Research Paper

Development of genome-wide PCR-based markers from insertion, deletion and single nucleotide polymorphisms for closely related Japanese rice cultivars and identification of QTLs for the appearance of cooked rice and polished rice

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Appearance of rice grain is an important property, affecting its acceptance by consumers. Moreover, appearance is a complex characteristic involving many components, including glossiness and whiteness. The genetic bases for the glossiness of cooked rice and the whiteness of polished rice (WPR) were determined using 133 recombinant inbred lines (RILs) derived from a cross between two closely related cultivars from Hokkaido, Joiku462, with high glossiness and whiteness, and Yukihikari, an ancestor of Joiku462 with low glossiness and whiteness. Analyses identified 167 genome-wide InDel markers, five cleaved amplified polymorphic sequences (CAPS) and eight derived CAPS markers differentiating the parental lines. The glossiness area (GLA) and glossiness strength (GLS) of cooked rice and WPR were determined for RILs in two locations, Pippu and Sapporo, Hokkaido. Four QTLs were detected. *qGLA10* and *qGLS9* were detected on chromosomes 10 and 9, respectively, with both being significant at both geographic locations. *qWPR1* on chromosome 1 was significant at Pippu, and *qWPR4* on chromosome 4 was significant at Sapporo. The Joiku462 alleles at all QTLs increased each trait. The PCR-based markers flanking these four QTLs may be useful for improvement of GLA, GLS and WPR.

Key Words: glossiness, whiteness, InDel marker, CAPS marker, dCAPS marker.

Introduction

Rice (*Oryza sativa* L.) is a food staple for more than half the world's population. The breeding of rice has produced new cultivars with favorable agronomic and economic characteristics, including biotic and abiotic stress resistance, high yield, and good eating quality. The breeding of temperate *japonica* rice for growth during the summer monsoon season at higher latitudes has a long history in Japan. Hokkaido, which is located at 45–42°N latitude, is the northernmost region of rice paddy cultivation in Japan and one of the northernmost limits of rice cultivation in the world. The alternative breeding history of rice cultivars grown in Hokkaido starts in the late 1800s and is relatively short. After rice production was improved so that Japan was almost completely

self-sufficient, the main breeding objective was changed from high yield to good eating quality (Horie *et al.* 2005). Preferences for eating quality of cooked rice vary depending on end-use, culture, and climate of each local region (Shinada *et al.* 2015). Consumers in Japan currently demand new rice cultivars with high eating quality, including rice that appears glossy and white, with good flavor and a soft and sticky consistency. Eating quality has been improved by inclusion of the elite Japanese cultivar Koshihikari, released for cultivation on Honshu, the main island of Japan, and related cultivars in the pedigree of modern Japanese rice cultivars (Yamamoto *et al.* 2010). In Hokkaido, the first good eating quality rice cultivar Yukihikari, released in 1981, was derived from the progeny of crosses between

Abbreviations: CAPS, cleaved amplified polymorphic sequence; dCAPS, derived cleaved amplified polymorphic sequence; GLA, glossiness area of cooked rice; GLS, glossiness strength of cooked rice; MAS, marker-assisted selection; NGS, next-generation sequencing; RIL, recombinant inbred line; WPR, whiteness of polished rice

Hokkaido landraces, without crossing with Koshihikari. The eating quality of Yukihikari was further improved by crosses with Koshihikari and other good eating quality cultivars. Among them, one recent Hokkaido cultivar, Joiku462, derived from the progeny of Yukihikari and released in 2009, has shown superior glossiness and whiteness.

Several quantitative trait loci (QTLs) for the appearance of cooked rice and polished white rice have been identified (Shinada *et al.* 2015, Takeuchi *et al.* 2007, Ujiie and Ishimaru 2014, Wada *et al.* 2008). The QTLs *qGL1*, *qGL3-1*, *qGL3-2*, *qGL6*, *qGL7* and *qGL10*, which are associated with the glossiness of cooked rice, were found by using back-crossed inbred lines (BILs), derived from a backcross of the Koshihikari/*indica* cultivar Kasalath with Koshihikari and chromosome segment substitution lines (CSSLs) derived from crosses between Koshihikari and Kasalath (Takeuchi *et al.* 2007). In addition, the QTLs *qGL1.1*, *qGL1.2*, *qGL2*, *qGL3*, *qGL6*, *qGL7*, *qGL10*, *qGL11*, *qGL12.1* and *qGL12.2* were identified using RILs derived from a cross between two closely related *japonica* cultivars, Moritawase and Koshihikari (Wada *et al.* 2008). DNA markers closely linked to these QTLs enhance the efficient development of rice cultivars with high glossiness. Although consumers make judgments on polished rice based largely on overall appearance, including whiteness, no QTL associated with WPR has yet been identified. Pedigree information on improved cultivars, including Joiku462, has been accurately recorded, but little is known about the genetic bases of the appearance of cooked and polished Hokkaido rice cultivars. Therefore, to introduce MAS and to efficiently improve the breeding of rice cultivars in Hokkaido, it is necessary to investigate the genetic basis of the glossiness of cooked rice and WPR.

