Genetic analysis of the low-amylose characteristics of rice cultivars Oborozuki and Hokkai-PL9

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Control of the amylose content is a major strategy for breeding rice with improved eating quality. To identify the low-amylose gene of the low-amylose rice cultivar Oborozuki, quantitative trait loci (QTL) and sequence analyses were conducted on 90 progeny obtained by crossing the two japonica cultivars Hokkai287 (a donor parent of Oborozuki) and Hokkai-PL9. One QTL for low amylose content was detected on the WX1 locus on the short arm of chromosome 6. This gene was designated Wx1-1 for the Hokkai287 allele, and is a novel allelic gene for the WX1 locus with a 37-bp deletion in intron 10. Homozygote genotype for Wx1-1 decreased the amylose content by 7.8%. Primer sets Wx-U1L3 designed to amplify the genome region containing the deletion clearly differentiated cultivars containing Wx1-1 from others. This DNA marker is useful for breeding cultivars with low amylose contents. Another QTL, qAC9.3, was detected on the short arm of chromosome 9. Hokkai-PL9-homozygote allele for qAC9.3 decreased the amylose content by 2.6%. QTL analyses showed that Wx1-1 and the Hokkai-PL9 allele of qAC9.3 have an additive effect in decreasing the amylose content. The two genes are useful tools in marker-assisted selection when breeding rice with a controlled amylose content for improved eating quality.

Key Words: Oryza sativa L., low amylose content, WX1 gene, eating quality, breeding, QTL analysis, DNA marker.

Introduction

A high eating quality is one of the most important objectives in rice breeding in Japan, where the aim is to produce rice that, when cooked, has a strong sticky texture and remains soft. The eating quality of cooked rice declines as the amylose content (AC) of the endosperm increases (Inatsu 1988, Kunihiro 1989). By selection for AC, the eating quality of rice cultivars has been improved in Japan, especially in Hokkaido. The development of mutants with a low AC has led to the breeding of practical cultivars, such as Aya (Kunihiro et al. 2003), Milky Queen (Ise et al. 2001), and Oborozuki (Ando et al. 2007), that produce rice that is sticky when cooked. Aya was the first low-AC cultivar to be recommended in Japan. It is derived from NM391, a mutant of Nihonmasari, and has an AC of about 12% when grown in Hokkaido. An improved cultivar Ayahime, derived from Aya, has been bred and released in Hokkaido (Kiuchi et al. 2009). Milky Queen, derived from a mutagenized Koshihikari, is widely cultivated in regions from Tohoku to Kyushu and shows a stable AC of about 10%. The cooked rice of Milky Queen rice is stickier and softer than that of Koshihikari. Oborozuki, a recommended cultivar for Hokkaido, was bred from Hokkai287, a low-amylose mutant of Kirara397. Its AC is about 14% in Hokkaido; this is about 6% lower than that of Hoshinoyume, one of the major recommended cultivars for Hokkaido with a good eating quality, but about 3% higher than that of Ayahime. The stickiness and softness of Oborozuki cooked rice are correspondingly intermediate between those of Hoshinoyume and Ayahime (Ando et al. 2007). Oborozuki is receiving a favorable reception in the market because of its excellent eating quality resulting from its moderately low-AC in Hokkaido; it was the most widely cultivated low-AC cultivars in Japan in 2009. Hokkai-PL9, which is a parental line for cold tolerance (Kuroki et al. 2007), has an AC that is about 3% lower...
than that of Hoshinoyume and about 2% more than that of Oborozuki (unpublished data).

Low ACs in cultivars are controlled by the WX and DU genes, some of which have been molecularly characterized. Aya has a single recessive gene [du(t)], which is independent of the WX1 locus (Kunihiro et al. 1993), but the position of the gene in the genome is unknown. The low-AC cultivars Norin-PL13 and Norin-PL14 contain du1 and du2 genes, respectively (Okuno et al. 1993). Sato et al. (2002) characterized Wx-mq on the WX1 locus as producing a low AC in Milky Queen. Chuba et al. (2006) identified Wx-y on the WX1 locus in Satonoyuki (Yukinomai), which has an AC of about 12%. Genetically analyzed low-amylose genes had not been characterized, we attempted to locate these genes for MAS.

