Breeding Science 68: 210–218 (2018)
doi:10.1270/jsbbs.17112

Research Paper

Identification of QTLs for rice grain size using a novel set of chromosomal segment substitution lines derived from Yamadanishiki in the genetic background of Koshihikari

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Grain size is important for brewing-rice cultivars, but the genetic basis for this trait is still unclear. This paper aims to identify QTLs for grain size using novel chromosomal segment substitution lines (CSSLs) harboring chromosomal segments from Yamadanishiki, an excellent sake-brewing rice, in the genetic background of Koshihikari, a cooking cultivar. We developed a set of 49 CSSLs. Grain length (GL), grain width (GWh), grain thickness (GT), 100-grain weight (GWt) and days to heading (DTH) were evaluated, and a CSSL-QTL analysis was conducted. Eighteen QTLs for grain size and DTH were identified. Seven (*qGL11*, *qGWh5*, *qGWh10*, *qGWt6-2*, *qGWt10-2*, *qDTH3*, and *qDTH6*) that were detected in F_2 and recombinant inbred lines (RILs) from Koshihikari/Yamadanishiki were validated, suggesting that they are important for large grain size and heading date in Yamadanishiki. Additionally, QTL reanalysis for GWt showed that *qGWt10-2* was only detected in early-flowering RILs, while *qGWt5* (in the same region as *qGWh5*) was only detected in late-flowering RILs, suggesting that these QTLs show different responses to the environment. Our study revealed that grain size in the Yamadanishiki cultivar is determined by a complex genetic mechanism. These findings could be useful for the breeding of both cooking and brewing rice.

Key Words: grain size, QTL, CSSLs, brewing-rice cultivar, QTL-by-environment interaction.

Introduction

Rice (*Oryza sativa* L.) is one of the most important crops in the world. It is not only a staple food for the Japanese population, but also the raw material for the alcoholic beverage known as sake. Brewing-rice cultivars have characteristic traits adapted to sake brewing, such as large grain size and a high percentage of white-core grain. These traits are favorable for high-grade grain polishing (Aramaki *et al*. 1995), fast water absorption (Horigane *et al*. 2014, Nagato and Ebata 1959) and amylolysis efficiency in the process of sake brewing (Yanagiuchi *et al*. 1997). As such they are important targets for the breeding of a brewing cultivar. Yamadanishiki is of very high quality and is the highest-yielding brewing-rice cultivar in Japan; therefore, Okada *et al*. (2017) and Yoshida *et al*. (2002) used Yamadanishiki as a crossing parent to conduct QTL analysis of favorable traits for sake brewing. These papers detected common QTLs for grain length on chromosomes 4 and 11, and found the QTL for grain width and weight on chromosome 5. However, it is still necessary to verify these putative QTLs, so they can be used in the breeding of sake-brewing rice. Because grain size is associated with yield, the QTLs for large grain size in brewing-rice cultivars may also facilitate the breeding of high-yield cooking cultivars.

A set of chromosomal segment substitution lines (CSSLs) has a genetic background that is almost completely homogenous to the recipient parent, except with one chromosomal segment from the donor parent. A complete CSSL set represents the entire genome of the donor reproduced in the background of the recipient. Therefore, the CSSLs can be used to evaluate the genetic effects derived from the donor in detail, and to elucidate the complex genetic

Communicated by Motoyuki Ashikari
Received September 6, 2017. Accepted November 13, 2017.
First Published Online in J-STAGE on March 30, 2018.
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mechanisms behind agronomic traits (Ebitani *et al*. 2005). A large number of CSSL sets have been developed to identify the QTLs for the agronomic traits of rice, such as grain size and heading date (Ando *et al*. 2008, Bian *et al*. 2010, Ebitani *et al*. 2005, Furuta *et al*. 2014, Murata *et al*. 2014).

Yamasaki and Ideta (2013) classified the Japanese paddy rice population into six subgroups: Kirara397, Reimei, Nipponbare, Koshihikari, Asahi, and Kamenoo. The brewing-rice cultivars, Omachi, Yamadanishiki, and Gohyakumangoku belong to the Kamenoo subgroup. Since Yamadanishiki has been the most popular and highest-yielding brewing cultivar in recent years, it was selected for the genetic analysis (Okada *et al*. 2017) and breeding of novel brewing cultivars (Kaji *et al*. 2013). There is a large genetic difference between the Koshihikari and Yamadanishiki cultivars in the Japanese rice population (Yamasaki and Ideta 2013); therefore, the use of a Koshihikari/Yamadanishiki segregating population to conduct genetic analysis of their agronomic traits could contribute to the identification of new QTLs and to next-generation rice breeding.

There are three elements that determine rice grain size: grain length (GL), grain width (GWh), and grain thickness (GT). Many QTLs for grain size have recently been detected (Huang *et al*. 2013). Moreover, Nagata *et al*. (2015) identified a large number of QTLs for GL and GWh despite using mapping populations of a single-crossing combination derived from Koshihikari and IR64. They indicated that grain shape was controlled by many QTLs, which suggests a complex genetic mechanism. Reviews by Zuo and Li (2014) and Zheng *et al*. (2015) indicate that rice grain size is determined by: a proteasomal degradation pathway related to genes such as *GW2* (Song *et al*. 2007) and *GW5*/*qSW5* (Shomura *et al*. 2008, Weng *et al*. 2008), a G-protein signaling pathway related to genes such as *GS3* (Fan *et al*. 2006, Takano-Kai *et al*. 2009), a phytohormone pathway related to genes such as *TGW6* (Ishimaru *et al*. 2013) and *OsBRI1* (Morinaka *et al*. 2006), and other pathways related to genes such as *GS5* (Li *et al*. 2011) and *GW8* (Wang *et al*. 2012a). Because the characteristics of these pathways are unclear, it is necessary to define the mechanisms controlling grain size.