Determination of molecular markers, through methods such as genotype fingerprinting, genetic diversity analysis, phylogenetic analysis, map-based gene cloning, variety identification and marker-assisted breeding, is useful for genetic research and breeding (Joshi *et al.* 2001, McCouch *et al.* 1997, Nagaraju *et al.* 2002, Ni *et al.* 2002). Many molecular markers have been identified in rice, including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs) and insertions-deletions (InDels) (Akagi *et al.* 1996, Chen *et al.* 2011, McCouch *et al.* 2002, Nasu *et al.* 2002, Panaud *et al.* 1996, Project IRGS 2005, Ren *et al.* 2005, Temnykh *et al.* 2001, Williams *et al.* 1990). Recently, InDel markers were successfully used for genetic studies in rice (Hayashi *et al.* 2006, Ji *et al.* 2010, Liu *et al.* 2012, 2015, Yonemaru *et al.* 2015). NGS has provided sequence data for multiple Japanese rice cultivars. Several Japanese rice cultivars have also been re-sequenced to identify genome-wide InDel polymorphisms and SNPs for analysis of genomes in the Japanese rice population (Arai-Kichise *et al.* 2011, 2014, Nagasaki *et al.* 2010, Takano *et al.* 2014, Yamamoto *et al.* 2010, Yonemaru *et al.* 2014). The NGS technique has resulted in the efficient and economical identification of large numbers of InDel polymorphisms and SNPs common to highly homologous rice genomes (Arai-Kichise *et al.* 2011, Liu *et al.* 2015). In the absence of abundant SNP markers, a relatively small number of InDel markers would be sufficient to discriminate among genotypes for rough mapping of QTLs and for marker-assisted introgression of a QTL into recipient cultivars. Although SSR markers have been widely used for this purpose, use of these markers requires screening for polymorphisms between each pair of parental cultivars due to unpredictable InDel sizes. Moreover, small InDels (≤5 bp) are difficult to distinguish by gel electrophoresis. Therefore, InDels of predictable size and a substantially polymorphic region (≥10 bp) are ideal for genotyping (Yonemaru *et al.* 2015). However, large InDels (10–100 bp) have rarely been verified for marker use.

The objectives of the present study were (1) to develop genome wide PCR-based markers, involving InDel, CAPS and dCAPS markers, distinguishing two closely related Japanese cultivars developed around 30 years apart, Yukihikari and Joiku462, using the previous NGS analysis (Takano *et al.* 2014); (2) to construct a molecular linkage map of a RIL of a cross between Yukihikari and Joiku462 using these PCR-based markers; and (3) to identify QTLs for the glossiness of cooked rice and the WPR.

Materials and Methods

Plant materials

Oryza sativa L. ssp. *japonica* cv. Yukihikari and Joiku462 were used as parental lines. Both were grown in Hokkaido, Japan, with Joiku462, released in 2009, being derived from Yukihikari, released in 1981 (**Supplemental Fig. 1**). The 133 RILs (F_9 and F_{10}) were developed by the single seed descent (SSD) method of progenies derived from a cross between Yukihikari and Joiku462. The F_9 RIL population was used for DNA marker analysis and the F_{10} RIL population for field trials.

Development of indel markers

Relative to the Yukihikari genome sequence, the Joiku462 sequence contains 4,900 insertions and 5,162 deletions, with a high sequence depth (DP ≥ 5 fold) and of lengths ranging from 1 bp to 78 bp (Takano *et al.* 2014). Of these 10,062 InDels, 6,137 (61%) were 1 bp in length, 2,755 (27%) were 2–4 bp, 652 (6%) were 5–9 bp, and 518 (5%) were ≥10 bp (Takano *et al.* 2014). The large size (≥10 bp) InDels were initially extracted at interval of 2–3 Mb to develop PCR-based markers, followed by extraction of the ≥5 bp InDel regions to encompass the sequence gaps between ≥10 bp InDel polymorphisms. The candidate primer sequences were selected to yield PCR products 60–400 bp in length, with each InDel between Yukihikari and Joiku462 located approximately in the middle of the product, and identical sequences in the two strains located outside the candidate sequence. To design primers for PCR validation,

sequences of 601 bp, including an InDel region and flanking sequences on both sides, were extracted. Primers 20–28 nucleotides (optimal length, 22 nucleotides) long, with melting temperatures (Tm) of 56–60°C (optimal Tm, 57°C) and yielding products 82–350 bp in length, were designed using Primer3.0 (v. 0.4.0) (Untergasser *et al.* 2012, http://bioinfo. ut.ee/primer3-0.4.0/) (**Supplemental Table 1**). DNA was extracted from fresh young leaves of parental plants and each RIL (F_9). Amplification reactions were carried out in a total volume of 10 μL, containing 40 ng template DNA, 1x PCR buffer, 0.2 mM of each dNTP, 1U Taq DNA polymerase (GoTaq® Green Master Mix, Promega) and 10 pmol of each forward and reverse primer. The amplification protocol consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, amplification at 50–60°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were electrophoresed on 1–4% agarose gels, which were stained with ethidium bromide and viewed under UV light.

Development of CAPS markers and dCAPS markers

To encompass the gaps between InDel markers, CAPS markers and dCAPS markers were developed based on SNPs. SNPs were converted to CAPS and dCAPS markers by using the web-based free software program dCAPS Finder 2.0 (Neff *et al.* 2002) to identify appropriate restriction enzymes to detect each SNP. Appropriate PCR primer sets flanking each target SNP were designed using Primer 3.0 (v. 0.4.0) software (Untergasser *et al.* 2012, http://bioinfo. ut.ee/primer3-0.4.0/) (**Supplemental Table 2**).

Amplification mixtures and protocols were identical to those shown above. The resulting PCR products were digested with suitable restriction endonucleases in a total volume of 10 μL according to the manufacturers' instructions. The digests were resolved by electrophoresis in 1–2% agarose gels, which were stained with ethidium bromide and visualized under UV light.

