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Research Paper

Quantitative trait loci for whiteness of cooked rice detected in improved rice cultivars in Hokkaido

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Improving the eating quality of cooked rice has been one of the most important objectives in rice breeding programs. Eating quality of cooked rice is a complex trait including several components, such as external appearance, taste, aroma, and texture. Therefore, dissection of these components followed by marker-assisted selection of detected QTL(s) may be a useful approach for achieving desirable eating quality in rice breeding. Whiteness of cooked rice (WCR) is an important factor related to the external appearance of cooked rice. WCR is known to be associated with the amylose and protein contents of the endosperm. However, the genetic basis of WCR remains unclear. In this study, we evaluated phenotypic variation in WCR among recently developed rice cultivars from Hokkaido, Japan. Then, we developed doubled haploid lines (DHLs) derived from a cross between two cultivars from Hokkaido, Joiku No. 462 (high WCR) and Jokei06214 (low WCR). Using the DHLs, we detected two QTLs for WCR, *qWCR3* and *qWCR11*, on chromosomes 3 and 11, respectively. We also examined the dosage effect of the two QTLs based on both the categorized segregants in the DHLs and the relationship between the WCR phenotype and inheritance around the QTL regions in cultivars from Hokkaido.

Key Words: whiteness of cooked rice, *Oryza sativa* L., eating quality, rice breeding programs.

Introduction

Rice is one of the most important staple foods in the world, eaten by 50% of the world's population. Improving the eating quality of cooked rice is one of the most important objectives to meet market demand. The eating quality of cooked rice is a complex trait including several components, such as external appearance, taste, aroma, and texture, and is usually evaluated by sensory tests. Although sensory tests are the most reliable method for evaluation of eating quality, these require a large amount of uniform polished rice samples, precise cooking methods and many well-trained panelists. Thus, sensory tests are not always applicable to all the progeny in rice breeding programs. Understanding the genetic basis of these components followed by marker-assisted selection (MAS) may be useful for cost and labor saving selection of improved cultivars.

Preferences for eating quality of cooked rice vary depending on end-use, culture, and climate in each local region. For example, rice with good external appearance (high whiteness and glossiness) and soft, sticky texture is preferred by Japanese consumers (Takeuchi *et al.* 2007). On the other hand, exquisite aroma is one of the most important quality traits of cooked rice that leads to high consumer acceptance in South Asia (Singh *et al.* 2007). A number of quantitative trait loci (QTLs) for glossiness, texture, taste, and aroma of cooked rice have been detected (Kobayashi and Tomita 2008, Singh *et al.* 2007, Takeuchi *et al.* 2007, 2008, Wada *et al.* 2008). These QTLs are useful for application in MAS for eating quality in each local rice breeding

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program as well as a means to further understand the genetic mechanism of each trait.

Whiteness of cooked rice (WCR) is an important factor related to the external appearance of cooked rice (Lv *et al.* 2009). Improving WCR is an important breeding objective in Japan. Although there is wide phenotypic variation in WCR among Japanese rice cultivars (Goto *et al.* 2014, Kogi *et al.* 2014), the genetic basis of WCR has not been clarified. Instead of sensory tests, evaluation methods for WCR use analytical equipment (such as a Mido Meter [MA-90-A, TOYO, Tokyo, Japan], a scaner or a spectrophotometer) as established in previous studies (Goto *et al.* 2014, Kogi *et al.* 2014). These methods are beneficial for the selection of WCR, but large uniform samples are still needed. In addition, amylose content (AC) and protein content (PC) of the endosperm influence WCR (Goto *et al.* 2014, Kinoshita 2013). Selection of breeding lines with low AC and PC may be useful for improving WCR. However, it is not clear whether these two factors are major determinants of WCR.

Hokkaido is the northern-most region of Japan and one of the northern limits of rice cultivation in the world. The environmental conditions in Hokkaido, low temperature and high nitrogen level, are not suitable for the production of rice with good eating quality for Japanese consumers (Inatsu 1988). Intensive selection pressures in Hokkaido rice breeding programs have focused on improving the eating quality of cooked rice for the last three decades. As a result, stable production of rice with good eating quality has been accomplished (Kinoshita 2013). During this history, there have been significant improvements in WCR among Hokkaido rice cultivars (Kinoshita 2013). Currently, there are no differences in WCR between Nanatsuboshi, a standard cultivar in Hokkaido, and elite rice cultivars from other regions in Japan, such as Koshihikari, Hitomebore, etc. Moreover, new cultivars with higher WCR than Nanatsuboshi were developed recently in Hokkaido (Hokkaido Agricultural Research Center 2005). The identification of genetic factors determining differences in WCR between Nanatsuboshi and these higher WCR cultivars will be useful for further improvement in WCR in Hokkaido rice.

