, Yamamoto *et al.* 2009). Among these populations, chromosome segment substitution lines (CSSLs) let us detect QTLs distributed throughout the genome with high sensitivity and by using a smaller number of plants than would be required in analyses using F_2 populations or RILs (Abe *et al.* 2013, Ando *et al.* 2008, Ebitani *et al.* 2005, Hori *et al.* 2010, Ishikawa *et al.* 2005, Kubo *et al.* 2002, Murata *et al.* 2014, Takai *et al.* 2009). Through the use of such populations, more than 40 QTLs have been detected that are associated with yield potential in rice, a complex trait whose evaluation requires much time and labor (Takai *et al.* 2014). Takai *et al.* (2014) also confirmed the importance of maintaining the sink–source balance in efforts to increase yield, and noted that this balance fluctuated in response to the nature of introgression of the donor's genome.

The size of the donor's chromosome segments in the respective CSSLs is generally not small enough to define the position of QTLs within a smaller chromosomal region relative to other mapping populations, so genetic mapping using CSSLs sometimes fails to assign a position to some QTLs (Ebitani *et al.* 2005). However, an advanced mapping population in which small chromosomal regions have segregated in a highly homogeneous background represents a promising alternative to locate such QTLs (Paterson *et al.* 1990). Recently, a genome-wide genetic analysis using such populations was conducted to uncover the genetic architecture underlying rice's heading date (K. Hori *et al.*, unpublished). In that study, 255 QTLs throughout the rice genome were detected by using 11 cross combinations, of which 127 were detected in regions that differed from those of the 650 previously identified QTLs for heading date that are listed in public databases (Yonemaru *et al.* 2010, Youens-Clark *et al.* 2011). The evidence strongly suggests that this type of advanced mapping population is useful to detect naturally occurring variations that underlie agronomic traits, including QTLs with small effects.

To enhance our understanding of the genetic control of grain shape in rice, we performed a QTL analysis for grain shape (i.e., grain length and width) in a cross between a *japonica* rice cultivar with short, round grains (Koshihikari) and an *indica* cultivar (IR64) with long, slender grains in

three mapping populations: an F_2 primary mapping population and reciprocal CSSL and BC_4F_2 populations as the advanced mapping populations. On the basis of the results, we compare the QTL detection power among these populations and comprehensively review the genetic control of grain shape in this *japonica × indica* cross.

Materials and Methods

Plant materials

We used three Koshihikari × IR64 populations in this study: an F_2 population and advanced reciprocal CSSL and BC_4F_2 populations (**Fig. 1**). We began with an F_1 population derived from the cross between Koshihikari and IR64 (IRGC66970), and repeatedly backcrossed the progeny with either Koshihikari or IR64 to produce BC_4F_1 plants. We conducted a whole-genome survey using 150 simple sequence repeat (SSR) markers in each backcrossed generation (BC_1F_1 to BC_4F_1) to select target chromosome segments in each CSSL and to minimize non-target chromosome segments from the donor, then selected plants that are homozygous for the target chromosome segments in the self-pollinated progeny (**Supplemental Table 1**). From BC_4F_4 or BC_4F_5 plants, we selected 42 CSSLs with a Koshihikari genetic background (IRK-CSSL) and 40 with an IR64 genetic background (KSI-CSSL), and grew the plants with their parents in 2011 (**Fig. 1**).

To further dissect grain shape, we grew 31 BC_4F_2 populations developed with Koshihikari as the recurrent parent (IRK-BC_4F_2), 37 BC_4F_2 populations developed with IR64 as the recurrent parent (KSI-BC_4F_2) and their parents in 2012 (**Fig. 1**). We used 44 plants for each IRK-BC_4F_2 line and 45 plants for each KSI-BC_4F_2 line in our genotyping and trait measurements. As a reference primary mapping population for QTL detection, we grew 247 F_2 plants and their parents in 2013. All plants were grown in an experimental field at
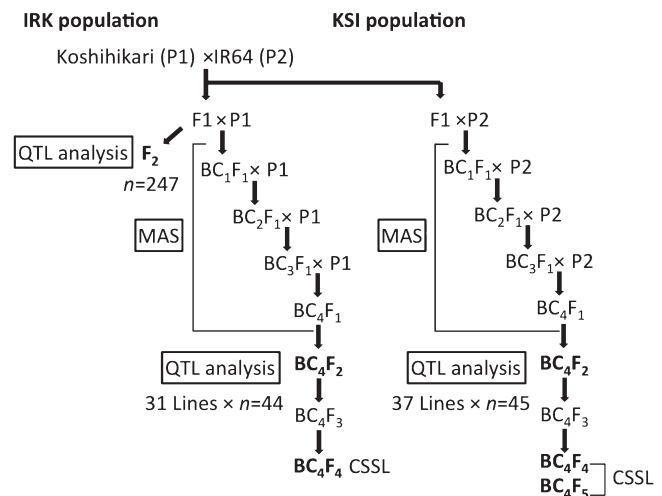
Fig. 1. Development of the plant materials used in this study. CSSL, chromosome segment substitution line; MAS, marker-assisted selection; QTL, quantitative trait locus.

the National Institute of Agrobiological Sciences, Tsukuba, Japan (36.03°N, 140.11°E). Basal fertilizer was applied at a rate of 56 kg/ha N, 56 kg/ha P, and 56 kg K/ha. Month-old seedlings were transplanted in mid-May at one seedling per hill in plots with a double row for each line, at 18 cm between plants and 36 cm between rows. Rice grains were harvested in September and were air-dried before analysis.