Field trials and assessments of traits

Seeds were sown in a greenhouse at Obihiro University of Agriculture and Veterinary Medicine on 26 April 2014. Seedlings aged 30 and 33 days were transplanted into the paddy fields of Kamikawa Agricultural Experiment Station (Pippu) and Hokkaido University (Sapporo), respectively, at densities of one plant per hill and a spacing of 30 × 15 cm (22.2 plants/m²) on 26 and 29 May, respectively. Forty plants of each triplicate parental line and of each RIL were grown in Pippu (43°51′N, 142°48′E) and 12 plants of each triplicate parental line and RIL were grown in Sapporo (43°07′N, 141°34′E). Plants in Pippu were fertilized with 8 kg N/10a, 9.7 kg P_2O_5/10a and 6.9 kg K_2O/10a, whereas plants in Sapporo were fertilized with 6.4 kg N/10a, 6.4 kg P_2O_5/10a and 4.8 kg K_2O/10a. The monthly accumulated air temperatures at each location during the growing seasons in 2014 and 2015, as determined by the Japan Meteorological Agency, are shown in **Supplemental Fig. 2**.

At maturity, the rice grains were harvested, bulked, air-dried, and dehulled. Brown rice grains smaller than 1.9 mm were removed by sieves. The remaining brown rice grains were polished with a rice-polishing machine (TOYO TESTER Seimaiki MC-90A, Toyo Rice Co., Ltd.) until 90% of the grains by weight were white. WPR was measured using a Rice Whiteness Tester C-300 (Kett Electric Laboratory Co., Ltd.). The glossiness of cooked rice was measured as described (Yanagihara 2000), with some modifications. Briefly, water weighing 1.5 times (w/w) the weight of white rice was added to 15 g white rice in a stainless petri dish. After 40 min at room temperature, the rice was cooked at 105°C for 20 min in an autoclave (TOMY ES-315) and allowed to stand for 30 min to steam the boiled rice. The appearance of the cooked rice was recorded with a CCD camera (SONY DXC 930) and its glossiness evaluated by an image analyzer (NIRECO Luzex-FS). GLA was calculated as the cumulative number of pixels from digital number 185 to digital number 255, and GLS was calculated as the average of the digital number of these pixels (Yanagihara 2000). Triplicate samples of each parental line and RIL were assayed.

Segregation analysis

The expected genotype ratio of the RIL population was 1:1 (Yukihikari type: Joiku462 type). The observed ratio of each marker was tested for deviation from its expected value using a chi-square goodness-of-fit test ($P < 0.05$).

Linkage and QTL analysis

A linkage map was constructed using JoinMap version 4.1 (van Ooijen 2011, http://www.kyazma.nl/), with a LOD threshold of 3.0. To map distances, recombination frequencies were converted using the Kosambi mapping function (Kosambi 1943). Interval mapping (IM) and multiple-QTL model (MQM) mapping were performed to identify putative QTLs using the established linkage map and the observed phenotypic traits. This method was run with MapQTL® version 6 (van Ooijen 2009); a $P < 0.05$ LOD score significance threshold was calculated by creating a group-wide distribution of the data based on testing of 1000 permutations. The positions of QTLs on the map were estimated using LOD peaks. Genetic parameters, including additive effects and variations explained by each QTL, were also estimated. Residual heterozygotes were considered to have missing data.

Results

Parental contribution, marker distortion and molecular linkage map

This study identified 165 InDels (147 of ≥10 bp and 18 of 5–9 bp), 5 CAPS and 8 dCAPS as markers (**Supplemental Tables 1**, **2**). Assessment of these 178 molecular markers in each RIL (F_9) yielded 23,674 genotypes; among these were 170 heterozygous alleles (0.7%), with an average of

1.3 loci for each RIL and 0.96 RILs for each locus. This heterozygosity ratio was close to the expected ratio of 0.52% (0.69 RILs) at each locus, indicating that each RIL was almost completely homozygous, with the genotype frequency being equivalent to the allele frequency at each locus. The parental contribution of Yukihikari to each RIL, calculated as the ratio of Yukihikari type markers to total markers, ranged from 26.4% to 72.5% (**Fig. 1**), with 97 (73%) of the RILs showing contributions from Yukihikari between 40.1% and 59.9%.

The two alleles at each of the 178 DNA loci should fit a 1:1 Mendelian ratio if there was no segregation distortion in the RIL population. Yukihikari allele frequencies at these 178 molecular markers ranged from 0.4135 to 0.6015 and averaged 0.4988 (**Fig. 2A**). Five markers, YJInDel-496 ($\chi^2 = 5.48$, $P = 0.02$), YJInDel-502 ($\chi^2 = 6.42$, $P = 0.01$), YJInDel-504 ($\chi^2 = 5.94$, $P = 0.01$), YJInDel-510 ($\chi^2 = 5.94$, $P = 0.01$) and YJInDel-515 ($\chi^2 = 4.77$, $P = 0.03$), were found to have segregation distortion (**Fig. 2B**), suggesting that non-Mendelian segregation occurred by chance at finite probability. These five markers, however, were present in a single genomic region, located between 18,160,055 bp and 25,439,854 bp, in the one-third terminal portion of the long arm of chromosome 12, with all five skewing towards
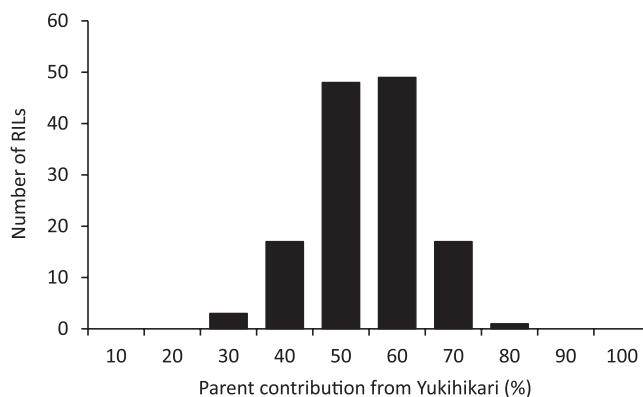

Fig. 1. Frequency distortion of the genetic contributions from parent *Oryza sative* spp. *japonica* cv. Yukihikari to each RIL.