In this study, we evaluated WCR among some of recent Hokkaido rice cultivars. Then, QTL analysis was carried out using doubled haploid lines (DHLs) derived from a cross between two cultivars from Hokkaido, Joiku No. 462 with high WCR, low PC and low AC, and Jokei06214 with low WCR. Based on the results of QTL analysis, we validated the genetic relationships between WCR and either AC or PC. Furthermore, we also analyzed inherited genome regions around the detected QTLs among rice cultivars from Hokkaido and discussed their contribution to WCR.

Materials and Methods

Plant material

Nine rice cultivars from Hokkaido were used to evaluate phenotypic variations in WCR (**Supplemental Table 1**). They were developed in Hokkaido between 1988 and 2010 and had close pedigree relationships (**Supplemental Fig. 1**). Five cultivars, Yukisayaka, Yumepirika, Joiku No. 462, Jokei06214, and Daichinohoshi were selected as cultivars developed after Nanatsuboshi. Three of them, Yukisayaka, Yumepirika, and Joiku No. 462 were developed as cultivars with better external appearance quality of cooked rice than that of Nanatsuboshi. Three cultivars, Kirara397, Hoshinoyume, Hokkai PL9 were selected as the progenitors of the above lines, and Nanatsuboshi was selected as a standard for the evaluation of WCR.

DHLs derived from the cross between Joiku No. 462 (high WCR) and Jokei06214 (low WCR) were developed as a mapping population. F_1 plants between Joiku No. 462 and Jokei06214 were used to produce A_0 plants by the anther culture method (Niizeki and Oono 1968), and a total of 88 A_0 plants were obtained. A_2 plants were developed by the single-seed descent method. These lines (A_2) and the nine cultivars were cultivated in an experimental paddy field at Kamikawa Agricultural Experiment Station, Pippu, Japan 43°51′N latitude in 2012 and 2013. Sowing and transplanting were performed in late April and late May, respectively. Fertilizer was applied at a rate of 0.8 N kg/acre. Eight plants of each line were harvested at maturity. The harvested products were dried and stored at room temperature for 5 months, then applied for each phenotypic evaluation as described below.

Sensory test of WCR

To evaluate WCR in a number of lines, we modified the evaluation method of cooked rice described as Takeuchi *et al.* 2008. One hundred grams of hulled grain was polished to a yield of ~90% in a rice mill SKM5B(1) (Satake, Hiroshima, Japan). Then, 20 g of polished rice was placed in a 50-cc iron bowl and washed four times with water. The washed rice was soaked in water for 30 min. Then 50-cc iron bowls were putted in a rice cooker kettle filled with 200 ml of water and then cooked for about 45 min at a 1.3 : 1 (v/w) ratio of water : polished rice in a rice cooker (Hitachi, Tokyo, Japan). The cooked rice was then steamed in the cooker for an additional 10 min. WCR was evaluated by 7–11 panelists. The WCR of each line was scored from –3 (extremely poor) to +3 (excellent) compared with that of Nanatsuboshi (score = 0). Then, the mean score from the panelists was calculated. We compared WCR scores obtained from two methods, normal evaluation method described as Takeuchi *et al.* 2008 and modified methods as described above (**Supplemental Fig. 2**). High positive correlation between WCR scores obtained by two methods was observed.

The appearance of the cooked rice and its WCR score in the eight elite cultivars are shown in **Supplemental Fig. 3**.

Evaluation of AC and PC

The AC of each line was evaluated in accordance with the method described by Ando *et al.* (2010). PC of polished

rice of each line was evaluated using infratec™1241 (Foss, Hillerød, Denmark). The values of arcsine-transformed AC and PC were used for QTL analysis.