Measurement of grain shape

We scanned approximately 250 per individual at 600 dpi on a GTX-820 scanner (Seiko Epson, Inc., Suwa, Japan). We measured the grain length (GL) and grain width (GW) of the seeds in the images using the "SmartGrain" grain shape analysis software (Tanabata *et al.* 2012). The "awn removal" function in the software was set at "intermediate", and other parameters used the default values. To calculate the mean and standard deviation (SD) of the grain length and grain width parameters for each population or line, we excluded any grains whose outlines lay outside the 95% confidence interval.

DNA extraction and marker analysis

Fresh leaves were harvested from field-grown plants, and total DNA was extracted from 1- to 3-cm sections of the fresh leaves by using the method of Takeuchi *et al.* (2008) for genotyping of the CSSLs and by crushing the tissue in 30 μL 0.5 M NaOH and diluting it in 120 μL of 1 M Tris·HCl (pH 8.0) for the F_2 and BC_4F_2 populations. We used a total of 129 SSR markers and a single-nucleotide polymorphism (SNP) marker for *GS3* (IDGS3_001_3), with an average marker interval of 2.7 Mb (**Supplemental Table 1**), to detect QTLs in the F_2 and BC_4F_2 populations. We added 97 SSR or insertion/deletion markers to the chromosomal regions around QTLs to determine their positions in IRK-BC_4F_2, and 100 for KSI-BC_4F_2 (**Supplemental Table 1**). The genotypes of the CSSLs, which we characterized by using 271 SNP markers (**Supplemental Tables 2, 3**), were determined by using 384-plex SNPs for the Illumina GoldenGate BeadArray technology platform (Illumina, Inc., San Diego, CA, USA) based on previously reported information (Ebana *et al.* 2010).

Substitution mapping of QTLs in CSSLs

We used Student's *t*-test in Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) to identify mean values for the grain-size parameters that differed significantly between the CSSLs and the recurrent parents, with significance at $P < 0.05$ (two-tailed). A CSSL whose mean for a grain size parameter differed significantly from the recurrent parent by the *t*-test was judged to carry one or more putative QTLs in one or more regions of the donor's chromosome segments in that CSSL. When the *t*-test was not significant, the CSSL was judged to lack a QTL in its donor's chromosome segments. Putative QTLs were assigned as described by Paterson *et al.* (1990) and Ishikawa *et al.* (2005). In brief, a small chromosomal region that carries one or more QTLs

was defined on the basis of the association among RILs between the phenotype and genotype within a certain chromosomal region. When a CSSL differed significantly from another CSSL that carried a single putative QTL and the recurrent parent for a trait, this line was judged to carry additional putative QTLs. Accordingly, putative QTLs were assigned to maximize the number of CSSLs whose phenotype could be explained. As a result, CSSLs whose phenotype differed significantly from the recurrent parent but for which the putative QTL's region was not supported by some other CSSLs were judged to be "not assigned".

QTL analysis in the F_2 and BC_4F_2 populations

We constructed a genetic map using MAPMAKER/EXP v. 3.0 software (Lander *et al.* 1987). QTL analysis for grain length and grain width was performed using version 2.5 of QTL Cartographer software (Basten *et al.* 2005), and the threshold was obtained by using 1000 permutations. QTLs were named according to the nomenclature rules of McCouch *et al.* (1997). Since the marker interval in the primary QTL analysis was 2.7 Mb, QTLs with a similar additive effect that were located less than 2.7 Mb apart were judged to be a single QTL.

Results

Variation of grain length and grain width in the F_2 population

To clarify phenotypic variation of seed shape in F_2 of the cross between Koshihikari and IR64, grain length and grain width in the 247 F_2 plants and their parents were measured. The mean grain length and grain width of Koshihikari grown in 2013 were 7.15 ± 0.35 mm (mean ± SD) and 3.48 ± 0.17 mm, respectively. Those of IR64 grown in 2013 were 9.64 ± 0.51 mm and 2.54 ± 0.16 mm, respectively. The difference in seed shape in these two cultivars was apparent in the two traits. Grain lengths in the F_2 population ranged from 6.81 to 9.34 mm and averaged 8.17 ± 0.38 mm, and grain widths ranged from 2.34 to 3.36 mm and averaged 2.86 ± 0.17 mm (**Supplemental Fig. 1A, 1B**). The distribution of these two traits in the F_2 population was continuous between the values of the parents except for a gap at a grain length of 7.1 mm.