Yukihikari alleles (**Fig. 2B**). These results suggested that Yukihikari alleles were transmitted at higher frequency than Joiku462 alleles in a 7.3 Mb region on the long arm of chromosome 12.

All 165 InDels, 5 CAPS and 8 dCAPS markers showed detectable linkage to other markers, yielding 12 linkage groups ranging in length from 105.4 cM to 223.3 cM (**Table 1**, **Fig. 3**). The total length of all linkage groups was
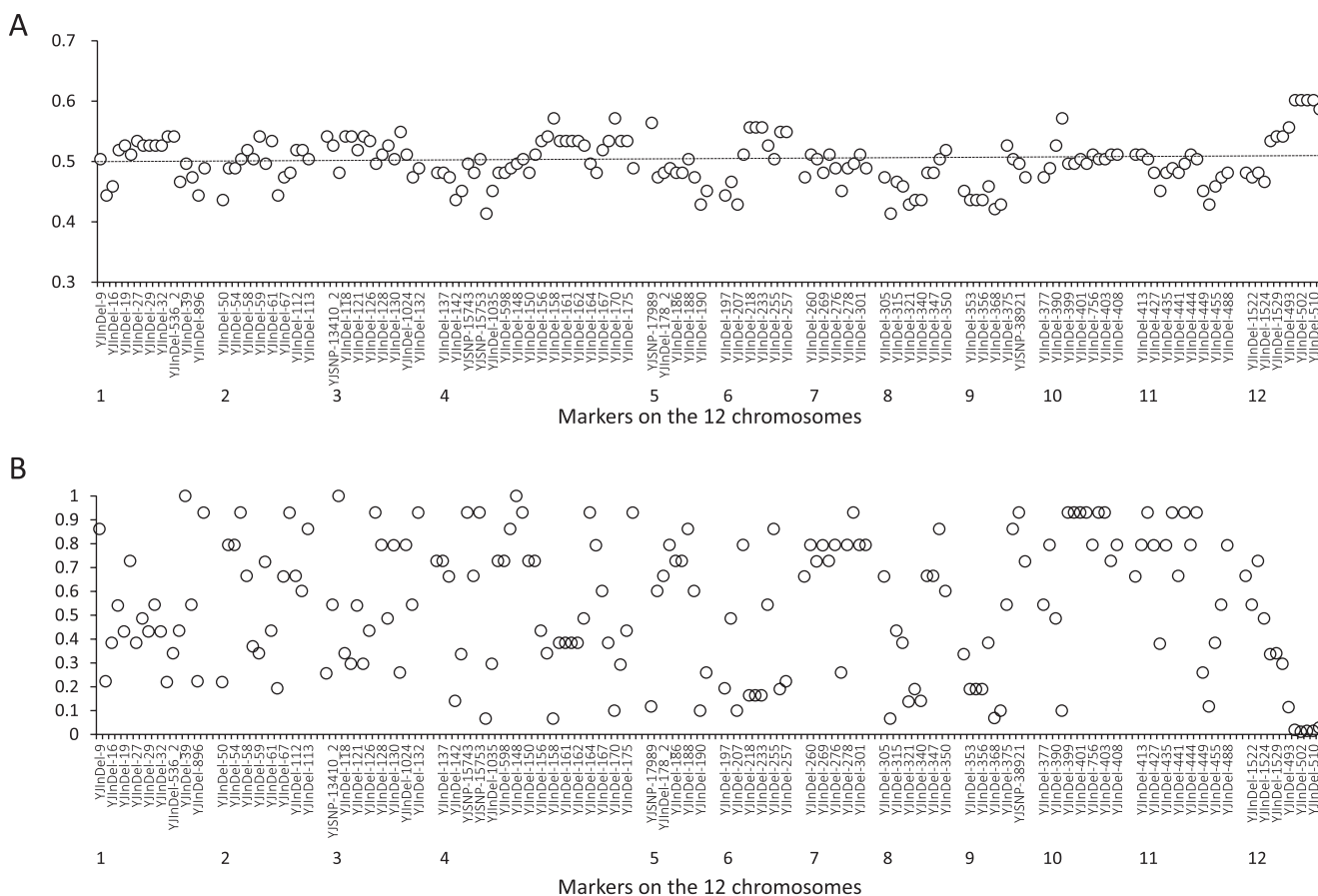

Fig. 2. Frequency of the allele from *Oryza sativa* spp. *japonica* cv. Yukihikari (A) and the segregation distortion test (B) in RILs resulting from crosses between Yukihikari and cv. Joiku462 at each marker locus.

Table 1. Summary statistics of the twelve linkage groups for the InDel and SNP-based map constructed in the present study