DNA analysis

Total DNA was isolated from young leaves using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). Polymorphic single nucleotide polymorphism (SNP) markers were selected from published genome-wide SNP information (Nagasaki *et al.* 2010). The DNA was genotyped using 768 selected SNPs with a GoldenGate BeadArray platform followed by Bead Station 500G system (Illumina, San Diego, CA, USA). All experimental procedures for SNP typing followed the manufacturer's instructions. In addition, we selected 38 primer sets of simple sequence repeat (SSR) sites from the list of McCouch *et al.* (2002) and IRGSP (2005) and these used for genotyping. PCR was carried out using a *Kapa2G Fast PCR kit* (Kapa Biosystems, Wilmington, MA, USA). Amplification was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) at 95°C for 1 minute, followed by 35 cycles of 95°C for 10 seconds, 55°C for 10 seconds, 72°C for one second, and followed by 72°C for 30 seconds. Amplified products were electrophoresed in 3% agarose gel with 0.5 × Tris-borate EDTA (TBE) buffer.

QTL analysis and determination of the pedigree haplotypes of QTL

A linkage map was constructed using MAPMAKER/EXP 3.0 (Lander *et al.* 1987). The detection of QTLs was conducted by composite interval mapping with QTL Cartographer 2.5 (Basten *et al.* 1998). The threshold to detect QTLs was determined by the 1000 per-mutation (Churchill and Doerge 1994) test at a probability level of 0.05 : LOD = 2.50 (WCR), 12.20 (AC) and 2.30 (PC). The pedigree haplotype of each QTL was defined as consecutive genotypes of four SNPs markers included in the interval with a reduction of 1.0 from each of the peak limit of detection (LOD) value.

Results

Phenotypic variation in WCR, AC and PC among Hokkaido rice cultivars

A wide range of phenotypic variation in WCR was observed among the nine Hokkaido rice cultivars (**Table 1**). The WCR score ranged from –0.54 to 0.50 (2012) and from –0.83 to 1.17 (2013). A difference in WCR between two years was observed. It was not clear which factors were responsible for this difference. However, the order of the quality of WCR scores among four cultivars evaluated in both years was almost same between two years. Thus, we judged that we could evaluate accurately phenotypic variation of WCR among nine cultivars. Four cultivars, Hoshinoyume, Yumepirika Yukisayaka, and Joiku No. 462, showed the same or a higher level of WCR than Nanatsuboshi. In particular, Joiku No. 462 and Yukisayaka showed the highest WCR scores.

Phenotypic variations in AC and PC among these cultivars

Table 1. WCR, AC, and PC score and haplotype diversity of eight SNP markers linked to *qWCR3* or *qWCR11* regions for nine cultivars from Hokkaido

						qWCR3[a]					*qWCR11*[a]			
						ac30493	aa32208	ac30537	aa32290		aa114500	aa114506	aa115083	aa116712
Year	Cultivar	WCR score*	AC (%)	PC (%)	Type[b]	17.9	22.1	22.6	23.2	Type[b]	23.6	24.0	25.8	26.6
2012	Joiku No. 462	0.5	18.0	5.9	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Ji}$	G	A	C	C
	Hoshinoyume	0	22.1	6.5	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Jk}$	A	G	A	T
	Nanatsuboshi*	0	19.5	6.0	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Jk}$	A	G	A	T
	Jokei06214	–0.21	23.1	6.2	$WCR3^{Jk}$	G	C	T	T	$WCR11^{Jk}$	A	G	A	T
	Kirara397	–0.43	20.4	6.7	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Jk}$	A	G	A	T
	HokkaiPL9	–0.54	–	–	$WCR3^{other}$	G	T	A	T	$WCR11^{Ji}$	G	A	C	C
2013	Joiku No. 462	1.17	18.3	6.1	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Ji}$	G	A	C	C
	Yukisayaka	1.17	18.2	5.7	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Ji}$	G	A	C	C
	Yumepirika	0.50	16.4	6.2	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Jk}$	A	G	A	T
	Hoshinoyume	0.50	20.7	6.0	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Jk}$	A	G	A	T
	Nanatsuboshi*	0	19.0	6.1	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Jk}$	A	G	A	T
	Kirara397	–0.33	20.2	6.6	$WCR3^{Ji}$	A	T	A	C	$WCR11^{Jk}$	A	G	A	T
	Daichinohoshi	–0.83	21.2	6.5	$WCR3^{other}$	G	T	T	T	$WCR11^{Ji}$	G	A	C	C

*Nanatsuboshi was used as a standard in the sensory test of WCR.