In this study, we report the development of novel CSSLs, i.e., chromosomal segments from the most popular brewing-rice cultivar, Yamadanishiki, in the genetic background of Koshihikari, an elite Japanese cooking cultivar. By examining these CSSLs for grain size and heading date, we identified novel QTLs and validated several other QTLs that had previously been detected using F_2 and recombinant inbred lines (RILs) derived from Koshihikari and Yamadanishiki crosses (Okada *et al*. 2017). In addition, one of the major QTLs for 100-grain weight (GWt) detected by the RILs was not identified in the CSSL-QTL analysis, suggesting that this QTL might be affected by the environment. To verify the effect of the environment on the QTLs for GWt, we re-analyzed the Koshihikari/Yamadanishiki RILs (Okada *et al*. 2017).

Materials and Methods

Development of CSSLs

The CSSL development process is illustrated in **Fig. 1**. Koshihikari was crossed with Yamadanishiki, and the resultant F_1 was backcrossed with Koshihikari. Two or four generations of backcrossing yielded BC_2F_1 and BC_4F_1. The BC_2F_1 population produced 156 plants in the BC_2F_4 generation after four generations of self-pollination. In the BC_4F_2 generation, 2,136 plants were generated from 89 plants from the BC_4F_1 generation. The leaves of the BC_2F_4 and BC_4F_2 populations were collected and DNA was extracted using the method described by Dellaporta *et al*. (1983) with minor modifications. One hundred and seventy-eight bulked BC_4F_1 DNA samples were produced by grouping the samples from BC_4F_2 plants in batches of 12, using an automated pipetting machine (epMotion 5070; Eppendorf, Hamburg, Germany). We performed a whole-genome survey (First MAS) of the BC_2F_4 and the bulked BC_4F_1 samples (**Fig. 1**), using 125 DNA markers including: 71 simple sequence repeat (SSR) markers, 36 cleaved amplified polymorphic sequence (CAPS) markers, 17 derived CAPS (dCAPS) markers, and one PCR-confronting two-pair primer (PCR-CTPP) marker (**Supplemental Table 1**). The average distance between adjacent markers was about 3.11 Mb. The CAPS and dCAPS markers were constructed from the linkage maps of Koshihikari/Yamadanishiki (Okada *et al*. 2017). After selecting heterozygous lines from the bulked BC_4F_1 samples, homozygous plants from BC_4F_2 were selected as candidate CSSLs (First MAS, **Fig. 1**). This resulted in 49 CSSL candidate plants derived from the BC_2F_4 and BC_4F_2 populations. Furthermore, the CSSLs cultivated in 2015 were selected to

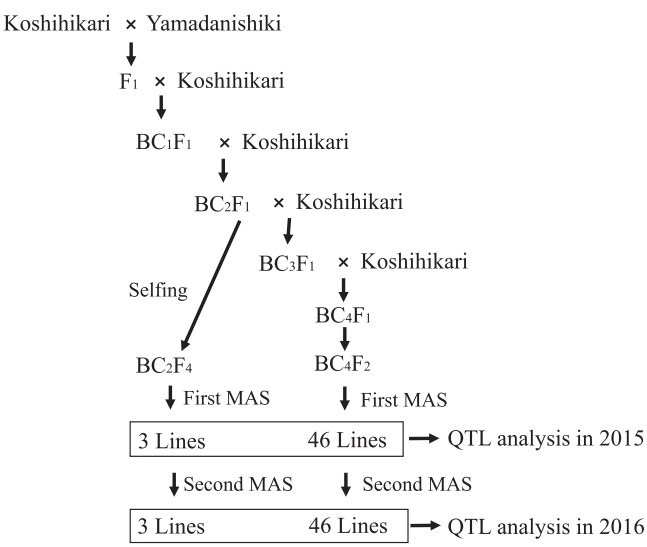

Fig. 1. Developmental scheme of the CSSLs for Yamadanishiki in the genetic background of Koshihikari. First MAS indicates the first round of marker-assisted selection (MAS), the whole-genome survey that was conducted using 125 DNA markers, while the second MAS was conducted to clear foreground and background heterozygosity.

decrease foreground and background heterozygosity (Second MAS, BC_2F_5 and BC_4F_3 generations, **Fig. 1**). The CSSLs from both years were genotyped using an array of 768 SNPs selected from Nagasaki *et al*. (2010) and Yamamoto *et al*. (2010), using the BeadStation 500G system (Illumina, San Diego, CA, USA) according to the manufacturer's instructions (**Supplemental Table 2**).

Trait evaluation

For trait evaluation, the 49 CSSLs were sown on 7 May 2015 and 6 May 2016, and 24 plants per line were transplanted to an experimental field at Kobe University, Food Resources Education and Research Center (Kasai City, Hyogo Prefecture Japan; 34.88°N, 134.86°E) on 6 June 2015 and 4 June 2016. We evaluated the grain traits and days to heading (DTH) of six plants per CSSL in both 2015 and 2016.

The plants were harvested over 45 days after their flowering date, and the harvested grains were air-dried for three days. After the grains were dehulled, we selected 100 grains per plant, excluding broken and immature grains, and measured the following grain traits: GL, GWh, GT, and GWt. The first three were measured using a RGQI20A rice grain analyzer (SATAKE Corporation, Higashi-Hiroshima City, Hiroshima Prefecture, Japan), and GWt was measured using an electric balance to an accuracy of 0.01 g. DTH was defined as the number of days from the sowing date to the initial flowering date.

