Verification of quantitative trait locus for stickiness of cooked rice and amylose content by developing near-isogenic lines

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We previously showed a major quantitative trait locus (QTL) for the eating quality of cooked rice (Oryza sativa L.) on the long arm of chromosome 2 using a population of doubled haploid lines derived from crosses between ‘Koshihikari’ and ‘Akihikari’. Here, we verified the effect of QTL by developing near-isogenic lines (NILs). We successfully developed six NILs on the genetic background of Akihikari in which different segments of chromosome 2 were introduced from Koshihikari. On the basis of the measurement of traits related to eating quality, we observed significant differences in the stickiness of cooked rice and amylose content (AC) of grains among NILs. The stickiness scores and AC values of the NILs were negatively correlated. Comparison of the introduced segments with stickiness revealed that the candidate genomic region of chromosome 2 that affects stickiness lies between 515 kbp (defined by the simple sequence repeat (SSR) markers RM13658 and RM3730) and 773 kbp (defined by the SSR markers KA43 and RM6933). These results clearly showed that a chromosome 2 segment introduced from Koshihikari increased stickiness and decreased AC in the NILs, and hence verified the result obtained from the doubled haploid lines.

Key Words: Oryza sativa L., stickiness of cooked rice, amylose content, substitution mapping, near-isogenic lines.

Introduction

In 1956, ‘Etsunan 17’, a line of japonica paddy rice (Oryza sativa L.) bred by Fukui Agricultural Experiment Station, officially became a new cultivar named ‘Koshihikari’. Since then, the area of land under Koshihikari cultivation has gradually increased: in 1979, Koshihikari was the most popular cultivar in Japan, covering about 300 000 ha, and has retained its position. In 2006, the area cultivated with Koshihikari had reached 627 800 ha, accounting for about 37% of the total paddy rice acreage in Japan (Ministry of Agriculture, Forestry and Fisheries, http://www.maff.go.jp/www/info/bunrui/bun02.html). The popularity of this cultivar is due to its wide adaptability to various climatic conditions and its excellent eating quality for Japanese consumers, such as the high stickiness of cooked rice. However, Koshihikari also has inferior agronomic traits, such as a long culm and low resistance to blast diseases.

In rice breeding programs in Japan, one of the most important goals has been to improve these inferior traits of Koshihikari, while retaining its eating quality. Selection for eating quality is essentially based on sensory tests, but it is not very efficient because it requires several generation alternations after crossing to start the selection. Eating quality is also influenced by environmental factors, such as air temperature during the ripening period (Nishimura et al. 1985) and the nitrogen level of the soil (Ishima et al. 1974). For more efficient selection for eating quality, researchers and rice breeders have attempted to develop an indirect selection system, such as marker-assisted selection (MAS), which makes it possible to reduce the labor intensity of testing, to exclude the effects of environmental conditions on the expression of eating quality, and to start in early generations, thereby providing faster results than with other approaches.

To use MAS for assessing eating quality, it is first necessary to identify DNA markers closely linked to the genes that control this quality. Ebiriki et al. (2002) detected a quantitative trait locus (QTL) for the “Mido” value, an index of eating quality, using backcrossed inbred lines derived from the cross between Koshihikari and ‘Kasalath’. Takeuchi et al. (2007) also detected QTLs for eating quality and amylose content (AC) using backcrossed inbred lines derived from the cross between Koshihikari and Kasalath. Li et al. (2003) detected QTLs for AC, as well as for the alkali spreading score and gel consistency, using backcrossed inbred lines derived from the cross between ‘Nipponbare’ and Kasalath. Wan et al. (2004) and Ogata et al. (1996) detected

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QTLs for eating quality, AC, and amylogetic characteristics using recombinant inbred lines derived from the cross between ‘Asominori’ and ‘IR24’. In these studies, putative QTLs for eating quality were detected in crosses between japonica and indica cultivars; however, genetic analysis of eating quality in japonica cultivars has been limited by the lack of polymorphic DNA markers. Recently, QTL analyses have been performed using recombinant inbred lines derived from the cross between japonica cultivars Koshikihari and ‘Moritawase’ and have detected QTLs for AC and the eating quality of cooked rice (Wada et al. 2006, 2007); however, no verification of these QTLs has been performed using near-isogenic lines (NILs).