[a] The same genotype as Joiku No. 462 is indicated in gray fill. Black fill indicates the nearest marker for each QTL.

[b] $WCR3^{Ji}$ and $WCR11^{Ji}$ indicate the same genotype pattern in all SNP markers around *qWCR3* and *qWCR11* as that of Joiku No. 462. $WCR3^{Jk}$ and $WCR11^{Jk}$ indicate the same genotype pattern in all SNP markers around *qWCR3* and *qWCR11* as that of Jokei06214. $WCR3^{other}$ indicates a different genotype pattern to both that of Joiku No. 462 and Jokei06214.

AC, amylose content; PC, protein content; WCR, whiteness of cooked rice.

were also observed (**Table 1**). The AC ranged from 18.0% to 23.1% (2012) and from 16.4% to 21.2% (2013). Joiku No. 462, Yukisayaka, and Yumepirika showed lower AC than that of Nanatsuboshi. On the other hand, Hoshinoyume, Kirara397, Daichinohoshi, and Jokei 06214 showed higher AC. In particular, Jokei06214 showed the highest AC. The PC ranged from 5.9% to 6.7% (2012) and from 5.7% to 6.6% (2013). Only two cultivars, Joiku No. 462 and Yukisayaka, showed lower PC than that of Nanatsuboshi.

Based on these results, we selected two cultivars, Joiku No. 462 as a cultivar with high WCR, low AC, and low PC, and Jokei06214 as a cultivar with low WCR, high AC, and high PC.

QTL analysis for WCR, AC and PC

The DHLs showed continuous variation in WCR from –0.31 to 0.63, with a mean score of 0.17 (**Fig. 1**). In addition, AC and PC were also evaluated in each line (**Supplemental Fig. 3**). PC among DHLs showed a continuous variation, from 5.35% to 7.63%, with a mean of 6.20%. AC among DHLs showed a binominal variation, from 16.2% to 23.7%, with a mean of 20.2%. Only AC showed a weak positive correlation with WCR (**Supplemental Fig. 4**).

Genome-wide screening revealed that 130 DNA markers (125 SNP markers out of 768, and 5 SSR markers) exhibited polymorphism between Joiku No. 462 and Jokei06214 (**Fig. 2**). Considering the fact that the two parents are of the same breeding pedigree (**Supplemental Fig. 1**), genome regions on the markers with no polymorphism implies that these are genetically identical.

Two putative QTLs for WCR were detected (**Table 2**, **Fig. 2**). The Joiku No. 462 allele at both detected QTLs increased WCR. *qWCR3* near ac00537 on chromosome 3 explained 11.7% of the total phenotypic variation. *qWCR11* near RM4601 on chromosome 11 explained 10.3% of the total phenotypic variation. Furthermore, one prominent QTL for AC was detected in chromosome 9. The Joiku No. 462 allele at this QTL decreased AC. This QTL region of Joiku No. 462 was derived from Hokkai PL9 (data not showed). Hokkai PL9 has a QTL for AC in this region, *qAC9* (Ando *et al.* 2010). Thus, we assigned this QTL as *qAC9*. Two QTLs for PC were detected on chromosome 2 (*qPC2*) and 3 (*qPC3*) (**Table 2**, **Fig. 2**). The Jokei06214 allele at *qPC2* increased PC, but at *qPC3*, the Joiku No. 462 allele increased it. The estimated region of *qPC3* overlapped with the *qWCR3* region.

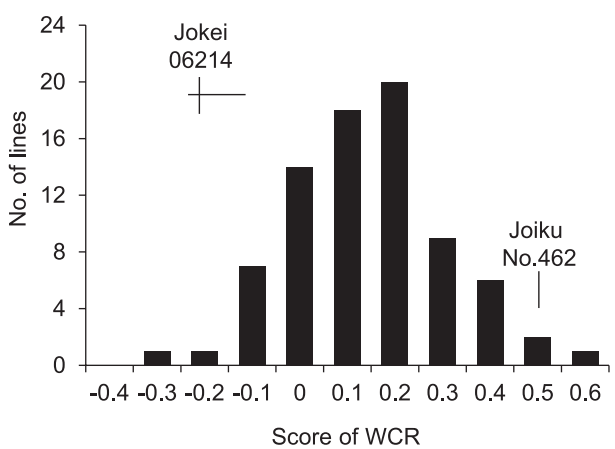

Fig. 1. Frequency distribution of WCR in a DHL population of Joiku No. 462 and Jokei06214. *Horizontal* and *vertical lines* represent standard deviation and mean values.