QTL identification by CSSL-QTL analysis

To identify QTLs, we first performed Dunnett's multiple comparisons test to compare Koshihikari with each CSSL at the significance level of $P < 0.05$, using R (ver. 3.2.0, R Core Team 2015). Next, a regression analysis was conducted with 51 lines (the CSSLs and their parents), using the BayesC model in the R package "VIGoR" (Onogi and Iwata 2016). BayesC is a variable selection method that infers the probability of being included in the regression model (i.e., inclusion probability) for each marker (Habier *et al*. 2011). The SNP genotypes were coded additively: 0 indicates homozygous Koshihikari alleles, 1 indicates heterozygosity, and 2 indicates homozygous Yamadanishiki alleles. Prior to analysis, missing genotypes were imputed as follows: when both of the genotypes adjacent to the missing genotype were the same, the missing genotype was imputed as the same genotype; when the adjacent genotypes differed, the missing genotype was imputed as the average of the adjacent genotypes; and when the chromosome end was missing, the missing genotype was imputed as the genotype before the chromosome end. In total, 0.11% of genotypes were missing and imputed. To ensure robust QTL detection, we conducted a sub-sampling procedure using BayesC. We randomly selected 80% of the lines and inferred the inclusion probability and the marker effect by fitting with BayesC. We repeated this subsampling 1,000 times and calculated the average of the inclusion probability and the marker effect for each marker. The prior distributions from BayesC were determined using the function "hyperpara" in the VIGoR package, with the assumption that 3% of markers were included in the model and that the included markers explained all the phenotypic variance observed. Statistical significance was assessed using permutation tests. First, we permutated the phenotypes, conducted the subsampling procedure 1,000 times as described above, and calculated the average inclusion probabilities for each marker. We then repeated this permutation test 1,000 times and obtained the null distribution of the average inclusion probability. Significance levels were set to 1% and 5%. We identified robust QTLs using both methods in both years, and named these QTLs according to the nomenclature guidelines by McCouch *et al*. (1997).

QTL reanalysis of RILs

Okada *et al*. (2017) performed a QTL analysis for grain traits using RILs derived from Koshihikari/Yamadanishiki. Since the CSSLs in the present study revealed unexpected reactivity in a major QTL for GWt on chromosome 5 that was detected by Okada *et al*. (2017) and Yoshida *et al*. (2002), we focused on the distribution of DTH. The histograms of DTH for these RILs revealed two peaks (**Supplemental Fig. 1**). The population of 190 RILs was divided into 88 early-flowering lines (eRILs), 92 late-flowering lines (lRILs), and 10 residual lines (rRILs; **Supplemental Fig. 1**). QTL analysis for GWt in the eRILs and lRILs from both years of the study (2013 and 2014) was conducted. Windows QTL cartographer 2.5 (Wang *et al*. 2012b) was used for QTL analysis, and QTLs were detected using the composite interval mapping method (Zeng 1994) with a window size and walk speed of 5 cM and 1 cM, respectively. The empirical threshold values as determined by 1,000 permutation tests were significant at the 5% level (Churchill and Doerge 1994).

Results

Characteristics of the CSSLs

The present study reveals the development of 49 CSSLs harboring chromosomal segments from Yamadanishiki in the Koshihikari genetic background (**Fig. 2**, **Supplemental Table 2**). The CSSLs contained the target chromosomal region, as well as non-target regions, from Yamadanishiki. Given that recombination events occur at the midpoint between two adjacent markers, the non-target regions ranged from 0–74.7 Mb with a mean length of 13.7 Mb; however, on average, each CSSL contained 93% of the Koshihikari genome (**Fig. 2**, **Supplemental Table 2**). When combined, the CSSLs covered more than 98% of the Yamadanishiki genome, although there were gaps in the target regions on chromosomes 2 (1.0 Mb), 7 (2.1 Mb and 0.5 Mb), 8 (2.0 Mb), and 11 (1.3 Mb). These gaps were partially covered by non-target regions; e.g., the gap on chromosome 2 was partially covered by CSSL3-1 or CSSL12-4 (**Fig. 2**, **Supplemental Table 2**).