We previously performed QTL analysis for eating quality (Tanaka et al. 2006) using doubled haploid lines derived from crosses between two japonica cultivars, Koshikihari and ‘Akihikari’, developed by Takeuchi et al. (2001). The cooked rice of Koshikihari is very sticky, glossy, and its eating quality is excellent, whereas that of Akihikari is less sticky and its eating quality is much lower than Koshikihari (Yamamoto and Ogawa 1992). Tanaka et al. (2006) showed four QTLs associated with the stickiness of cooked rice on chromosomes 1, 2, 3, and 6. Among them, the Koshikihari allele seemed to decrease stickiness at the QTLs on chromosomes 1 and 3; therefore, these QTLs were not considered to relate to the high stickiness of Koshikihari. In contrast, at the QTLs on chromosomes 2 and 6, the Koshikihari allele seemed to increase stickiness and decrease AC. The QTL on chromosome 2 was repeatedly detected across the two-year experiments, whereas the QTL on chromosome 6 was detected in only one year. In addition, the percentage of variance explained by the QTL for stickiness and AC on chromosome 2 was the highest; therefore, Tanaka et al. (2006) suggested that the QTL region on the long arm of chromosome 2 exerted a notable influence on the excellent eating quality of Koshikihari.

Here, we attempted to verify the QTL detected on chromosome 2 for stickiness and AC by developing six NILs, in which a chromosomal segment from Koshikihari was successfully introduced into the genetic background of Akihikari. Then, we evaluated the eating quality of the cooked rice by measuring its stickiness and AC values of NILs. Based on these results, the effect of QTL is discussed. In addition, comparison of the substituted chromosomal segments allowed us to delimit the candidate genomic region of the QTL for stickiness and AC.

Materials and Methods

Plant materials

Figure 1 shows the breeding scheme used for the development of the NILs. DH92 (a doubled haploid line, Takeuchi et al. 2001) was chosen for further backcrossing with Akihikari. Successive backcrossings were conducted using Akihikari as the recurrent parent. During backcrossing, appropriate individuals or lines were selected by MAS based on the presence of a target region on chromosome 2, while minimizing other chromosomal regions. During the selection of candidate NILs, 12 simple sequence repeat (SSR) markers (Temnykh et al. 2001, McCouch et al. 2002) and three single nucleotide polymorphism (SNP) markers (Plant Genome Center, http://www.pgcDNA.co.jp/snps/) were used for successive selection. To determine the size of the substituted segments, we also developed six new SSR markers using genomic sequence information (International Rice Genome Sequencing Project, 2005; http://rgp.dna.afrgc.go.jp/E/IRGSP/index.html). The primer sequences of these newly developed SSR markers are shown in Table 1 and the 21 markers are listed in Figure 2. The genetic background of NIL-1, 3, 4, and 6 was surveyed in 2004 using 21 restriction fragment length polymorphism (RFLP) markers for BCf1 generation. Lines with more than three Koshikihari segments were excluded. In 2006, the whole genome of each NIL was also surveyed using 89 SSR markers (Temnykh et al. 2001, McCouch et al. 2002) distributed across 12 chromosomes (Fig. 3A) to evaluate the isogenic status of the selected NILs. Days-to-heading (DTH)-based selection was also conducted after BCf2 generation. As a result of this work, we developed six NILs in which the chromosomal segment of the QTL region of Koshikihari was introduced into the Akihikari genetic background.