To elucidate genetic interactions between the two QTLs for WCR, segregants of DHLs were classified into four-genotype classes (*qWCR3* + *qWCR11*, *qWCR3*, *qWCR11*, no QTLs for WCR) based on the genotype of the nearest marker for each QTL. Although two-way ANOVA did not show a significant effect on WCR between the two QTLs ($P = 0.90$), a trend for increasing mean score of WCR with an increase in the number of QTLs was observed (**Fig. 3**).

Inheritance of two QTLs among the Hokkaido rice cultivars

To elucidate the role of the two QTLs among rice cultivars from Hokkaido, pedigree haplotypes around the QTLs among the nine cultivars from Hokkaido were compared.

Haplotypes and their inheritance of the *qWCR3* and *qWCR11* regions among the nine cultivars from Hokkaido are summarized in **Table 1** and **Supplemental Fig. 1**. Five cultivars, Hoshinoyume, Nanatsuboshi, Yumepirika, Yukisayaka, and Kirara397, had same haplotype as Joiku No. 462 around the *qWCR3* region. Three of these cultivars, Hoshinoyume, Yumepirika, and Yukisayaka, showed higher WCR. On the other hand, the other three cultivars, Daichinohoshi, Hokkai PL9, and Jokei06214, with different haplotypes from that of Joiku No. 462 showed lower WCR. Daichinohoshi, Hokkai PL9, and Yukisayaka possessed the same haplotype as Joiku No. 462 around the *qWCR11* region. Yukisayaka showed higher WCR.

Joiku No. 462 was derived from a cross between Yukisayaka and Joiku No. 452. Only Yukisayaka possessed the same genotype as Joiku No. 462 in both QTL regions (**Supplemental Fig. 1**). The haplotype around *qWCR3* and *qWCR11* in Joiku No. 462 was inherited from Yukisayaka.

Discussion

Several QTLs for components of eating quality are present in clusters (Kobayashi and Tomita 2008, Takeuchi *et al.* 2007, 2008, Wada *et al.* 2008). Although the individual components of eating quality seem to be different characteristics, they may be related to each other. In this study, *qWCR3* was detected on the long arm of chromosome 3. Previously, Kobayashi and Tomita (2008) detected a QTL for stickiness of cooked rice, *qST3-2*, in this region. Furthermore, a QTL for PC (*qPC3*) was also detected in the same region. This region may effect not only WCR but also the texture of cooked rice. To verify this, evaluation of effect of *qWCR3* region for texture (stickiness and hardness) of cooked rice would be necessary. On the other hand, two