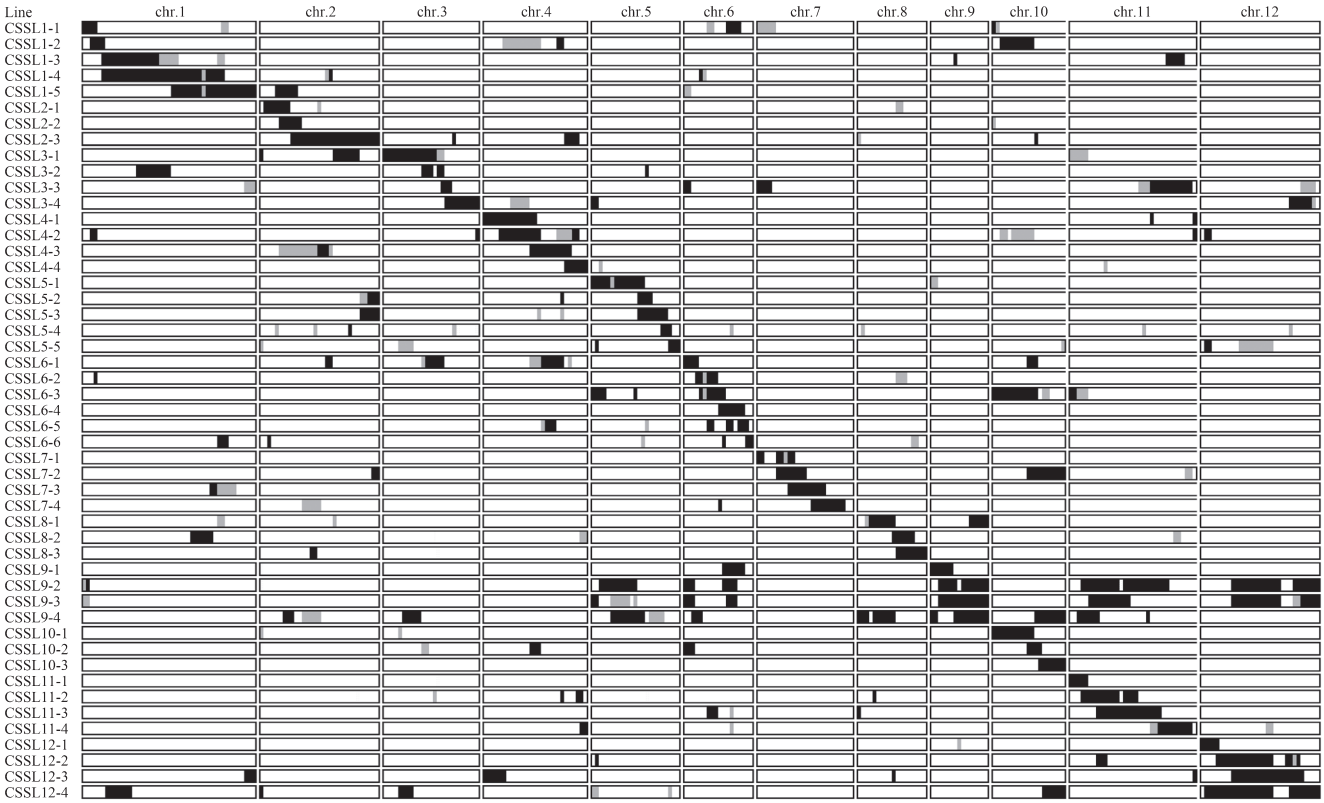

Fig. 2. Graphical genotypes of the 49 Yamadanishiki CSSLs in the Koshihikari genetic background, from 2016. Black and white bars indicate fragments homozygous with Yamadanishiki and Koshihikari, respectively. Gray bars represent heterozygous segments, and striped bars indicate missing data.

Trait evaluation and QTL identification using the CSSLs

Nine and four of the CSSLs had significantly longer and shorter average GLs than Koshihikari, respectively (**Table 1**). To consider the genetic effects of both target and non-target chromosomal regions, we identified robust QTLs using two methods: Dunnett's multiple comparison and BayesC model regression analysis (**Fig. 2**, **Table 1**, **Supplemental Fig. 2**). Four QTLs on chromosomes 6 (*qGL6-1* and *qGL6-2*), 10 (*qGL10*), and 11 (*qGL11*) were identified (**Table 2**). All these QTLs increased GL in the CSSLs carrying the Yamadanishiki allele. However, we did not identify any QTLs in the Yamadanishiki allele that decreased GL (**Table 2**, **Supplemental Fig. 2B**). CSSL11-3, which harbors *qGL11*, showed the longest GL among the CSSLs carrying the Yamadanishiki allele in the target QTL regions (CSSL6-3, 6-4, 6-5, 10-1, and 11-3). Thus, we assumed that *qGL11* has the largest effect on GL (**Table 1**). *qGL4-2* on chromosome 4, which was one of the major QTLs for GL as reported by Okada *et al*. (2017) was unstable in the CSSLs.

The GWh of CSSL2-3 was significantly smaller than that of Koshihikari, whereas eight CSSLs exhibited greater grain width (**Table 1**). Four QTLs located on chromosomes 2 (*qGWh2*), 4 (*qGWh4*), 5 (*qGWh5*), and 10 (*qGWh10*; **Table 2**) were identified. The latter three increased GWh in the CSSLs carrying the Yamadanishiki allele, while *qGWh2* decreased GWh (**Tables 1**, **2**, **Supplemental Fig. 2B**). Of these QTLs, it appears that *qGWh10* has the largest effect on GWh (**Tables 1**, **2**).

Most of the CSSLs had greater GT than Koshihikari, and GT in CSSL10-3 was similar to Yamadanishiki (**Table 1**). Three QTLs were identified on chromosomes 3 (*qGT3*) and 10 (*qGT10-1* and *qGT10-2*; **Table 2**). The CSSLs carrying the Yamadanishiki allele increased GT (**Tables 1**, **2**, **Supplemental Fig. 2B**).

The GWt of half the CSSLs was significantly greater than that of Koshihikari plants, but CSSL8-1 had significantly lower GWt than Koshihikari in 2016 (**Table 1**). Five QTLs were identified on chromosomes 6 (*qGWt6-1* and *qGWt6-2*), 7 (*qGWt7*) and 10 (*qGWt10-1* and *qGWt10-2*; **Table 2**). The CSSLs carrying the Yamadanishiki allele increased GWt (**Tables 1**, **2**, **Supplemental Fig. 2B**). Interestingly, *qGWt10-2* was located in a similar region to *qGWh10* and *qGT10-2* (**Table 2**).