| Chromosome	Number of markers	Length		Average spacing		Maximum spacing		Coverage
		(cM)	(Mb)	(cM)	(Mb)	(cM)	(Mb)	(%)
1	18	200.3	37.7	11.8	2.1	29.8	7.8	83.8
2	15	116.1	24.0	8.3	1.6	23.3	3.6	65.2
3	16	223.3	32.3	14.9	2.0	40.9	5.7	86.6
4	33	212.4	34.6	6.6	1.0	24.3	6.5	95.9
5	10	136.0	22.5	15.1	2.2	58.9	5.8	74.7
6	11	122.3	26.0	12.2	2.4	27.9	7.2	81.0
7	11	133.4	28.3	13.3	2.6	36.9	10.9	93.1
8	11	115.8	22.3	11.6	2.0	31.5	8.4	78.1
9	11	120.6	19.9	12.1	1.8	37.0	5.1	83.4
10	13	114.9	21.5	9.6	1.7	22.2	8.6	90.9
11	16	140.9	29.9	9.4	1.9	35.1	5.0	96.0
12	13	105.4	23.1	8.8	1.8	19.2	5.0	83.4
Total	178	1741.5	322.2	11.1	1.9	32.3	6.6	84.3
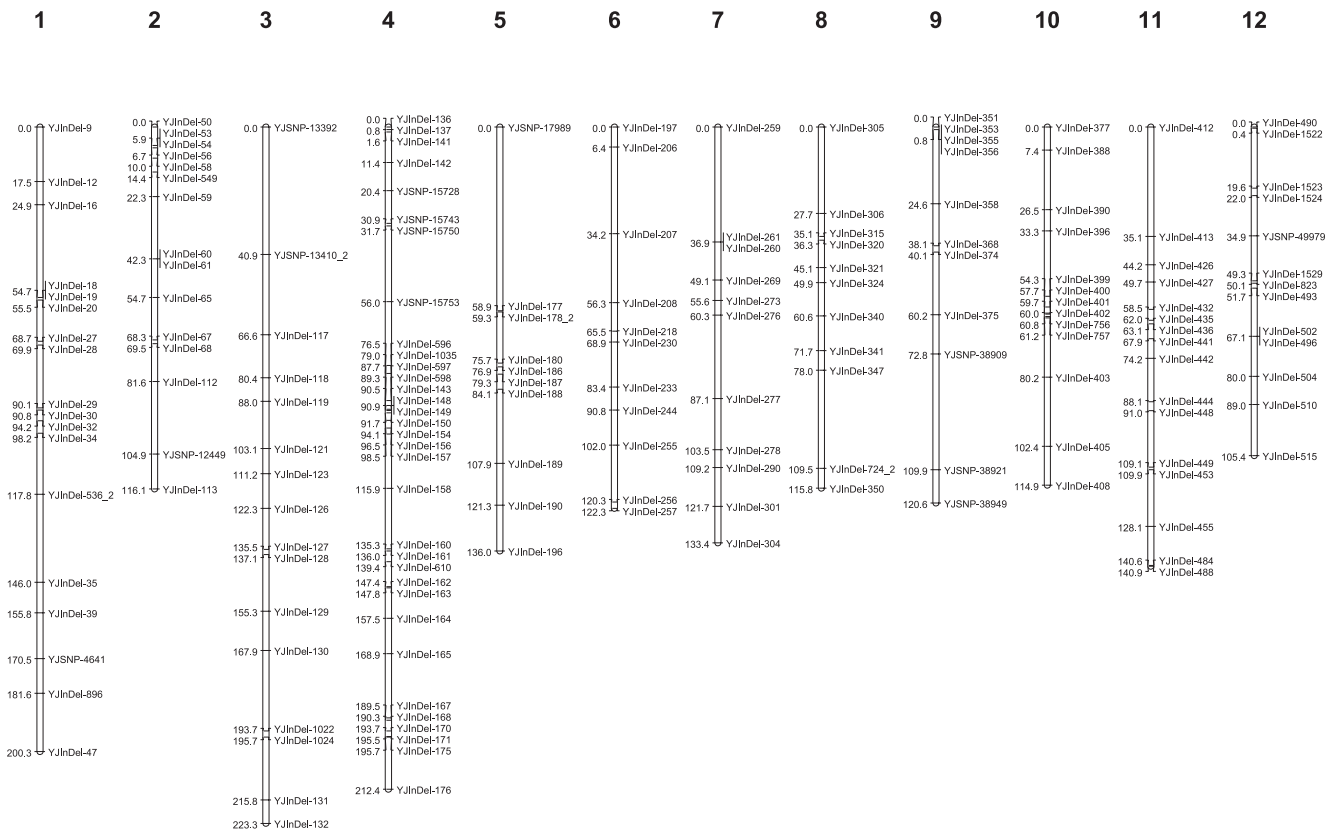
Fig. 3. Genetic linkage map of the 165 InDel markers, five CAPS markers and eight dCAPS markers constructed in RILs (F$_9$) derived from crosses between *Oryza sativa* ssp. *japonica* cv. Yukihikari and Joiku462.

1,741.5 cM. The average interval between markers ranged from 6.6 cM (1.0 Mb) to 13.3 cM (2.6 Mb), with an average of 11.1 cM (1.9 Mb). There were two gaps of ≥40.1 cM on chromosomes 3 and 5.