In 2006 and 2007, we cultivated these six NILs, Akihikari, and Koshikihari in a paddy field at Fukui Agricultural Experiment Station (36°03'N) to evaluate traits related to eating quality and agronomic characteristics. We used BCfF6 and BCfF7 generations of NIL-1, 3, 4, and 6 in 2006 and 2007, respectively, and BCfF3 and BCfF4 generations of NIL-2 and 5 in 2006 and 2007, respectively. Fifty hills were planted per line and the planting density was 20.7 hills/m2. As the base manure, 6.0 g m−2 of nitrogen fertilizer was applied, with 3.6 g m−2 of nitrogen fertilizer applied as ear manure, divided into two applications. Major agronomic characteristics (DTH, culm length, panicle length, and panicle number) were measured in both years.

Evaluation of stickiness from sensory tests, and measurement of AC and protein content

Rice grains of Akihikari and NILs, and Koshikihari were harvested 31 and 36 days after heading, respectively, and then air-dried and polished to yield approximately 90% in a Clean One-Pass rice miller (Satake, Tokyo, Japan). A total of 150 g of polished rice was washed for 2 min with a Hayatogi washer (Crescent, Hiroshima, Japan) and transferred into a 500-mL beaker. The rice was soaked in water for 90 min and then cooked in an ERC-9F SP electric rice cooker (Nichiwa Electric, Hyogo, Japan) at a 1.26 (w/w) ratio of water to polished rice. Stickiness was evaluated by six people (4 men and 2 women; age range, 24–47 years) who had been trained to distinguish differences in stickiness. Stickiness scores were given as −1 (less sticky than Akihikari), 0 (same as Akihikari), +1 (stickier than Akihikari), and +2 (much stickier than Akihikari). Stickiness was
Verification of QTL for stickiness and amylose content

Table 1. Primer sequences of the markers developed

<table>
<thead>
<tr>
<th>Marker name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>KA4</td>
<td>cacacagctgctgaaatta</td>
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<tr>
<td>KA35</td>
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<td>tgcagatgctactccaga</td>
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<td>KA80</td>
<td>tctagctctgctctctcctc</td>
<td>gtacagctctgctctgctctt</td>
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A total of 100 g of polished rice grains were crushed with a test mill (Brabender, Duisburg, Germany) and sieved through 0.15-mm mesh, and then AC and protein content were measured with an Auto Analyzer II and an InfraFaker 450 (both from Bran+Luebbe, Norderstedt, Germany), respectively, with six replications per sample.

DNA isolation and marker analyses

Total DNA was extracted from the leaves by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). PCR was performed in a 10-μL volume of reaction mixture containing 10 ng of genomic DNA, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 μM of each forward and reverse primer, 1.5 mM MgCl2, 2 mM of dNTPs, and 0.25 U of Taq ExHIS polymerase (TaKaRa, Kyoto, Japan). The amplification profile was as follows: 1 min at 94°C, 35 cycles of 30 sec at 94°C, 1 min at 55°C, and 2 min at 72°C, followed by 7 min at 72°C for the final extension. We used an iCycler Thermal Cycler PCR system (Bio-Rad Laboratories, CA, USA). PCR products were fractionated on a 2.5% agarose TBE gel.

Results

Graphical representation of NIL genotypes

The genotypes of the developed NILs are shown in Figure 2A (foreground) and Figure 3A (background). In NIL-1, a Koshihikari segment for the target region (from RM3316 to RM8029) was introduced into the genetic background of Akihikari. Different segments, but sharing the same target region, were also introduced into NIL-2 (from S0651 to RM3535), NIL-3 (two segments, from S0299 to KA68 and from KA80 to RM8029), NIL-4 (from KA35 to RM8029), NIL-5 (from KA68 to RM8029), and NIL-6 (from S0299 to KA80).