Most of the CSSLs had slightly shorter DTH than Koshihikari (**Table 1**). In particular, the DTH of both CSSL6-1 and CSSL10-2 were notably shorter than that of Koshihikari, by approximately six days in 2015 and eight days in 2016 (**Table 1**). In contrast, the DTHs of CSSL2-3 and CSSL3-4 were longer than that of Koshihikari (**Table 1**). Although four chromosomal substituted regions in the four CSSLs were found on chromosomes 2, 3, 6, and 10, we confirmed that CSSL3-4 and CSSL2-3 carried the Yamadanishiki

Table 1. The phenotypic average values of each CSSL and parents and comparison between Koshihikari and each CSSL in 2015 and 2016

Line	Grain length (mm)		Grain width (mm)		Grain thickness (mm)		100-grain weight (g)		Days to heading (days)	
	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
CSSL1-1	5.34 ***	5.27	3.04	2.99	2.01 ***	2.04 ***	2.37 ***	2.26 ***	92 **	91 ***
CSSL1-2	5.25	5.18 **	3.10 ***	3.01	2.02 ***	2.04 ***	2.37 ***	2.25 ***	92 **	90 ***
CSSL1-3	5.28	5.23	3.05	2.96	2.01 ***	2.01 ***	2.34 ***	2.19	92 *	91 ***
CSSL1-4	5.23	5.17 ***	3.04	3.00	2.02 ***	2.05 ***	2.30 ***	2.21	94	93
CSSL1-5	5.24	5.23	3.06 *	2.98	2.02 ***	2.01 ***	2.32 ***	2.20	92 **	91 ***
CSSL2-1	5.22	5.21	3.04	2.97	2.00 ***	2.01 ***	2.24	2.18	92 *	91 ***
CSSL2-2	5.24	5.23	3.05	2.97	2.02 ***	2.03 ***	2.31 ***	2.23 *	92 **	91 ***
CSSL2-3	5.32 ***	5.19	2.93 ***	2.90 ***	1.98 ***	2.04 ***	2.26	2.22	102 ***	101 ***
CSSL3-1	5.30	5.29 *	3.05	2.99	2.03 ***	2.10 ***	2.37 ***	2.34 ***	99 ***	91 ***
CSSL3-2	5.21	5.14 ***	3.09 ***	3.00	2.05 ***	2.04 ***	2.38 ***	2.19	92 **	91 ***
CSSL3-3	5.28	5.24	3.05	2.97	2.02 ***	2.03 ***	2.34 ***	2.21	92 ***	91 ***
CSSL3-4	5.22	5.20	3.11 ***	3.00	1.98 ***	1.97	2.30 ***	2.21	117 ***	113 ***
CSSL4-1	5.26	5.20	3.03	2.96	2.02 ***	2.02 ***	2.31 ***	2.18	92 **	91 ***
CSSL4-2	5.26	5.18 **	3.08 ***	3.00	2.02 ***	2.02 ***	2.40 ***	2.23 *	91 ***	89 ***
CSSL4-3	5.19 *	5.16 ***	3.12 ***	3.07 ***	2.04 ***	2.03 ***	2.40 ***	2.28 ***	91 ***	90 ***
CSSL4-4	5.36 ***	5.29 *	3.04	2.97	2.02 ***	2.01 ***	2.40 ***	2.24 **	91 ***	91 ***
CSSL5-1	5.28	5.23	3.04	2.94 *	1.98 ***	2.01 ***	2.33 ***	2.16	93	92 **
CSSL5-2	5.27	5.19	3.06 *	2.95	2.02 ***	2.03 ***	2.37 ***	2.18	92 ***	91 ***
CSSL5-3	5.25	5.20	3.06 *	2.96	2.00 ***	2.01 ***	2.33 ***	2.16	92 **	91 ***
CSSL5-4	5.28	5.23	3.07 ***	2.98	2.02 ***	2.00 **	2.38 ***	2.20	91 ***	90 ***
CSSL5-5	5.22	5.18 **	3.11 ***	3.03 **	2.05 ***	2.06 ***	2.39 ***	2.25 ***	91 ***	90 ***
CSSL6-1	5.16 ***	5.07 ***	3.13 ***	3.03 **	2.05 ***	2.03 ***	2.41 ***	2.19	89 ***	86 ***
CSSL6-2	5.33 ***	5.24	3.09 ***	3.01	2.00 ***	2.01 ***	2.41 ***	2.24 **	93	93
CSSL6-3	5.44 ***	5.34 ***	3.14 ***	3.01	2.06 ***	2.04 ***	2.58 ***	2.32 ***	93	94
CSSL6-4	5.40 ***	5.30 ***	3.05	2.96	2.01 ***	1.99	2.42 ***	2.20	92 **	92 **
CSSL6-5	5.36 ***	5.30 **	3.10 ***	2.99	2.04 ***	2.00 *	2.44 ***	2.24 **	92 ***	92 **
CSSL6-6	5.34 ***	5.26	3.16 ***	3.02	2.06 ***	2.00 *	2.52 ***	2.23 *	91 ***	92 **
CSSL7-1	5.29	5.20	3.08 ***	3.00	2.01 ***	2.02 ***	2.38 ***	2.25 ***	92 *	92 **
CSSL7-2	5.27	5.15 ***	3.12 ***	3.05 ***	2.04 ***	2.06 ***	2.46 ***	2.28 ***	98 ***	91 ***
CSSL7-3	5.26	5.19	3.10 ***	2.99	1.98 ***	2.00 **	2.40 ***	2.19	92 *	93
CSSL7-4	5.20 *	5.15 ***	3.06 **	3.00	1.95 *	2.04 ***	2.30 ***	2.21	93	92 *
CSSL8-1	5.29	5.21	3.01	2.88 ***	1.94	1.99	2.26	2.05 ***	92 **	92 ***
CSSL8-2	5.27	5.22	3.08 ***	2.98	2.03 ***	2.05 ***	2.39 ***	2.21	91 ***	90 ***
CSSL8-3	5.26	5.18 **	3.08 ***	2.99	2.00 ***	2.03 ***	2.35 ***	2.18	92 **	91 ***
CSSL9-1	5.30	5.28	3.03	3.01	1.98 ***	2.03 ***	2.30 ***	2.24 **	92 **	92 ***
CSSL9-2	5.48 ***	5.43 ***	3.00	2.97	1.97 ***	2.00 **	2.38 ***	2.25 ***	93	91 ***
CSSL9-3	5.43 ***	5.38 ***	3.06	3.00	1.96 ***	2.03 ***	2.39 ***	2.26 ***	92 ***	91 ***
CSSL9-4	5.20 *	5.15 ***	3.17 ***	3.06 ***	2.03 ***	2.05 ***	2.42 ***	2.26 ***	90 ***	86 ***
CSSL10-1	5.32 **	5.28 *	3.09 ***	3.02	2.02 ***	2.06 ***	2.46 ***	2.31 ***	92 **	92 *
CSSL10-2	5.25	5.18 *	3.11 ***	3.05 ***	2.00 ***	2.05 ***	2.38 ***	2.25 ***	89 ***	86 ***
CSSL10-3	5.26	5.21	3.14 ***	3.04 **	2.05 ***	2.08 ***	2.47 ***	2.30 ***	91 ***	90 ***
CSSL11-1	5.30	5.24	3.08 ***	2.99	1.99 ***	2.04 ***	2.39 ***	2.23 *	93	90 ***
CSSL11-2	5.28	5.22	3.08 ***	2.96	2.02 ***	2.02 ***	2.36 ***	2.18	92 ***	91 ***
CSSL11-3	5.44 ***	5.36 ***	3.06 *	3.01	1.97 ***	2.01 ***	2.39 ***	2.26 ***	93	93
CSSL11-4	5.36 ***	5.28	3.03	2.98	2.01 ***	2.03 ***	2.38 ***	2.23 *	92 **	91 ***
CSSL12-1	5.30	5.30 **	3.09 ***	3.05 ***	2.02 ***	2.04 ***	2.41 ***	2.31 ***	92 *	90 ***
CSSL12-2	5.35 ***	5.28 *	3.04	2.97	1.99 ***	2.02 ***	2.36 ***	2.21	93	90 ***
CSSL12-3	5.35 ***	5.20	3.04	2.96	1.96 ***	2.00 **	2.34 ***	2.14	93	91 ***
CSSL12-4	5.28	5.22	3.01	2.98	1.99 ***	2.03 ***	2.30 ***	2.21	92 *	90 ***
Koshihikari	5.25	5.24	3.02	2.98	1.92	1.97	2.20	2.16	95	94
Yamadanishiki	5.59	5.54	3.27	3.23	2.07	2.07	2.80	2.72	110	108