Phenotype distribution and correlation

Differences between parental lines were consistent across the two locations, with Joiku462 having higher GLA, GLS and WPR values than Yukihikari ($P < 0.05$) (**Table 2**). In the RIL population, transgressive levels differed for these three characteristics (**Table 2**, **Fig. 2**). **Fig. 4** shows the phenotype frequency distributions of these three characteristics in the RILs and the two environments (Pippu and Sapporo). The three traits in the RIL population segregated continuously in both environments. GLA ($r = 0.46$, $P < 0.01$), GLS ($r = 0.51$, $P < 0.01$) and WPR ($r = 0.58$, $P < 0.01$) were positively correlated in the RILs grown in the two locations. The pairwise correlation coefficients in each environment are shown in **Table 3**. GLA was positively correlated with GLS at both locations ($P < 0.01$), as were WPR and GLA ($P < 0.01$).
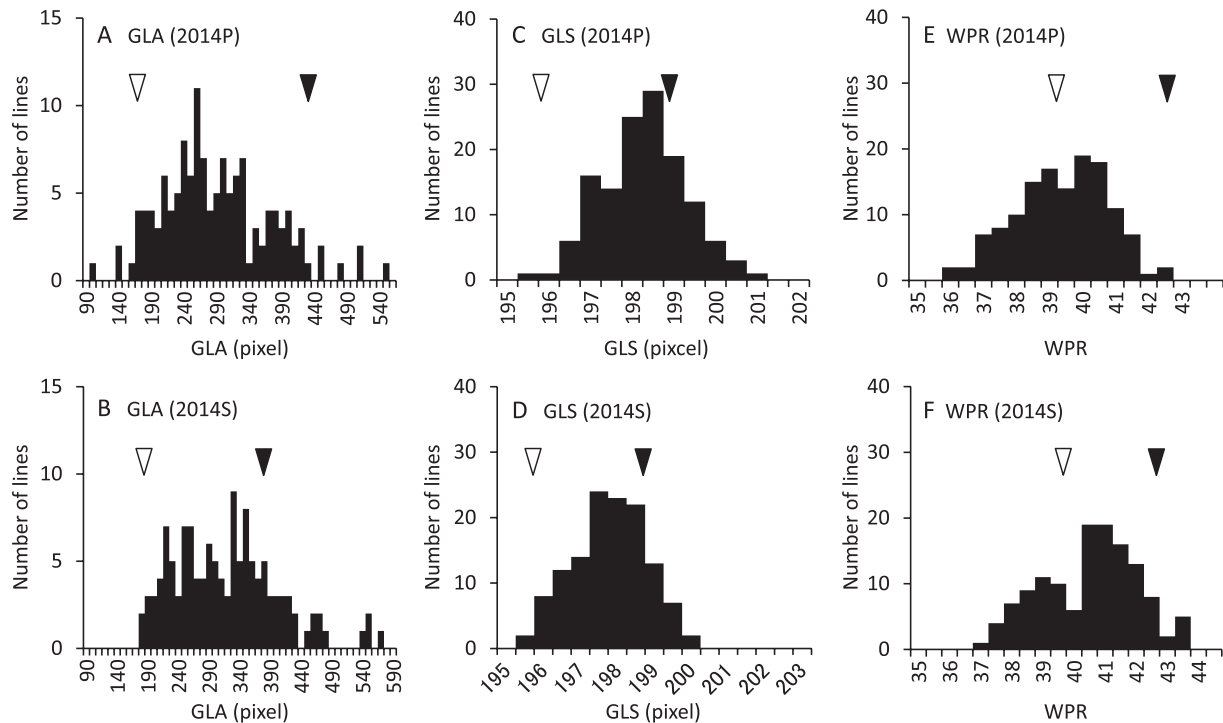
Fig. 4. Frequency distributions of (A, B) GLA, (C, D) GLS, and (E, F) WPR in RILs (F_{10}) derived from crosses between Yukihikari and Joiku462 and cultivated in (A, C, E) Pippu and (B, D, F) Sapporo. The *white* and *black* arrows indicate the mean values for Yukihikari and Joiku462, respectively.

Table 2. Trait names with parental and RIL population averages of GLA, GLS and WPR

Trait	Environ- ment	Parents			RILs		
		Yuki- hikari	Joiku462	Differ- ence	Mean	Min	Max
GLA	2014P	160.3	432.9	272.6**	280.7	108.3	555.3
	2014S	183.6	385.6	202.0*	319.3	182.0	578.3
GLS	2014P	196.2	199.6	3.5*	198.5	196.0	201.3
	2014S	195.9	198.7	2.8*	198.1	195.7	200.3
WPR	2014P	39.4	42.9	3.5*	39.7	36.3	42.9
	2014S	40.7	42.6	1.9*	40.9	37.4	43.7

* $P \leq 0.05$ and ** $P \leq 0.01$

Table 3. Correlation coefficients among GLA, GLS and WPR in RILs (F_{10}) from crosses between Yukihikari and Joiku462

2014P			2014S		
	GLS	WPR		GLS	WPR
GLA	0.57**	0.33**	GLA	0.64**	0.64**
GLS		0.13	GLS		0.12

** $P \leq 0.01$

QTLs for the appearance of cooked rice and polished white rice

LOD thresholds for each trait estimated from 1000-fold permutation tests ranged from 2.8 to 3.0, depending on characteristic and location (**Table 4**, **Fig. 5**). A single QTL was detected for GLA on chromosome 10. *qGLA10* showed positive additive effects at both geographic locations, Pippu

and Sapporo, indicating that the allele from Joiku462 increased GLA. *qGLA10* had LOD scores ranging from 3.00 to 3.94 and explained 9.9–13.3% of the variation in phenotypic mean in each environment. Similar results were observed for GLS, in that a single QTL for GLS was detected on chromosome 9. *qGLS9* showed positive additive effects in both environments, indicating that the allele from Joiku462 increased GLS. *qGLS9* had LOD scores ranging from 4.25 to 4.53, accounting for 14.3–14.5% of the variation in phenotypic mean in each environment.

In contrast to GLA and GLS, two QTLs were detected for WPR, one on chromosome 1 and the other on chromosome 4 (**Table 4**). *qWPR1* had positive additive effects in Pippu, indicating that the allele from Joiku462 increased WPR. *qWPR1* had LOD scores ≥3.12, explaining 10.2% of the variation in phenotypic mean in Pippu. *qWPR4* had positive additive effects in Sapporo, indicating that the allele from Joiku462 increased WPR. In Sapporo, *qWPR4* had LOD scores ≥4.49, explaining 15.0% of the variation in phenotypic mean at this location.