QTLs for grain length and grain width in the F_2 population

To identify QTLs for grain length and grain width in a primary mapping population, QTL analysis was performed in the F_2 population of the cross between Koshihikari and IR64. Five QTLs whose IR64 allele increased grain length and three whose IR64 allele decreased grain width were detected, as expected from the trait values of the parents and the distribution in the F_2 population (**Fig. 2A, Supplemental Fig. 1**). The number of QTLs detected is comparable to that in the previous studies using the F_2 (1 to 32, 8.7 on average) (Huang *et al.* 2013). A QTL for grain length on chromosome 3 accounted for 40% of the phenotypic variation
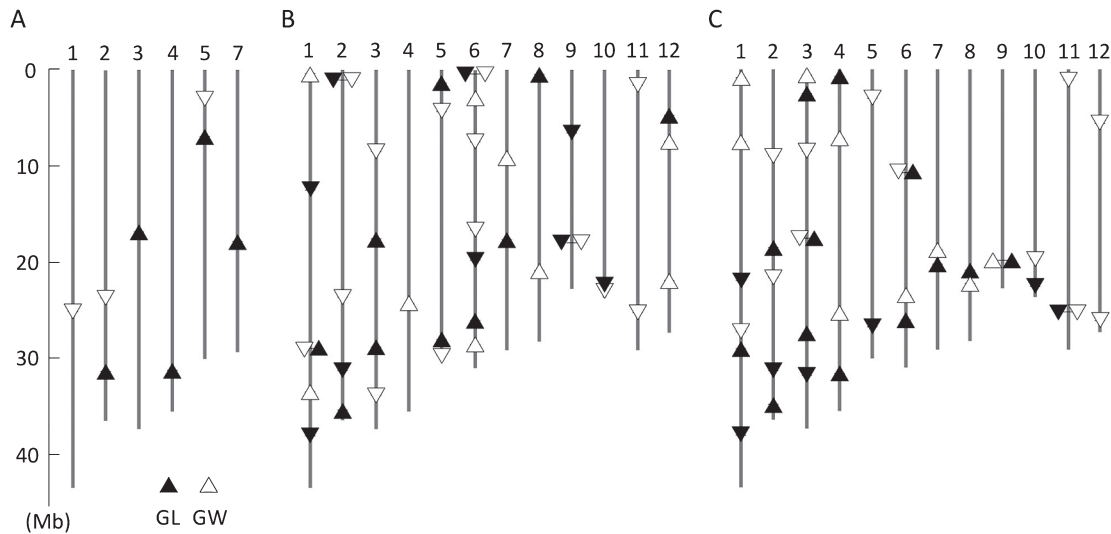
Fig. 2. Chromosome locations of the QTLs for grain length and grain width detected in this study. (A) F_2 population (Koshihikari × IR64), (B) IRK-BC$_4$F$_2$ population (see **Fig. 1** for details), (C) KSI-BC$_4$F$_2$ (see **Fig. 1** for details). Upward-pointing triangles (△, ▲) indicate that the IR64 alleles increase the parameter value, whereas downward-pointing triangles (▽, ▼) indicate that the IR64 alleles decrease the parameter value.

and a QTL for grain width on chromosome 5 accounted for 24% of the phenotypic variation, but the other QTLs had small effects, and each accounted for less than 10% of the phenotypic variation (**Table 1**).

Genomic constitution of CSSLs

To determine grain shape in advanced mapping populations of the same cross combination, 42 CSSLs with a Koshihikari genetic background (IRK-CSSL) and 40 with an IR64 genetic background (KSI-CSSL) were developed and their genomic constitution was characterized. Each of the 42 IRK-CSSLs contained at least one large substituted segment of a particular target chromosomal region from IR64 and additional small segments in non-target regions in the Koshihikari genetic background (**Supplemental Table 2**). If we assume for simplicity that each recombination occurred midway between the two surrounding markers, the size of the target IR64 chromosomal segments in each CSSL ranged from 1.3 to 22.3 Mb and averaged 11.1 Mb (**Supplemental Table 2**). Although several non-target regions from IR64 (a mean size of 2.2 Mb) and heterozygous regions (a mean size of 0.8 Mb) remained, each CSSL contained approximately 95.9% of the Koshihikari genome (**Supplemental Table 2**). Similarly, each of the 40 KSI-CSSLs contained at least one large substituted segment of a particular target chromosomal region from Koshihikari and additional small segments in non-target regions in the IR64 genetic background (**Supplemental Table 3**). The size of the target Koshihikari chromosomal segments in each CSSL ranged from 2.9 to 27.8 Mb and averaged 12.3 Mb, with several non-target regions from Koshihikari (a mean of 2.3 Mb) and heterozygous regions (a mean of 2.6 Mb), and each CSSL contained approximately 95.4% of the IR64 genome (**Supplemental Table 3**).

QTL detection in the CSSLs

To identify putative QTLs and their chromosomal locations in the IRK-CSSLs and KSI-CSSLs developed in the present study, grain length and grain width in these CSSLs and their parents were measured and substitution mapping for these traits was performed. In the IRK-CSSLs, grain lengths ranged from 6.95 to 8.27 mm, versus 7.24 mm in Koshihikari and 9.80 mm in IR64 in 2011 (**Fig. 3**A). Grain widths ranged from 3.09 to 3.55 mm, versus 3.44 mm in Koshihikari and 2.55 mm in IR64 (**Fig. 3**B). Twenty-nine IRK-CSSLs differed significantly from 'Koshihikari' in grain length (**Fig. 3**A), and 31 differed in grain width (**Fig. 3**B). Putative QTLs were assigned at 20 chromosomal regions for grain length and 16 for grain width (**Fig. 4**A). We could not assign putative QTLs in one line (SL2022) that differed significantly from Koshihikari in grain length and in four lines (SL2012, SL2033, SL2039, and SL2042) that differed in grain width (**Fig. 4**A). Seven CSSLs carried multiple putative QTLs for either grain length or grain width (**Fig. 4**A, footnote c).

In the KSI-CSSLs, grain lengths ranged from 8.91 to 10.18 mm, versus 7.15 mm in Koshihikari and 9.83 mm in IR64 (**Fig. 3**C). Grain widths ranged from 2.39 to 2.84 mm, versus 3.41 mm in Koshihikari and IR64 in 2.56 mm (**Fig. 3**D). Twenty-six KSI-CSSLs differed significantly from IR64 in grain length (**Fig. 3**C), and 29 differed in grain width (**Fig. 3**D). Putative QTLs were assigned at 14 chromosomal regions for grain length and 15 for grain width (**Fig. 4**B). We could not assign putative QTLs in one line (SL2109) that differed significantly from IR64 in grain length and in two lines (SL2126 and SL2127) that differed in grain width (**Fig. 4**B). Three CSSLs were judged to be carrying multiple putative QTLs for either grain length or grain width (**Fig. 4**B, footnote c).