We tried to minimize the introgression of Koshihikari segments for non-target chromosomes during backcross generations; however, several other Koshihikari segments were introduced into the NILs (Fig. 3A). This resulted from the lack of polymorphic RFLP markers during selection in earlier generations; however, recent progress in the entire sequencing of the rice genome has enabled us to develop a sufficient number of polymorphic SSR markers in all chromosomes between Koshihikari and Akihikari. We used 89 SSR markers that showed polymorphisms between Koshihikari

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Fig. 1. Breeding scheme for development of NILs.

evaluated with three replications per sample. The average stickiness scores were compared with those of Akihikari rice, randomly distributed throughout the sensory tests, by t-test (P < 0.05).
and Akihikari to determine the status of the genotype in the whole genome of the NILs. Five small Koshihikari segments (two on chromosome 5, and one each on chromosomes 4, 6, and 10) were detected in NIL-1 in addition to the target region on chromosome 2. Additional segments were also detected for other NILs: three regions on chromosomes 5, 9, and 11 in NIL-2; five on chromosomes 1, 3, 4, 7, and 11 in NIL-3, one on chromosome 5 in NIL-4, five (two on chromosome 3 and one each on chromosomes 4, 7, and 10) in NIL-5, and five on chromosomes 3, 4, 5, 7, and 11 in NIL-6. Although several non-target segments were substituted into the NILs, their sizes were relatively small, and no segment was shared among all NILs other than the segments on chromosome 2.

**Fig. 2.** Graphical genotypes of the long arm of chromosome 2 of developed NILs and QTL regions for eating quality detected in previous studies. (A) Graphical genotypes of the long arm of chromosome 2 of developed NILs with statistics for the difference in stickiness and amylase content (AC) between NILs and Akihikari. To graphically represent the genotypes of NILs, the recombination point was arbitrarily determined at the mid-point between markers, which showed different genotypes. White and black blocks denote the homozygous allele of Akihikari and Koshihikari, respectively. **, * significant at 1% and 5%, respectively; ns not significant. (B) Putative QTL regions for stickiness and AC previously detected by Tanaka et al. (2006) and putative QTL region for overall evaluation detected by Wada et al. (2007). Arrows on right of QTL regions denote positions of LOD peaks. The relative positions of RFLP and SSR markers used in those QTL analyses are also shown on the left of QTL regions. Physical distance (kbp) of adjacent DNA markers is shown on the left of DNA markers.

**Stickiness, AC and protein content of NILs**

The average stickiness scores and AC values obtained in 2006 and 2007, and protein content obtained in 2007 are shown in Table 2. The stickiness scores of Koshihikari were significantly higher than those of Akihikari in both years. The stickiness scores of NIL-1 and NIL-2 did not differ significantly from those of Akihikari, but those of NIL-3, 4, 5, and 6 were significantly higher than those of Akihikari in both years; however, the stickiness scores of all NILs were significantly lower than those of Koshihikari (*P*<0.01, data not shown). The AC value of Koshihikari was significantly lower than that of Akihikari in both years. The AC values of NIL-1 and NIL-2 did not differ significantly from that of Akihikari, but the AC values of NIL-3, 4, 5, and 6 were significantly lower than those of Akihikari. The AC values of NIL-3 and NIL-5 were not significantly different from
Koshihikari, and those of NIL-4 and NIL-6 were significantly lower than Koshihikari \( (P<0.05, \text{ data not shown}) \). These results clearly suggest that the QTLs for stickiness and AC are attributable to a particular region of chromosome 2. Protein content of the NILs was not significantly different from that of Akihikari.

**Delimitation of the candidate genomic region for the QTL controls stickiness and AC**

From the analysis of substituted segments on chromosome 2, together with the phenotypic values for each NIL, the candidate genomic region was narrowed down for the QTL that controls stickiness and AC. The stickiness scores for NIL-3, 4, 5, and 6 were significantly higher than those of Akihikari, whereas those of NIL-1 and NIL-2 were not significantly different. In NIL-3, 4, 5, and 6, several small Koshihikari segments were detected other than chromosome 2; however, no Koshihikari segment was shared in NIL-3, 4, 5, and 6, except for the target region on chromosome 2. These results suggested that the target QTLs for stickiness and AC were located on a region in chromosome 2 that was common to these four lines.