Discussion

Improvements in the glossiness of cooked rice and WPR over the past 30 years

Available genetic information on the appearance of polished and cooked *japonica* rice cultivars is currently limited, although several studies have attempted to identify QTLs associated with these traits (Shinada *et al.* 2015,

Table 4. QTLs for cooked rice appearance involving GLA, GLS and WPR. QTLs detected in the Yukihikari/Joiku462 RIL population at Pippu and Sapporo in 2014

| Trait	QTL	Environ-ment	LOD threshold	Chr.	Nearest marker		Marker interval		LOD	PVE (%)	Additive effect	Donor of possitive allele
					Marker	Physical position (Mb)	Marker	Physical position (Mb)				
GLA	*qGLA10*	2014P	2.9	10	YJInDel-388	2.5	YJInDel-377–YJInDel-388	1.5–2.5	3.00	9.9	27.36	Joiku462
		2014S	2.8	10	YJInDel-388	2.5	YJInDel-377–YJInDel-390	1.5–11.2	3.94	13.3	30.74	Joiku462
GLS	*qGLS9*	2014P	2.9	9	YJInDel-358	9.4	YJInDel-356–YJInDel-358	5.7–9.4	4.53	14.5	0.42	Joiku462
		2014S	3.0	9	YJInDel-358	9.4	YJInDel-356–YJInDel-358	5.7–9.4	4.25	14.3	0.48	Joiku462
WPR	*qWCR1*	2014P	3.0	1	YJInDel-9	7.1	YJInDel-9–YJInDel-12	7.1–8.7	3.12	10.2	0.4	Joiku462
	qWCR4	2014S	2.9	4	YJSNP-15753	17.5	YJSNP-15750–YJSNP-15753	11.0–17.5	4.49	15.0	0.7	Joiku462
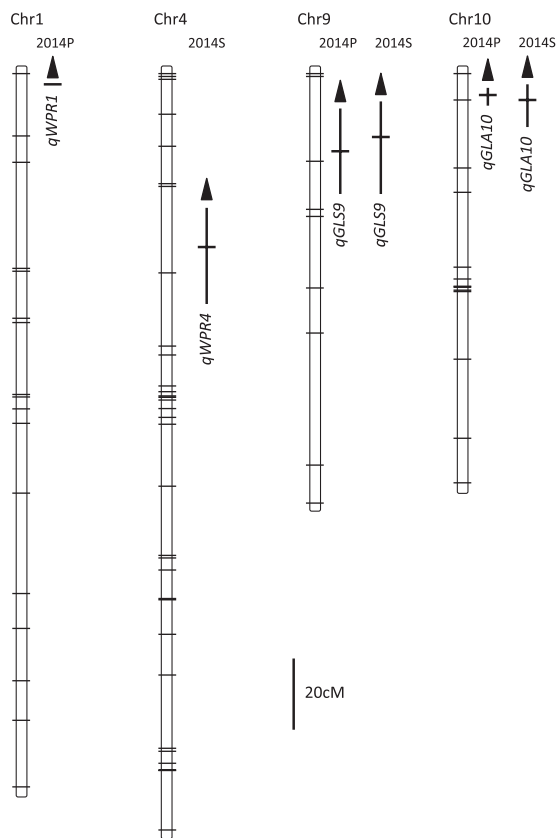
Fig. 5. Localization of the QTLs for GLA, GLS and WPR on the linkage map of chromosomes 1, 4, 9 and 10. Vertical and horizontal bars on the right hand side of each linkage map represent 95% confidence intervals and peak LOD scores, respectively.

Takeuchi *et al.* 2007, Ujiie and Ishimaru 2014, Wada *et al.* 2008). Sixteen QTLs on eight chromosomes have been found associated with the glossiness of cooked rice (Takeuchi *et al.* 2007, Wada *et al.* 2008). The QTL *qGL10* was found associated with the SSR marker RM2887 at around 8.9 Mb on the short arm of chromosome 10 (Wada *et al.* 2008). A second *qGL10* was also identified, located near the RFLP markers R1877, R716 and G127 (around 19.1 Mb) on the long arm of chromosome 10 (Takeuchi *et al.* 2007). Koshihikari alleles at both QTLs were associated with increased glossiness (Takeuchi *et al.* 2007, Wada *et al.* 2008),

indicating that Koshihikari has two superior QTLs on chromosome 10 associated with the glossiness of cooked rice. The present study detected the QTL *qGLA10* at the nearest marker, YJInDel-388, located at 2.5 Mb on chromosome 10, suggesting that *qGLA10* is allelic or closely linked to *qGL10* (Wada *et al.* 2008). If they are allelic, the high glossiness of Joiku462 may be derived from Koshihikari, resulting from the transmission of *GL10/qGLA10* at the distal region of the short arm of chromosome 10. If they are closely linked, *qGLA10* likely derives from other cultivars. In contrast, no QTLs/genes for the glossiness of cooked rice had been reported on chromosome 9, indicating that *qGLS9* is a novel QTL for the glossiness of cooked rice. The glossiness of cooked rice correlated negatively with amylose content (Juliano *et al.*1965, Takeuchi *et al.* 2007, Tanaka *et al.* 2006). In addition, QTLs for glossiness mapped to approximately the same region as that of QTLs for amylose content on chromosomes 2, 3 and 6 (Takeuchi *et al.* 2007, Tanaka *et al.* 2006). The amylose content QTL *qAC9.3*, located on the short arm of chromosome 9 (Ando *et al.* 2010), and Joiku462 were associated with the low amylose allele at *qAC9.3* (Shinada *et al.* 2015). Development of near isogenic lines (NILs) and fine mapping of these QTLs may help clarify the relationship between *qGLS9* and *qAC9.3*.