Materials and Methods

Plant materials

A population of 90 progeny was developed from a cross between Hokkai287 and Hokkai-PL9. Hokkai287 is a low-amylose line selected from somaclonal mutants (Araki et al. 1996) and is the donor parent responsible for the low-amylose characteristics of Oborozuki (Ando et al. 2007). Hokkai-PL9 is a cold-tolerant breeding line (Kuroki et al. 2001). A population of 90 progeny was developed from a cross of Hokkai287 with the glutinous rice cultivar Hakuchomochi was planted, and F1 seeds were harvested in the growth chamber and matured at 23°C (daytime) and 18°C (nighttime) in 2009. Brown rice seeds were divided into normal nonglutinous, dull+ (light), dull++ (heavy), and glutinous grains by the naked eyes. Cross-sections of the kernel were stained with 0.02%/0.35%KI iodine solution, and were divided into glutinous and nonglutinous grains. To estimate the amount of WX1 protein in rice grain of Hokkai287, Kirara397, and Oborozuki, seeds were grown in the field, National Institute of Crop Science (Tsukubamirai, Ibaraki, Japan); both parent cultivars and Oborozuki were also grown for comparison. In 2004, the F3 lines were seeded on April 30th and transplanted on May 30th and transplanted on May 27th. Seeds were harvested at maturation for measurements of their AC. For genetic analysis of the low-amylose characteristics of Hokkai287, an F1 plant derived from a cross of Hokkai287 with the glutinous rice cultivar Hakuchomochi was planted, and F2 seeds were harvested in the growth chamber and matured at 23°C (daytime) and 18°C (nighttime) in 2009. Brown rice seeds were divided into normal nonglutinous, dull+ (light), dull++ (heavy), and glutinous grains by the naked eyes. Cross-sections of the kernel were stained with 0.02%/0.35%KI iodine solution, and were divided into glutinous and nonglutinous grains. To estimate the amount of WX1 protein in rice grain of Hokkai287, Kirara397, and Oborozuki, seeds were grown in a greenhouse and matured at approximately 30°C (daytime) and 20°C (nighttime) in 2006.

DNA extraction and PCR amplification

Genomic DNA from the F3 lines was extracted from the leaves of the plants by using the CTAB method (Murray and Thompson 1980). The genotypes of the F1 lines were determined for 168 simple sequence repeat (SSR) markers covering the 12 chromosomes (Temnykh et al. 2001, McCouch et al. 2002, IRGSP 2005, Kuroki et al. 2007) and a primer pair Wx-U1L3 designed from the sequence data for WX1 loci, as described below. The reaction mixture (6 μL total volume) consisted of 1 μL of template DNA, 0.7 μL of 10× PCR buffer (Promega, Madison, WI, USA), 0.4 μL of 25 mM MgCl2, 0.7 μL of a solution containing 2 mM of each deoxyribonucleotide triphosphate (dNTP; Boehringer Mannheim, Mannheim, Germany), 0.1 μL 5 U Taq DNA polymerase (Promega), 0.3 μL of a 20 pM solution of each primer, and 2.8 μL H2O. Amplification was performed for 30 cycles of 94°C (1 min), 55°C (2 min), and 72°C (3 min), followed by a final hold at 72°C for 7 min. The amplified DNA product was separated by electrophoresis on 3.5% agarose gel.

Analysis of amylose contents

To analyze its AC, polished rice was crushed with a TM5 mill (Satake Co. Ltd., Tokyo, Japan). The resulting rice flour was diluted with 0.5 N aqueous NaOH and left overnight at room temperature. The solution was diluted to 0.05 N with H2O, and AC was determined by using an Auto Analyzer II (Bran+Luebbe Co. Ltd., Norderstedt, Germany). The AC of each line was determined by using three different samples of rice from the individual line. The average AC value was used for the analysis of quantitative trait loci (QTL).