Table 1. QTLs for grain length and grain width identified in an F_2 population and in the BC_4F_2 populations

Chr.	Postion (Mb)		QTL name		Additive effect (BC_4F_2)[e] IRK	KSI	Additive effect (F_2)[e]	R^2 (%) (F_2)[f]
1	12.49		*qGL1-1*		0.102	–	–	–
1	21.90		*qGL1-2*		–	0.083	–	–
1	28.90		*qGL1-3*		–0.117	–0.156	–	–
1	38.01		*qGL1-4*	c	0.116	0.258	–	–
1	0.30–0.75	a	*qGW1-1*		–0.055	–0.054	–	–
1	7.40		*qGW1-2*		–	–0.014	–	–
1	25.07	b	(W)		–	–	0.060	4.9
1	27.19–28.90	a	*qGW1-3*		0.026	0.059	–	–
1	33.48		*qGW1-4*	c	–0.015	–	–	–
2	0.98		*qGL2-1*		0.076	–	–	–
2	18.46		*qGL2-2*		–	–0.168	–	–
2	31.20		*qGL2-3*		0.080	0.091	–	–
2	31.20	b	(L)		–	–	–0.020	3.4
2	34.69–35.26	a	*qGL2-4*	c	–0.023	–0.236	–	–
2	0.98		*qGW2-1*		0.064	–	–	–
2	8.98		*qGW2-2*		–	0.052	–	–
2	21.52–23.57	a	*qGW2-3*		0.099	0.058	0.074	8.5
3	2.45		*qGL3-1*		–	–0.161	–	–
3	17.42		*qGL3-2*		–0.304	–0.347	–0.315	40.2
3	27.32–28.78	a	*qGL3-3*		–0.102	–0.131	–	–
3	31.74		*qGL3-4*	c	–	0.162	–	–
3	0.54		*qGW3-1*	c	–	–0.038	–	–
3	8.39		*qGW3-2*		0.028	0.057	–	–
3	17.42		*qGW3-3*		–	0.071	–	–
3	33.80		*qGW3-4*	d	0.044	–	–	–
4	0.69		*qGL4-1*		–	–0.105	–	–
4	31.56		*qGL4-2*		–	–0.176	–0.127	4.4
4	7.10		*qGW4-1*	d	–	–0.054	–	–
4	24.24–25.22	a	*qGW4-2*		–0.022	–0.036	–	–
5	1.23		*qGL5-1*		–0.159	–	–	–
5	6.83	b	(L)		–	–	–0.124	4.5
5	26.65		*qGL5-2*	c	–	0.173	–	–
5	28.02		*qGL5-3*		–0.092	–	–	–
5	2.89–4.26	a	*qGW5-1*		0.150	0.163	0.127	24.4
5	29.54		*qGW5-2*	c	0.019	–	–	–
6	0.38		*qGL6-1*	c	0.093	–	–	–
6	10.65		*qGL6-2*		–	–0.384	–	–
6	19.70		*qGL6-3*	c	0.069	–	–	–
6	26.00		*qGL6-4*		–0.096	–0.127	–	–
6	0.38		*qGW6-1*	c	0.026	–	–	–
6	2.85		*qGW6-2*	c	–0.041	–	–	–
6	7.41		*qGW6-3*		0.027	–	–	–
6	10.65		*qGW6-4*		–	0.066	–	–
6	16.58		*qGW6-5*		0.037	–	–	–
6	23.34		*qGW6-6*		–	–0.044	–	–
6	28.47		*qGW6-7*		–0.039	–	–	–
7	17.69–20.11	a	*qGL7*		–0.105	–0.134	–0.157	7.6
7	9.08		*qGW7-1*		–0.052	–	–	–
7	18.66		*qGW7-2*		–	–0.031	–	–
8	0.42		*qGL8-1*		–0.078	–	–	–
8	20.82		*qGL8-2*	c	–	–0.112	–	–
8	20.82–22.21	a	*qGW8*		–0.035	–0.044	–	–
9	6.59		*qGL9-1*		0.051	–	–	–
9	18.04		*qGL9-2*		0.072	–	–	–
9	19.79		*qGL9-3*	c	–	–0.025	–	–
9	18.04		*qGW9-1*	d	0.037	–	–	–
9	19.79		*qGW9-2*	d	–	–0.034	–	–
10	22.43		*qGL10*	c	0.116	0.082	–	–
10	19.69		*qGW10-1*		–	0.045	–	–
10	22.83		*qGW10-2*		0.025	–	–	–
11	25.13		*qGL11*	d	–	0.134	–	–
11	1.08–1.46	a	*qGW11-1*		0.028	0.060	–	–
11	25.13		*qGW11-2*		0.043	0.018	–	–
12	4.75		*qGL12*		–0.058	–	–	–
12	5.51		*qGW12-1*	d	–	0.031	–	–
12	7.57		*qGW12-2*	c	–0.018	–	–	–
12	22.02		*qGW12-3*	c	–0.050	–	–	–
12	26.00		*qGW12-4*	c	–	0.047	–	–

a, The positions of the QTLs in the two BC_4F_2 populations fell within 2.7 Mb of each other, and they were judged to be the same QTL.

b, The positions of the QTLs in the F_2 population fell >2.7 Mb from that in the two BC_4F_2 populations, but they were judged to be the same on the basis of their additive effects.

c, The QTLs that were not detected in the F_2 or CSSL populations.

d, The QTLs that were detected only in the BC_4F_2 population of the same genetic background.

e, Additive effect of Koshihikari allele on grain length or grain width.