Dunnett's multiple comparison test was conducted for each trait to compare Koshihikari with each CSSL, and "*", "**" and "***" represented significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

allele at *Hd6* (Takahashi *et al*. 2001), and CSSL6-1 and CSSL10-2 carried the Yamadanishiki allele at *Hd17* (Matsubara *et al*. 2012, **Supplemental Table 2**). Therefore, two QTLs on chromosomes 3 (*qDTH3*) and 6 (*qDTH6*) were verified (**Table 2**). These results corresponded with the report by Okada *et al*. (2017).

QTL reanalysis for GWt using RILs derived from a Koshihikari/Yamadanishiki cross

Okada *et al*. (2017) reported that *qGWt5*, which is in a similar region of *qGWh5* that was identified in the present study, was detected as a major QTL in F_2 and in RILs derived from a Koshihikari/Yamadanishiki cross. However,

Table 2. Identified QTL for grain traits

Trait	QTL	Position (Mb)	Marker interval	Allelic effect[a]	Refference[b]	Representitive CSSL[c]
GL	*qGL6-1*	18.9–23.3	ac06000665-RM1340	↑	12	CSSL6-3
	qGL6-2	27.39–27.88	aa06001119-aa06001139	↑	6	CSSL6-4, 6-5
	qGL10	2.15	aa10000749	↑		CSSL10-1
	qGL11	16.96–21.06	aa11003403-aa11004494	↑	5, 16	CSSL11-3
GWh	*qGWh2*	25.59	aa02002928	↓	2	CSSL2-3
	qGWh4	20.03–23.13	RM1359-ab04001157	↑	5	CSSL4-3
	qGWh5	28.22–28.99	ac05000341-aa05001022	↑	5, 14, 16	CSSL5-5
	qGWh10	18.01–20.36	RM6704-aa10003274	↑	2, 8, 15, 16	CSSL10-2, 10-3
GT	*qGT3*	9.29–18.88	ac03000229-aa03002121	↑	9	CSSL3-1, 3-2
	qGT10-1	12.45–13.06	aa10002652-ac10000368	↑		CSSL10-1, 10-2
	qGT10-2	18.55	ac10000429	↑	7	CSSL10-2, 10-3
GWt	*qGWt6-1*	9.14–11.84	ac06000397-ac06000592	↑	1	CSSL6-2, 6-3
	qGWt6-2	30.97	RM5753	↑	13, 16	CSSL6-6
	qGWt7	4.77–7.18	aa07001807-aa07001842	↑	3	CSSL7-1, 7-2
	qGWt10-1	2.15–10.26	aa10000749-aa10001539	↑		CSSL10-1
	qGWt10-2	18.55–21.00	ac10000429-aa10003332	↑	15, 16	CSSL10-2, 10-3
DTH	*qDTH3*	29.09–36.35	aa03002463-aa03002773	↑	4, 11	CSSL3-4
	qDTH6	0.78–6.09	aa06000024-ac06000103	↓	10	CSSL6-1