Additional analysis showed that the introduced segments in the target region in NIL-3 were disrupted by the substitution of an Akihikari segment (from RM3730 to KA43). This gap showed that two small segments of Koshihikari but not a whole region were responsible for the stickiness and AC of NIL-3, 4, 5, and 6 (Fig. 2A) from RM13658 to RM3730 (515 kbp maximum) and from KA43 to RM6933 (773 kbp maximum). These results strongly suggested that the QTLs for stickiness and AC were located in one or both of these small intervals.

**Agronomic characteristics of NILs**

Table 2 shows the major agronomic characteristics of the NILs, Akihikari and Koshihikari. DTHs of all NILs were not significantly different from that of Akihikari, and were about 12 days shorter than that of Koshihikari. The culm lengths of NIL-2 and NIL-6 were significantly shorter than that of Akihikari. The panicle length of NIL-3 was significantly longer than that of Akihikari, and the panicle numbers of NIL-3 and NIL-4 were significantly smaller than that of Akihikari. All other NILs did not differ significantly from Akihikari in these parameters.

**Discussion**

To verify the effect of QTLs on stickiness and AC that had been identified in the previous study (Tanaka et al. 2006, shown in Figure 2B), we developed NILs of Akihikari in which Koshihikari target segments were introduced. Previously, it had not been easy to develop DNA markers
showing polymorphism between two closely related Japanese cultivars, but with the aid of data from International Rice Genome Sequencing Project 2005 (http://rgp.dna.affrc.go.jp/E/IRGSP/index.html), we successfully developed several informative SSR markers and eventually developed six NILs with appropriately introduced Koshihikari segments. Among these NILs, a few lines showed agronomic characteristics that differed significantly from those of Akihikari, such as culm and panicle lengths. This may have resulted from the presence of Koshihikari segments in non-target chromosomal regions (Fig. 3A); however, the DTHs of the NILs were the same as in Akihikari, which is an important trait for possible alterations in AC and the amylopectin structure in response to temperature changes during the ripening period. The QTL regions for stickiness and AC on chromosomes 1, 3, 5, and 6, which were previously detected by Tanaka et al. (2006), as shown in Figure 3B, did not appear to be present in all NILs, except for the target QTL region on chromosome 2 (Fig. 3A); therefore, we decided that these NILs can be used to evaluate eating quality.

Evaluation of the eating quality of cooked rice relies on several characteristics, such as external appearance, taste, aroma, stickiness, hardness, as well as overall evaluation in a standard sensory test (Fukui and Kobayashi 1996). Osato et al. (1998) evaluated the reliability of the sensory test and reported that overall evaluation and stickiness showed significant difference in cultivars but were less significant for external appearance, taste, and hardness, and overall evaluation tended to be influenced by the taste preference of each judge; therefore, stickiness was considered to be the most reproducible and distinct element for assessing eating quality. In addition, we found that the eating quality of Koshihikari is characterized primarily by the high stickiness of this cultivar (Tanaka et al. 2006). On this basis, stickiness was chosen as the primary criterion for eating quality.

On the basis of comparing stickiness and AC values, and analysis of the introduced segments in NILs, the effect of QTL, which controls stickiness and AC, was confirmed; furthermore, the candidate genomic region was narrowed. The candidate genomic regions of the QTL were determined by two intervals: RM13658 to RM3730 (515 kbp maximum) and KA43 to RM6933 (773 kbp maximum). It is most likely that the QTLs for stickiness and AC are located in one or both of these intervals, although it was not clear which segment was responsible for the respective trait, stickiness or AC of Koshihikari. Further analysis would provide the necessary evidence.