f, Percentage of phenotypic variance explained by QTL.
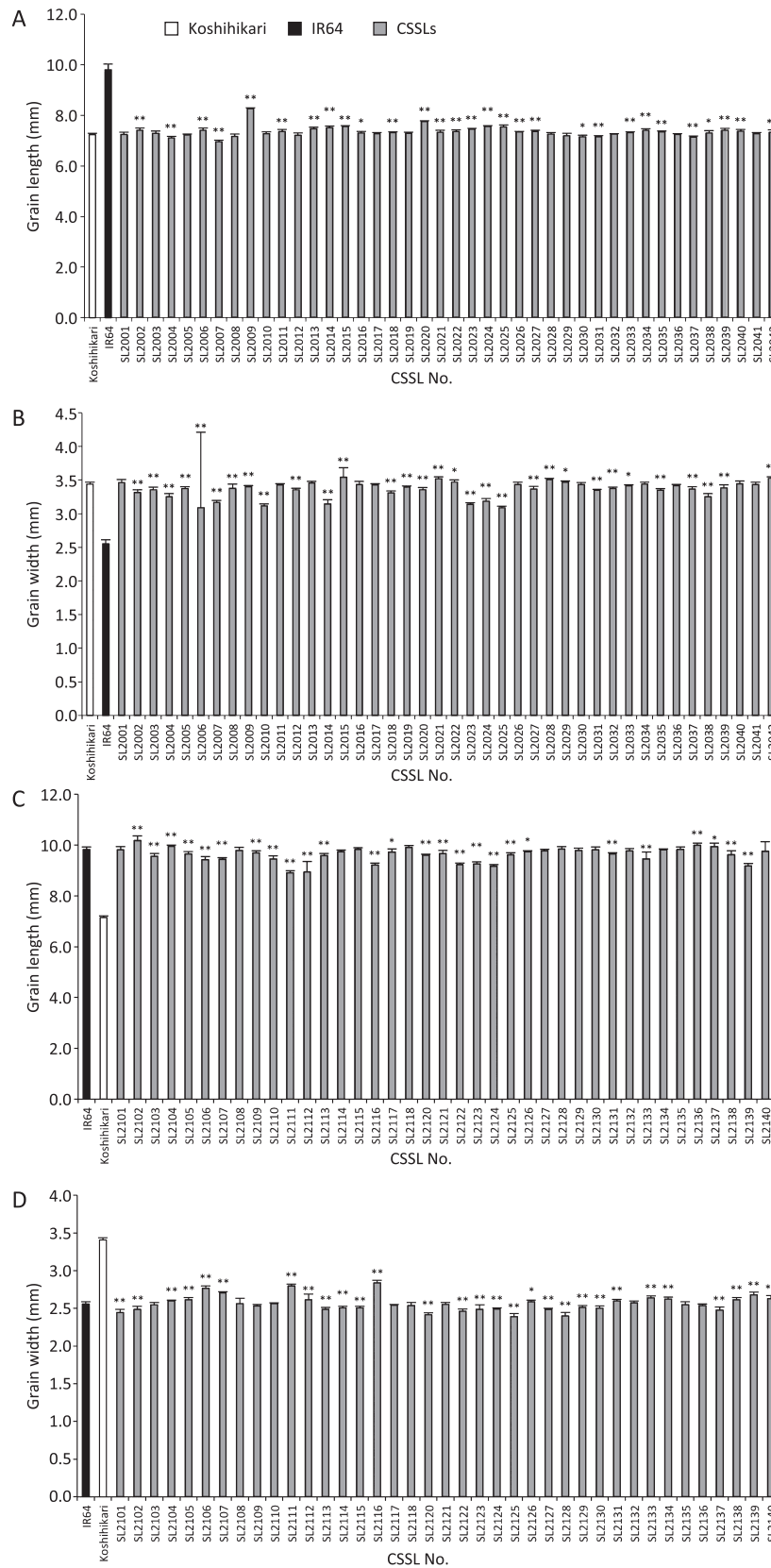
Fig. 3. Grain length and grain width in two sets of CSSLs: (A, B) IRK-CSSLs, with IR64 introgression in a Koshihikari background; (C, D) KSI-CSSLs, with Koshihikari introgression in an IR64 background. Values are means ± SD. Bars labeled with asterisks differed significantly from the means for (A, B) Koshihikari and (C, D) IR64: *, $P < 0.05$; **, $P < 0.01$ (two-tailed t-test). Data for SL2119 were excluded from this figure because this line headed too late for us to harvest seeds under natural field conditions.

Nine chromosomal regions that carried putative QTLs for grain length were shared in the two sets of CSSLs (**Fig. 4**A, **4**B, footnote a), but another 16 putative QTLs were found in only one of the sets (**Fig. 4**A, **4**B, footnote d). Five chromosomal regions that carried putative QTLs for grain width were shared in the two sets of CSSLs (**Fig. 4**A, **4**B, footnote a), but another 17 putative QTLs were only found in one of the sets (**Fig. 4**A, **4**B, footnote d). In total, 47 putative QTLs for the two traits, nearly six times that in our analysis using the F_2 population, were identified and assigned a location in the two sets of CSSLs (**Fig. 4**A, **4**B).

Variation of grain length and grain width in the BC_4F_2 populations

To further clarify grain shape in another advanced mapping population, grain length and grain width were measured in 31 BC_4F_2 populations developed with Koshihikari as the recurrent parent (IRK-BC_4F_2) and 37 BC_4F_2 populations developed with IR64 as the recurrent parent (KSI-BC_4F_2). The mean grain length and grain width of Koshihikari grown in 2012 were 7.03 ± 0.32 mm and 3.41 ± 0.16 mm, respectively. Those of IR64 grown in 2012 were 9.49 ± 0.51 mm and 2.50 ± 0.16 mm, respectively. Grain length in IRK-BC_4F_2 ranged from 6.55 to 8.65 mm and averaged 7.12 ± 0.21 mm, whereas that in KSI-BC_4F_2 ranged from 8.27 to 10.54 mm and averaged 9.35 ± 0.28 mm (**Supplemental Fig. 1**C, **1**E). Grain width in IRK-BC_4F_2 ranged from 2.97 to 3.82 mm, and averaged 3.38 ± 0.09 mm, whereas that in KSI-BC_4F_2 ranged from 2.07 to 3.10 mm, and averaged 2.52 ± 0.10 mm (**Supplemental Fig. 1**D, **1**F). The range of variation of both traits in the BC_4F_2 populations was smaller than that in the F_2 population, and their averages were closer to those of the recurrent parents than in the F_2 population.