[a] ↑ and ↓ represented increase and decrease of trait values at Yamadanishiki allele, respectively.
[b] The number of refference report followed as 1: Lu *et al.* 1996, 2: Huang *et al.* 1997, 3: Li *et al.* 2000, 4: Takahashi *et al.* 2001, 5: Yoshida *et al.* 2002, 6: Aluko *et al.* 2004, 7: Bai *et al.* 2010, 8: Nelson *et al.* 2011, 9: Lu *et al.* 2013, 10: Matsubara *et al.* 2012, 11: Hori *et al.* 2013, 12: Huang *et al.* 2013, 13: Dang *et al.* 2015, 14: Nagata *et al.* 2015, 15: Zhen *et al.* 2017 and 16: Okada *et al.* 2017.
[c] Representitive CSSL indicated CSSL having a listed QTL in the foreground region.

Table 3. QTLs detected by QTL reanalysis for GWt of eRILs and lRILs

Population	QTL	Year	Peak (cM)	LOD	AE[a] (g)	PVE[b] (%)
eRILs	*qGWt5*[c]	2013		0.4	–0.016	0.9
		2014		0.5	–0.018	1.1
	qGWt10-2	2013	61.6	8.5	–0.078	25.7
		2014	58.9	4.5	–0.057	13.8
lRILs	*qGWt5*	2013	126.7	4.5	–0.057	14.6
		2014	126	10.5	–0.086	31.4
	qGWt10-2[c]	2013		1.4	–0.028	3.5
		2014		0.4	–0.016	1.1

[a] Additive effect.
[b] Phenotypic variance expressed.
[c] The data of non-significant QTLs represented the values at peak positions of *qGWt5* detected in lRILs and *qGWt10-2* detected in eRILs.

qGWt5 was not detected in the present CSSL analyses (**Tables 1**, **2**, **Supplemental Fig. 2**). We therefore conducted QTL reanalysis for GWt in the RIL population, divided into eRILs and lRILs based on flowering date (**Supplemental Fig. 1**). We only obtained significant logarithm of odds values for *qGWt10-2* in the eRILs and for *qGWt5* in the lRILs (**Table 3**, **Supplemental Fig. 3**). In addition, the linkage maps for chromosomes 5 and 10 of the eRILs and lRILs were almost identical (**Supplemental Fig. 4**).

Discussion

A set of CSSLs carries genomic segment(s) from a donor parent placed in the genetic background of a recipient parent, and manipulating these facilitates the comprehension of the whole genome of the donor parent by allowing the precise assessment of the genetic effects of the segments from the donor parent. In this study, we developed novel CSSLs in the genetic background of Koshihikari, a cooking-rice cultivar, with substituted chromosomal fragments from Yamadanishiki, a brewing-rice cultivar. Koshihikari and Yamadanishiki are distantly related Japanese cultivars (Yamasaki and Ideta 2013) and differ in many traits, such as grain size and heading date (Okada *et al*. 2017). In this study, we evaluated the grain size and heading date of the CSSLs that we developed, which enabled us to identify relevant QTLs.

We identified a total of 16 QTLs for grain traits: four QTLs for GL, four QTLs for GWh, three QTLs for GT, and five QTLs for GWt (**Table 2**). Of these, 15 QTLs caused an increase in the corresponding grain traits in the CSSLs carrying the Yamadanishiki allele, and only *qGWh2* caused a decrease in its trait (**Tables 1**, **2**, **Supplemental Fig. 2B**). This suggests that the grain size of Yamadanishiki is controlled by complex genetic mechanisms. Of the 13 QTLs identified for GL, GWh, and GWt, five (*qGL11*, *qGWh5*, *qGWh10*, *qGWt6-2*, and *qGWt10-2*) were similarly detected in RILs from the same crossing combination, whereas eight were newly identified in the CSSL-QTL analysis. The QTLs for GT were not identified in this manner because GT was not included in the RIL analysis. The results indicate that CSSLs can be used to identify QTLs that have relatively small genetic effects (Howell *et al*. 1996, Nagata *et al*. 2015). However, *qGL4-2*, one of the major QTLs on chromosome 4 that was detected using the RILs (Okada *et al*.

2017), was detected in only one of the two years of the present study using the CSSLs. Therefore, *qGL4-2* may be affected by the environment; for example, inter-year variation in mean temperature or rainfall patterns may modify its effect. We propose that the combination of the previously identified major QTLs and the newly identified QTLs results in the large grain size of Yamadanishiki. In addition, many of the QTLs identified in the present study might overlap to some extent with previously reported QTLs (**Table 2**), because the QTLs associated with grain size have been detected in large loci (Huang *et al* 2013, Nagata *et al*. 2015).

Two QTLs (*qDTH3* and *qDTH6*) for DTH that were identified on chromosomes 3 and 6 correspond to the known genes *Hd6* and *Hd16* on chromosome 3 and *Hd17* on chromosome 6, respectively (Hori *et al*. 2013, Matsubara *et al*. 2012, Takahashi *et al*. 2001). In this study, we validated and confirmed the effects of 15 previously identified QTLs (**Table 2**), and identified three novel QTLs (*qGL10*, *qGT10-1*, and *qGWt10-1*). Of the QTLs identified in this study, seven (*qGL11*, *qGWh5*, *qGWh10*, *qGWt6-2*, *qGWt10-2*, *qDTH3* and *qDTH6*) had previously been detected by using F_2 and RILs derived from Koshihikari/Yamadanishiki (Okada *et al*. 2017). These QTLs had relatively large genetic effects, suggesting that they were particularly important in regulating the grain size and heading date of Yamadanishiki.