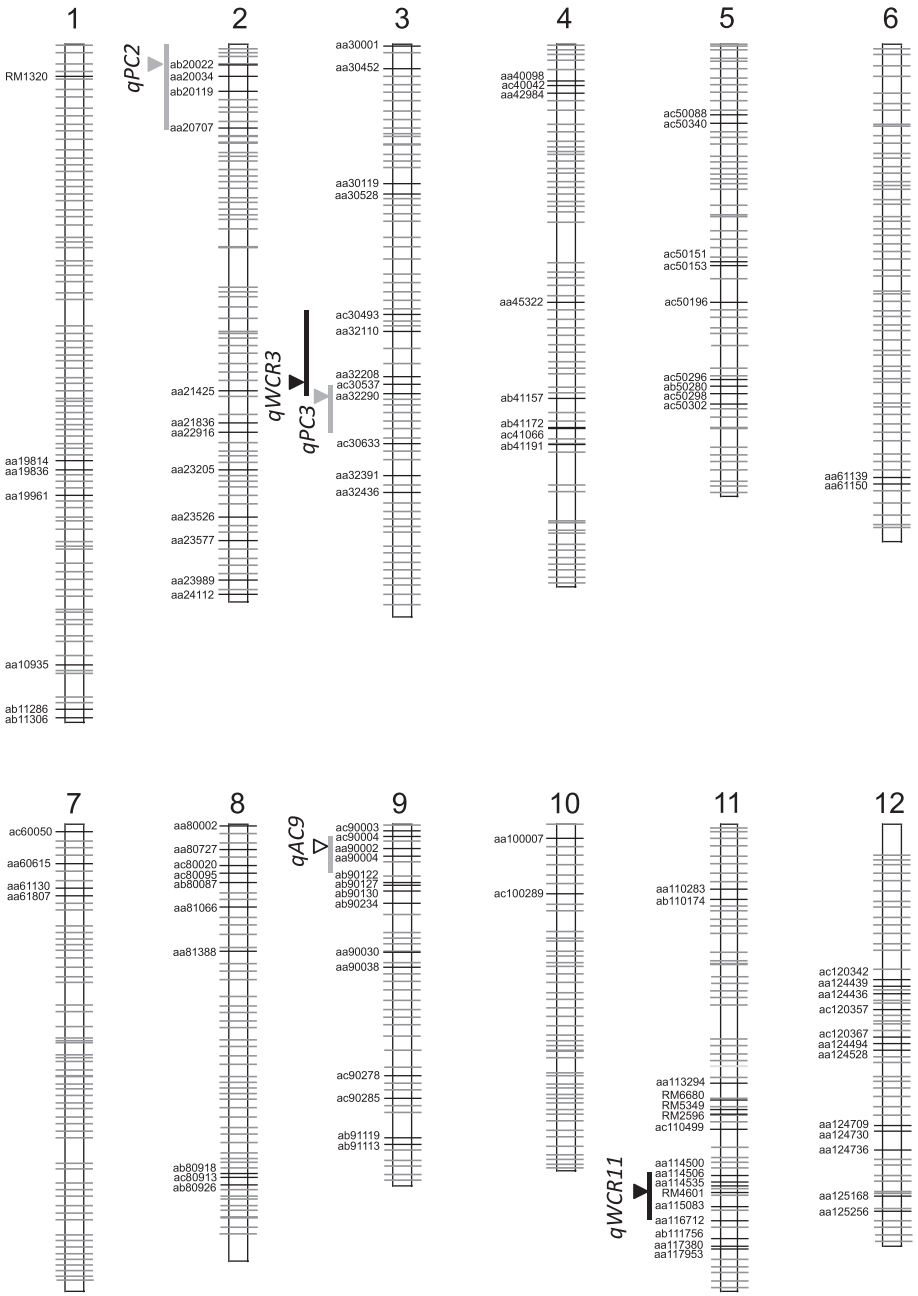

Fig. 2. Construction of a linkage map for the DHL population between Joiku No. 462 and Jokei06214. In this study, a total of 768 SNP markers and 38 SSR markers were used. The positions of 125 SNPs and 5 SSR polymorphism markers are shown as black vertical lines. Other non-polymorphism markers are shown as gray vertical lines. The location of each marker was based on the genomic sequence of the cultivar Nipponbare in IRGSP build 5 in RAP-DB (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html). The black, white, and gray triangles show QTL regions for WCR, AC, and PC, respectively, identified using mapping population. *Vertical lines* indicate the interval with a reduction of 1.0 from the peak LOD value.

QTLs, *qTA11* for taste of cooked rice and *qGL11* for glossiness of cooked rice were detected on chromosome 11, which are distinct from the *qWCR11* region (Takeuchi *et al.* 2008, Wada *et al.* 2008).

It is well known that chemical properties affect the eating quality of cooked rice. A previous study suggested a relationship between WCR and AC of the endosperm (Goto *et al.* 2014). Phenotypic correlations between WCR and PC were also suggested among Hokkaido rice cultivars (Kinoshita 2013). In this study, a weak relationship between WCR and AC was observed in the DHL population (**Supplemental Fig. 4**). However, no significant QTL for WCR was detected with the most prominent QTL for AC on chromosome 9. On the other hand, even though *qPC3* and *qWCR3* were detected in the same region, no significant correlation was observed between WCR and PC in the same

Table 2. QTLs involved in WCR, AC, and PC detected in the DHL population

Trait	Chromosome	Position of nearest QTL marker[a]	Marker interval	LOD	PVE (%)[b]	Ae[c]
WCR	3	22,664,880	ac30537*–aa30290	3.23	11.8	0.06
	11	24,664,880	RM4601*–aa115083	2.86	10.3	0.06
AC	9	1,689,083	ac90004-aa90002*	29.23	67.9	0.25
PC	2	1,389,486	–ab20002*	2.53	9.3	–0.20
	3	28,715,718	Ac30633–aa302391*	3.42	12.9	0.25

[a] Position of nearest marker based on location of the start of simple sequence repeat in IRGSP build 5 in RAP-DB (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html).
[b] Percentage of total phenotypic variance explained by the QTL.
[c] Additive effect of Joiku No.462 allele. Positive direction indicates Jokei462 allele increases the value.
*Nearest marker for QTL.