QTLs for grain length and grain width in the BC_4F_2 populations

QTL analysis for grain length and grain width was performed in each of 31 IRK-BC_4F_2 and 37 KSI-BC_4F_2 populations and the number of QTLs and their positions and effects in BC_4F_2 were compared with those in other mapping populations. In IRK-BC_4F_2, we detected QTLs in 23 out of 31 populations (**Supplemental Table 4**): 19 for grain length and 23 for grain width (**Fig. 2**B). In KSI-BC_4F_2, we detected QTLs in 26 out of 37 populations (**Supplemental Table 5**): 20 for grain length and 21 for grain width (**Fig. 2**C). We detected a total of 65 QTLs for the two traits in the two populations (**Table 1**). This number is 8 times that in our analysis using the F_2 and 1.4 times that in our analysis using the CSSLs.

Of these 65 QTLs, 8 (6 for grain length and 2 for grain width) were mapped at the same marker intervals, and another 10 (3 for grain length and 7 for grain width) were mapped within 2.7 Mb of each other between the IRK-BC_4F_2 and KSI-BC_4F_2 populations (**Table 1**, footnote a). Another 17 (9 for grain length and 8 for grain width) were not detected in the F_2 or CSSL populations (**Table 1**, footnote c). Among the 33 putative QTLs that were detected in one set of CSSLs (**Fig. 4**A, **4**B, footnote d), 6 were detected only in the BC_4F_2 population of the same genetic background (**Fig. 4**A, **4**B, footnote b, **Table 1**, footnote d), and the other 27 were detected in the BC_4F_2 of the other genetic background (**Table 1**). Among the 47 putative QTLs detected in the CSSLs, 7 were not detected in the BC_4F_2 populations (**Fig. 4**A, **4**B, footnote c). Our analysis confirmed that most of the 65 QTLs detected using BC_4F_2 were found in both the Koshihikari and IR64 genetic backgrounds.

All 8 of the QTLs detected in the F_2 population were validated in the BC_4F_2 populations, although 3 of the BC_4F_2 QTLs were located more than 2.7 Mb from the corresponding QTL in the F_2 population (**Table 1**, footnote b). For example, in the F_2 population, a QTL whose IR64 allele increases grain length (additive effect [AE] = –0.02) was detected at 31.20 Mb on chromosome 2. But in IRK-BC_4F_2, QTL *qGL2-3*, with the opposite additive effect (AE = 0.09), was detected at the same position, and *qGL2-4*, whose additive effect was in the same direction (AE = –0.03) as in the F_2 population, was detected at 34.69 Mb. The positions of the two QTLs in IRK-BC_4F_2 were almost same as those in KSI-BC_4F_2. Notably, the additive effects of *qGL2-4* in the KSI-BC_4F_2 population (AE = –0.24), in which only *qGL2-4* is segregating, was 8 times that in the IRK-BC_4F_2 population (AE = –0.03) and more than 8 times that in the F_2 population (AE = –0.02), in which both *qGL2-3* and *qGL2-4* are segregating (**Fig. 5**).

Collectively, BC_4F_2 population exhibited highest sensitivity in detecting QTLs among three populations, yet our analysis underestimated in the additive effect of some QTLs.