In previous studies associated with grain size, *GS5* (3.4 Mb) and *GW5*/*qSW5* (5.3 Mb) on chromosome 5, and *TGW6* (25.1 Mb) and *GW6a* (26.6 Mb) on chromosome 6 have been cloned (Ishimaru *et al*. 2013, Li *et al*. 2011, Shomura *et al*. 2008, Song *et al*. 2015, Weng *et al*. 2008). However, *qGWh5* and *qGWt6-2* were clearly different from the cloned genes, because they were located in the distal regions of the long arms of chromosomes 5 and 6, respectively (**Table 2**). Using Yamadanishiki as a crossing parent, Yoshida *et al*. (2002) and Okada *et al*. (2017) detected a major QTL for GWh that was in the same region as *qGWh5*. In addition, based on an advanced backcrossed population with substituted IR64 genomic segments in a Koshihikari background, Nagata *et al*. (2015) reported that this QTL has a very small decreasing effect on GWh in the IR64 allele, and is located at 29.54 Mb on chromosome 5. We can therefore conclude that the QTL reported by Nagata *et al*. (2015) is identical to *qGWh5* (**Table 2**), and we infer that the alleles of Koshihikari, Yamadanishiki, and IR64 exhibit different genetic effects.

qGWh10 is located in the same region as *qGT10-2* and *qGWt10-2* (**Table 2**), suggesting that these QTLs are associated with a single gene. Recently, Zhen *et al*. (2017) confirmed that *qGS10* is associated with grain size, and that it affects GL, GWh, and grain weight. They suggested that *qGS10* was identical to *qGWh10*; in the present study, the latter affected GT, but had no genetic effect on GL. The region around *qGWh10* has also been detected via QTL analysis of populations derived from *japonica* × *indica* and *indica* × *indica* crosses (Huang *et al*. 1997, Nelson *et al*. 2011, Zhen *et al*. 2017), suggesting that this QTL is highly conserved across Asian rice cultivars. We found that *qGL11* had the largest effect on GL (**Table 2**), which was consistent with previous research (Okada *et al*. 2017, Yoshida *et al*. 2002). The present study presents the first validation of this QTL.

Genotype-by-environment interaction and QTL-by-environment interaction are observed in many crops; in the context of breeding, it is important to understand how QTLs respond environmental conditions (Moreau *et al*. 2004, Nelson *et al*. 2011, Wang *et al*. 2016, Zheng *et al*. 2010). Okada *et al*. (2017) reported that *qGWh5* and *qGWh10* were major QTLs for not only GWh but also GWt. However, CSSL-QTL analysis in the present study did not detect *qGWt5* as a robust QTL for GWt, but instead detected *qGWt10-2* (**Table 2**). Three hypotheses were considered: the effect of the genetic background, QTL-by-QTL interaction, and QTL-by-environment interaction. We then performed a reanalysis of these QTLs, using two RIL populations divided according to flowering date, to examine QTL-by-environment interaction (Okada *et al*. 2017, **Supplemental Figs. 1**, **3**, **4**). The results reveal that *qGWt5* and *qGWt10-2* have different responses to the environment (**Table 3**, **Supplemental Fig. 3**). *qGWt5* had an important genetic effect in the late-flowering plants (lRILs: 11 August–1 September), whereas *qGWt10-2* had a large effect in the early-flowering plants (eRILs: 23 July–9 August). *qDTH3*, a QTL for DTH, would have impacted these results, and CSSL3-4, which carries the Yamadanishiki allele, showed an approximately 20-day increase in DTH compared to Koshihikari (**Table 1**). Because most CSSLs exhibited early flowering, the CSSL-QTL analysis was unlikely to have detected *qGWt5*. Since the average air temperature of the 30 days of the ripening term was 27.3°C (2013) and 25.9°C (2014) for the eRILs, and 24.1°C (2013) and 23.5°C (2014) for the lRILs, it is possible that the different effects of these two QTLs were caused by differences in the ripening temperature. Nevertheless, the effect of genetic background and QTL-by-QTL interactions should be also considered. Okada *et al*. (2017) reported that *qGWh10* corresponded to the QTL for white-core expression, but no QTL for white core was detected around *qGWh5*. Therefore, these two QTLs may have different functions in grain development during ripening. Evaluation of the effect of these QTLs under environmental changes, e.g., changes in flowering date, is essential for future breeding. It is also important to understand the effect of *qGWh5* on GWt in Yamadanishiki, since this cultivar flowers around 20 August.

In conclusion, 18 QTLs for grain size and DTH were identified using CSSLs, and six major QTLs for grain trait and two QTLs for DTH that were previously detected by Okada *et al*. (2017) appear to be particularly important for Yamadanishiki. In addition, *qGWt5* and *qGWt10-2* appear to have different functions and exhibit different responses to the environment. This information could potentially be used not only to improve the breeding of brewing-rice cultivars, but also to increase the yield of cooking-rice cultivars.

Acknowledgments

We thank Prof. Shigeo Takumi (Kobe University, Japan) for use of the epMotion 5070 pipetting machine. This work was supported by JSPS KAKENHI Grant Number 17J01082 and Council for Science, Technology and Innovation (CSTI), Cross-ministerial Strategic Innovation Promotion Program (SIP), "Technologies for creating next-generation agriculture, forestry and fisheries" (funding agency: Bio-oriented Technology Research Advancement Institute, NARO).

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