Because the genomic sequence of Nipponbare that corresponds to the candidate regions of the QTL (515 and 773 kbp) is available in a database (The Rice Annotation Project Database; Ohyanagi et al. 2006, Tanaka et al. 2007), we were able to look for the predicted genes in the QTL region. Although 40 and 67 gene models could be identified in two candidate genomic regions, respectively, no probable candidate gene for this QTL was found based on the existing annotations (data not shown). Further delimitation of the candidate region will thus be required to find a novel gene for the QTL.

Wada et al. (2007) also detected a QTL for the overall evaluation of cooked rice in sensory tests on chromosome 2, as shown in Figure 2B. This QTL was detected in a region between SSR markers RM3515 and RM5470, and the presence of the Koshihikari allele in this region increased the overall evaluation of eating quality. This QTL region corresponds to the introduced segments in NIL-3, 4, 5, and 6 in the present study; these NILs had significantly higher stickiness than Akihikari. Additional analysis will be needed to clarify whether the two QTLs are the same or have instead resulted from different but tightly linked loci. Mitsueda et al. (2004) also reported a QTL controlling AC on chromosome 2. This QTL does not correspond to the introduced segments in NILs because it was detected on the short arm of chromosome 2.

The fact that the stickiness scores and AC values of the NILs were negatively correlated (Table 2) agrees with previous findings (Juliano 1971, Inatsu 1988, Sato et al. 2002,
Suzuki et al. 2003). Thus, it is possible that the QTL region verified here to control stickiness includes one or more genes related to those encoding AC. Genes that encode for low AC, including $du1$, $du4$, and $lam(t)$ located on chromosomes 10, 12, and 9, respectively (Yano et al. 1988, Kikuchi and Kinoshita 1987), and $dh2$, 3, 5, 6(t) (Yano et al. 1988, Tomita and Nakagahara 1990) have been identified so far. However, no gene related to AC has been identified on chromosome 2, suggesting that the QTL region that controls AC is novel.

AC values of NIL-3, 4, 5, and 6 were almost the same as or lower than that of Koshihikari, whereas their stickiness scores were lower. The decreases in DTH in NILs compared with Koshihikari might be responsible for the difference in stickiness due to the different environmental conditions (mainly temperature) during the ripening period. This should be verified by additional experiments, such as the evaluation of stickiness in NILs grown under different environmental conditions during the ripening period. Alternatively, chromosomal regions in addition to the target region on the long arm of chromosome 2 may affect stickiness. Additional verification will be required by using NILs into which other QTLs have been introduced, such as the QTL for stickiness and AC on chromosome 6, which was detected by Tanaka et al. (2006).

Finally, it is necessary to discuss in which stage the DNA markers linked to the QTL for stickiness and AC can be used in a practical breeding program. Koshihikari and its descendants have often been used to develop cultivars with excellent eating quality in rice breeding programs in Japan. As a result, many cultivars currently being cultivated in Japan already have excellent eating quality, comparable to Koshihikari. Therefore, it is necessary to verify whether the QTL region can be used for selection to increase eating quality in rice breeding of Japanese cultivars by conducting association analysis. On the other hand, in introgression breeding with more distantly related lines (such as indica cultivars or wild relatives), many studies have focused on the introgression of genes related to disease and insect resistance or environmental stress tolerance, while less attention has been paid to eating quality. Several major QTLs associated with eating quality, for example, the $Wx$ gene (Takeuchi et al. 2007, Li et al. 1999) and $alk$ region (Li et al. 2003), have been reported using populations derived from crosses between a Japanese cultivar and a distantly related line or cultivar. However, it seems rather difficult to retain eating quality in introgression breeding. In these cases, information on the QTL for stickiness and AC on chromosome 2 tagged by several DNA markers, if combined with such major QTLs, will be effective to retain good eating quality similar to Koshihikari.

Acknowledgments

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Literature Cited


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