Discussion

Our results clearly indicate that the genetic control of grain shape, an important agronomic trait of rice, is complex, as we found 65 QTLs involved in genetic variation of grain shape in a single cross combination. Extensive genetic research on grain shape previously identified nearly 200 QTLs for grain length and grain width, of which 16 have been cloned in rice (Huang *et al.* 2013). The two QTLs with major effects detected here, *qGL3-2* and *qGW5-1*, probably correspond to *GS3* and *GW5/qSW5*, respectively (Fan *et al.* 2006, Mao *et al.* 2010, Shomura *et al.* 2008, Weng *et al.* 2008). The three QTLs (*qGL1-4*, *qGW2-2*, and *qGW8*) were located in genomic regions harboring previously cloned or finely mapped gene loci (Singh *et al.* 2012, Song *et al.* 2007, Xie *et al.* 2006). Nevertheless, some QTLs detected here (*qGW1-1*, *qGL4-1*, *qGL9-1*, *qGL12-1*, and *qGL12-2*) appear to be new on the basis of the map locations of the previously reported QTLs (Huang *et al.* 2013). The allelic effects of these QTLs need to be validated as potential sources of alleles for modulating grain shape. In addition, further screening of QTL alleles for grain shape in other
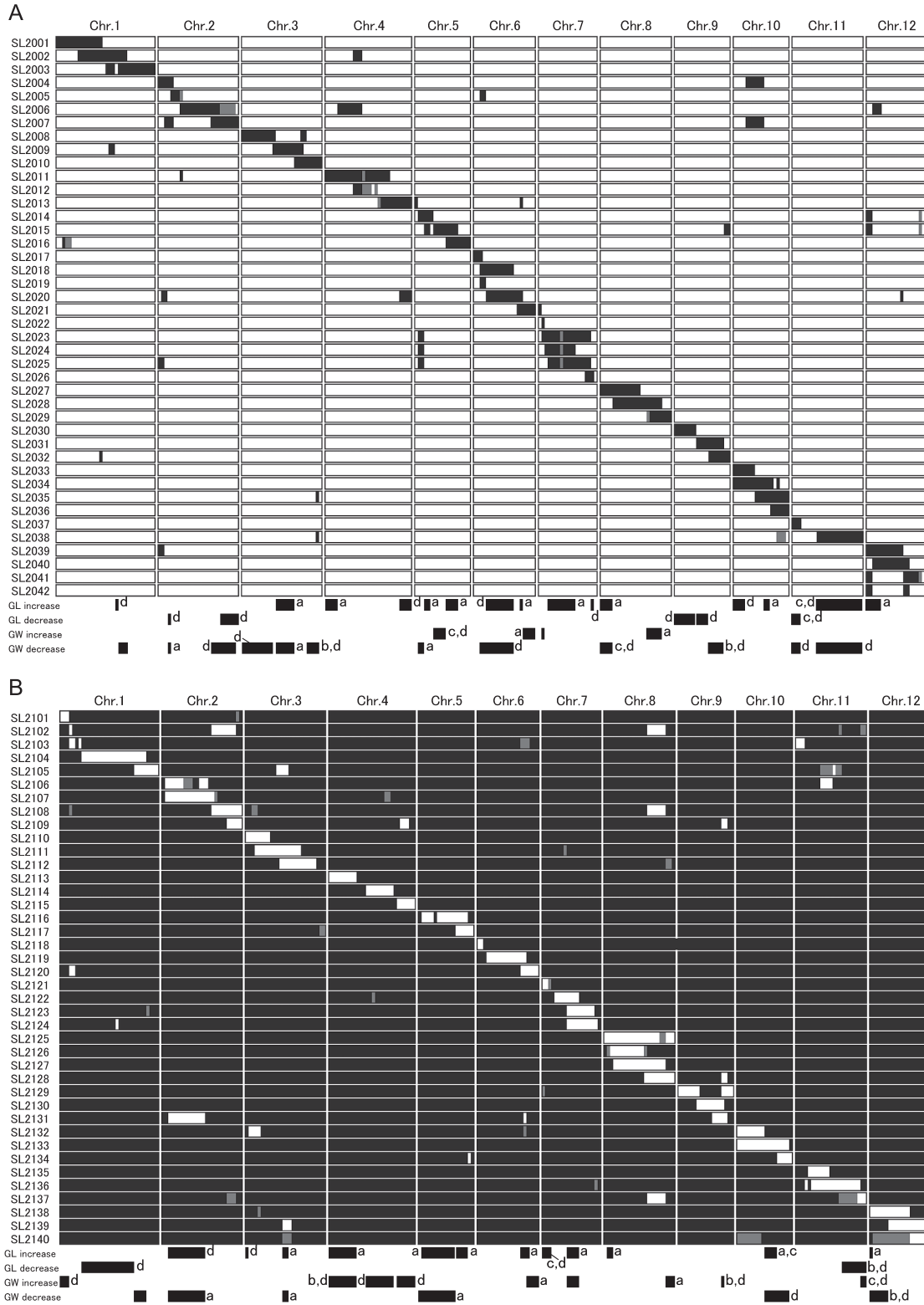
Fig. 4. (A) Graphical genotypes and positions of the putative QTLs in the IRK-CSSLs, with IR64 introgression in a Koshihikari background. Black regions, homozygous for IR64 alleles; white, homozygous for Koshihikari alleles; gray, heterozygous. (B) Graphical genotypes and positions of the putative QTLs in the KSI-CSSLs, with Koshihikari introgression in an IR64 background. Black regions, homozygous for IR64 alleles; white, homozygous for Koshihikari alleles; gray, heterozygous. a, Putative QTLs that were detected in both the IRK- and KSI-CSSLs; b, putative epistatic QTLs that were not detected in the other genetic background in the CSSLs or in the BC_4F_2 population; c, putative QTLs that were not detected in the BC_4F_2 population. d , putative QTLs that were detected in one set of CSSLs; Chr., chromosome number; GL, grain length; GW, grain width.
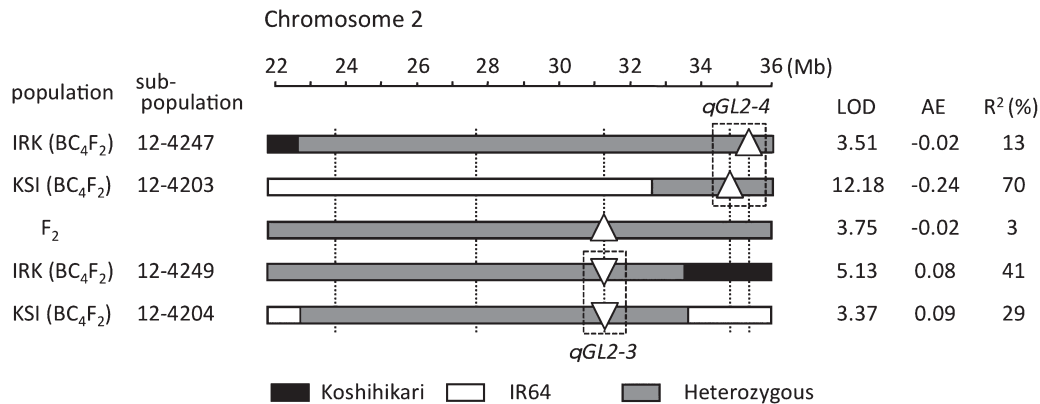
Fig. 5. Graphical genotypes and positions of the QTLs for grain length that were detected in the region from 30 to 36 Mb on chromosome 2 in the F_2 population and in two BC_4F_2 sub-populations in the genetic background of Koshihikari (IRK) or IR64 (KSI). Black boxes, homozygous for IR64; white, homozygous for Koshihikari; gray, heterozygous. AE, additive effect. Upward-pointing triangles (△) indicate that the IR64 alleles increase values, and downward-pointing triangles (▽) indicate that the IR64 alleles decrease values.

cultivars by using advanced mapping populations and fine-scale genetic mapping would provide a more comprehensive elucidation of the genetic control of grain shape in Asian rice cultivars.