The present study demonstrated that *qWPR1* and *qWPR4* were novel QTLs for WPR. *qWPR1* had a significant effect in Pippu and *qWPR4* had a significant effect in Sapporo, suggesting that interactions between these QTLs and the environment affected WPR. During the grain filling period (August), the lowest temperature during the day was higher in Sapporo than in Pippu (**Supplemental Fig. 2**). Elevated nighttime temperatures during the grain filling period increased the whiteness of head rice (Lanning and Siebenmorgen 2013). To evaluate the interactions of temperature during the grain filling period with each QTL, NILs for each QTL should be grown at different temperatures during the grain filling period. At present, we are developing NILs for all combinations with each QTL. Recently, two QTLs for whiteness of cooked rice (WCR), *qWCR3* and *qWCR11* on chromosomes 3 and 11, respectively, were found to contribute to the superior WCR of Joiku462 (Shinada *et al.* 2015). The present study suggested that genetic control of WPR is independent of that of WCR.

Whole genome re-sequence data from NGS yielded 1,842 non-synonymous SNPs in 948 genes and 141 protein-altering indels between Yukihikari and Joiku462 (Takano *et al.* 2014). A total of 1,089 genes have the potential to generate strong functional effects associated with differences in the agronomic traits of Yukihikari and Joiku462. Thus, these 1,842 nonsynonymous SNPs and 141 protein-altering InDels will facilitate not only fine mapping but QTL cloning.

Development of PCR-based genome wide InDel and SNP markers distinguishing closely related cultivars

DNA polymorphisms between closely related cultivars can provide clues to the molecular basis of targeted breeding traits, because these cultivars are frequently crossed multiple times during crop development. InDels have become increasingly important DNA markers for studying genetic variation (Liu *et al.* 2015, Yonemaru *et al.* 2015). In our previous report, assessment of Yukihikari and Joiku462, two closely related Japanese cultivars grown in Hokkaido, identified genome-wide InDel polymorphisms ranging in size from 1 to 78 bp, as well as SNPs (Takano *et al.* 2014). This study used the Illumina HiSeq system to evaluate re-sequencing data from NGS, with an average 10-fold sequencing depth, by paired-end sequencing with read lengths of 101 bp. A total of 76,480 SNPs were identified, with an average density of 4.4/kb, resulting in 518 InDels with sequences ≥10 bp, which were distributed on individual chromosomes at a mean density of 739.0/kb. Based on these InDel polymorphisms, we developed genome-wide InDel, CAPS and dCAPS markers and identified InDel and/or SNP markers closely linked to QTLs for GLA, GLS and WPR. Although we could not exclude the possibility that of QTLs underlying GLA, GLS and WPR in the gaps on chromosomes 3 and 5, these flanking PCR-based DNA markers will be used in MAS to improve GLA, GLS and WPR in breeding programs.

Segregation distortion of the long arm of chromosome 12

Segregation distortion is defined as a deviation of the observed genotypic frequency from the expected Mendelian segregation ratio. This study, which used SSD methods without artificial selection, identified five InDel markers, located between 18,160,055 bp and 25,439,854 bp on chromosome 12 and skewed towards Yukihikari alleles ($P < 0.05$) in RILs. Segregation distortion may be due to genetic, physiological, and/or environmental factors (Liu *et al.* 2008, Matsushita *et al.* 2003, Wang *et al.* 2009, Xu *et al.* 1997).

Seed dormancy is highly associated with segregation distortion (Gu *et al.* 2006), which favors alleles from the nondormant parent in germinated populations and from the dormant parent in nongerminated subpopulations across generations. In the present study, however, harvested seeds of each RIL throughout its development were incubated at 42°C for 10 days to break seed dormancy before planting, indicating that seed dormancy was not associated with seg-

regation distortion in this study.

Biological mechanisms for distorted segregations include the preferential fertilization of male or female gametes or zygotic differences in viability resulting from gene or chromosomal mutations (Lyttle 1991, Oka 1988, Taylor and Ingvarsson 2003). In rice, several gametophytic and zygotic barriers causing deviations in allele frequencies from Mendelian ratios have been observed during interspecific or intersubspecific crosses (Harushima *et al.* 1996, 2002, Koide *et al.* 2008, Reflinur *et al.* 2014, Wang *et al.* 2005, 2009). A gametophyte gene locus, *ga-13*, on rice chromosome 12 was identified by distorted segregation of the linked isozyme gene *Acp1* during an *indica*/*japonica* cross (Rha *et al.* 1994). The *Acp1* locus is located at 67 cM on the classical genetic map (http://www.shigen.nig.ac.jp/rice/oryzabase/genes/symbolDetailAction.do?mutantGeneId=966). The *ga-13*-caused distortion favored the *indica* allele, the distortion pattern did not change across three environments, and the distortion was found due to genotypic differences of male gametes competing for fertilization (Rha *et al.* 1994). Female segregation distortion flanking four DNA markers at 19.4–25.0 Mbp on chromosome 12 was also observed during another *indica*/*japonica* cross (Reflinur *et al.* 2014). Because both genomic regions overlapped the present segregation distortion region, the distortion mechanism occurring in the progeny of closely related cultivars remains to be clarified. Using reciprocal BC_1F_1 and F_2 populations, we intend to determine whether the segregation distortion identified in this study was caused by the preferential fertilization of male or female gametes or by zygotic differences.

Conclusion

Over the past 30 years, the introgression of four QTLs, *qGLA9*, *qGLS10*, *qWPR1* and *qWPR4*, was shown essential for improvements in the appearance of cooked rice and polished rice of the Hokkaido rice cultivar Joiku462. Flanking molecular markers may be useful for MAS to improve GLA, GLS and WPR. Additional studies, including the isolation of each QTL, are needed to clarify environmental interactions, as well as the molecular basis of GLA, GLS and WPR.

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