DHL population. We should consider that genetic variation of WCR, AC and PC in these DHLs may not reflect those in the total range of rice cultivars in Hokkaido. However, it was also noted that AC and PC are not the only determinants of WCR. Thus, there may be limitations in the indirect selection of high WCR progeny by selection of high AC or PC progeny.

In this study, we detected two QTLs for WCR, *qWCR3* and *qWCR11*, on chromosomes 3 and 11, respectively. For improvement in quantitative traits in breeding programs, validation of the combination effects of QTLs is very important. In this study, we observed a trend of stacking effects of detected QTLs for WCR in the DHLs examined, which indicated that WCR in *qWCR3* + *qWCR11* is highest, and that in *qWCR3* and *qWCR11* higher than no QTLs for WCR (**Fig. 3**). This stacking effect of *qWCR* loci was also implicated in the Hokkaido cultivars evaluated (**Table 1**). More specifically, Yukisayaka, which possessed the same two QTLs as Joiku No. 462, showed the highest WCR, with the same level as Joiku No. 462. These results suggested that the combination of *qWCR3* and *qWCR11* is useful to achieve a higher WCR level. Utilization of these two QTLs by MAS could be substitutable for sensory tests in the selection of WCR.

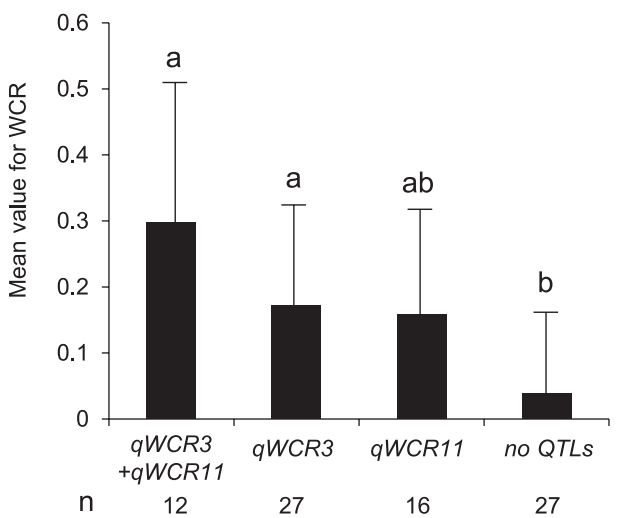

Fig. 3. Mean values for WCR with different combination of two QTLs. n: represents the number of individuals. Groups with different letters are significantly different ($P < 0.05$, Tukey-Kramer HSD test).

Genetic diversity among rice breeding materials in each local region is very small. Therefore, resolution of previous DNA markers, such as SSR or Indel, was not good enough to identify specific regions of interest inherited during the breeding process. Genome-wide SNPs allowed us to investigate detailed information of inherited haplotype even in a closely related population such as Hokkaido rice cultivars. In this study, using genotype data of SNP markers and pedigree information, we found inheritance of detected QTLs with phenotypic changes in WCR among recent Hokkaido rice cultivars (**Table 1**, **Supplemental Fig. 1**). Considering our data, we can explain the genetic basis of historical improvements in WCR in Hokakido rice as follows. 1) $WCR3^{Ji}$ is effective by itself and distributed in recent Hokkaido rice such as Yumepirika, Hoshinoyume, Nanatsuboshi, and Kirara397, which has been a first step in WCR improvement. 2) Recent accomplishments of the highest WCR in Joiku No. 462 and Yukisayaka were caused by a combination of two better alleles of QTLs, $WCR3^{Ji}$ from Hoshinoyume or Akiho and $WCR11^{Ji}$ from Hokkai PL9. To substantiate our hypothesis, further study is necessary, but we think these two regions play an important role and are applicable for MAS for WCR improvement in Hokkaido rice cultivars at the current level.

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