QTL alleles for grain length were co-localized with those for grain width in 18 regions (**Table 1**). Previous studies detected QTLs for either grain length or grain width in a given chromosomal region, but complementation testing of the cloned genes (*GW2*, *GS3*, *GS5*, and *GW6a*) revealed pleiotropic effects of single genes for grain length or grain width (Li *et al.* 2011, Mao *et al.* 2010, Song *et al.* 2007, 2015). Thus, some of our examples (i.e., *qGL3-2* and *qGW3-3*, *qGL9-2* and *qGW9-2*, and *qGL11* and *qGW11-2*) might result from pleiotropic effects of single genes, whereas others might represent close linkage of independent genes, because the two QTLs were not located in the same marker interval in a single mapping population (i.e., *qGL5-3* and *qGW5-2*, *qGL6-4* and *qGW6-6*, and *qGL10* and *qGW10-1*). Therefore, fine genetic mapping and functional characterization of the genes that control grain shape will be indispensable for uncovering the complexity of the traits and identifying loci that have been mapped in similar regions in different cross combinations.

Previous studies detected 1 to 32 QTLs (8.7 on average) for grain length and grain width per single cross combination (Huang *et al.* 2013). This number is comparable to that in our analysis using the F_2 population; we therefore believe that QTL mapping using such a primary mapping population cannot reliably detect QTLs with small effects, probably owing to noise from the QTLs with large effects (Yamamoto *et al.* 2009). In addition to the QTLs with large effects, epistasis between pairs of QTLs with small effects potentially involves complicated genetic control of yield-related traits, including grain shape (Lei *et al.* 2008, Li *et al.* 1997, Li *et al.* 2008). Most of the 65 QTLs in the present study were detected under both the Koshihikari and IR64 genetic backgrounds, and some (*qGW5-1*, *qGL8-2*, and *qGW8*) were located in chromosomal regions that harbored a QTL

with an epistatic effect in previous studies. Thus, we hypothesize that a larger proportion of the genetic variation than that expected on the basis of previous studies could be explained by an additive model in the cross combination we used. To further clarify non-additive effects of the QTL alleles, the combined effect of QTL alleles should be evaluated in a homogeneous genetic background by using crosses between near-isogenic lines.

The case of *qGL2-3* and *qGL2-4*, two QTLs for grain length that lie about 4 Mb apart on chromosome 2 (**Fig. 5**), provides an important insight into the genetic control of agronomic traits. The position of *qGL2-3* in the F_2 population might not have been estimated correctly, possibly owing to the heterogeneous genetic background. Even in the analysis using the BC_4F_2 populations, linkage of the QTL alleles in repulsion decreased our ability to detect QTLs and resulted in underestimation of the additive effect when the two QTLs are segregating (**Fig. 5**). Six putative QTLs detected in our analysis using CSSLs were not detected in the analysis using the BC_4F_2 populations for similar or other reasons (**Fig. 4**A, **4**B, footnote c). The cases in which multiple QTL alleles for a trait are linked in coupling might result in overestimation of the effect of a single QTL in primary mapping studies, as has been reported previously (Ashikari *et al.* 2005, Fukuoka *et al.* 2012, Song *et al.* 2015, Yu *et al.* 2008). From these observations, both a homogeneous background and the number of recombination events in a mapping population are important factors when dissecting agronomic traits that are under complicated genetic control.

By using fewer than 50 genotypes per population, our analysis using CSSLs efficiently assigned a greater number of putative QTLs for grain length and grain width than our analysis using the F_2 population. CSSLs have been used to detect QTLs for other traits such as heading date, yield, and preharvest sprouting resistance (Abe *et al.* 2013, Ando *et al.* 2008, Ebitani *et al.* 2005, Hori *et al.* 2010, Ishikawa *et al.* 2005, Takai *et al.* 2009, 2014). Previous studies and the present study together suggest that CSSLs are useful genetic

materials for dissecting complex agronomic traits in rice, but the number of recombinations in these lines determines the resolution of the genetic mapping, as was suggested previously (Ebitani *et al.* 2005, Xu *et al.* 2010). Koshihikari is a leading cultivar in temperate areas, and IR64 is a leading cultivar in tropical areas. Reciprocal CSSLs from the cross between these cultivars, which are being released by researchers from the Rice Genome Resource Center at the National Institute of Agrobiological Sciences (http://www.rgrc.dna.affrc.go.jp/index.html), will be useful genetic materials for analyzing various agronomic traits to enhance cultivars in both tropical and temperate areas.

We identified a large number of QTL alleles that will be useful for modulating grain shape in the genetic background of the two improved cultivars, Koshihikari and IR64. These QTL alleles mostly had smaller effects than those that have been cloned so far, but will nonetheless be useful for increasing grain yield, because a drastic increase of grain size usually does not increase grain production (Peng *et al.* 2008, Takai *et al.* 2013, Takita 1983). Thus, it is necessary to change the traits related to grain production while retaining a balance between sinks and sources. Some QTL alleles that increase the 1000-grain weight are co-localized with QTLs that reduce the number of grains per panicle (Takai *et al.* 2014). This example suggests that breeders should choose QTL alleles for grain shape that lack negative associations with other agronomic traits or should remove tightly linked undesirable traits to increase grain production in a practical breeding program.

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