Sequence analysis and WX1 protein determination

WX1 genes from the genomic DNA of Hokkai287 and Kirara397 were amplified by the polymerase chain reaction (PCR) using two primer pairs, B-E and N-Y (Inukai et al. 2000), and additional primer pairs E14UL (Table 1). PCR products were purified by using exonuclease I and alkaline phosphatase (shrimp) (Takara Bio Inc., Shiga, Japan). DNA sequences were determined by direct sequencing using an Applied Biosystems 3730xl DNA Analyzer (Life Technologies Corporation, NY, USA). The sequencing output was

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primera</th>
<th>Strand</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For sequencing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>CAACCACCATGTCGGCTCTCACCA</td>
</tr>
<tr>
<td>E</td>
<td>−</td>
<td>ACCCTGAACACACACCGATCA</td>
</tr>
<tr>
<td>N</td>
<td>+</td>
<td>TCAAGGCATGGGAGAGAAGTA</td>
</tr>
<tr>
<td>Y</td>
<td>−</td>
<td>AGACATTTCCGGTTCTCCGCA</td>
</tr>
<tr>
<td>E14U</td>
<td>+</td>
<td>GAAAGGAGGGAGTAAAAAC</td>
</tr>
<tr>
<td>E14L</td>
<td>−</td>
<td>AACCAGATACATAACTAAAA</td>
</tr>
<tr>
<td>For detecting the deletion in WX1 locus of Hokkai287</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx-U1</td>
<td>+</td>
<td>CAGGCCTGGAGGACAGAGG</td>
</tr>
<tr>
<td>Wx-L3</td>
<td>−</td>
<td>TCACCTTGCCCCGATCTTC</td>
</tr>
</tbody>
</table>
Genetic analysis of low-amylose characteristics in rice cultivars

Seeds from the materials planted in a greenhouse in 2006 were harvested at 10 and 13 days after pollination, the time when expression of the \( W_x \) gene was observed by Hirano and Sano (1991). The amount of \( WX_1 \) protein in materials was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting with antiserum against \( WX_1 \) protein (Suzuki et al. 2002).

Construction of a linkage map and QTL analysis

In total, 2011 SSR markers covering all 12 chromosomes were tested for parental polymorphisms in the analysis of the population. Linkage maps were constructed from the genotype data of polymorphic 168 SSR and \( W_x \)-U1L3 markers by using MAPMAKER/EXP 3.0 (Lander et al. 1987). Putative QTLs were detected by using the composite interval mapping (CIM) function of QTL Cartographer 2.0 (Wang et al. 2001–2003). The CIM threshold was determined by means of 1000 permutation tests at the 5% level of significance.

Results

Frequency distribution of the amylose content of the progeny

Figure 1 shows the frequency distributions of AC for progeny derived from the cross between Hokkai287 and Hokkai-PL9 at \( F_6 \) in 2004 and at \( F_7 \) in 2005. The values of the AC for Hokkai287 and Hokkai-PL9 were 9.3% and 16.8%, respectively, in 2004, and 7.7% and 15.3%, respectively, in 2005. The progeny showed a binomial distribution of AC, suggesting that a single major gene controls low AC in Hokkai287. Minor genes may also make some contribution, because each peak showed a wider distribution than the variance for each parent.
QTL and sequence analysis

By means of QTL analysis using genotype data for 168 SSR markers, one QTL for AC (qAC6) was detected near RM8119 on the short arm of chromosome 6, and another (qAC9) was detected near RM23804 on the short arm of chromosome 9. As there are strong suggestions that qAC6 is located in the WX1 locus, where several low-amylose genes have been reported (Mikami et al. 1999, Sato et al. 2002, Chuba et al. 2006), we determined the nucleotide sequences of the coding regions in the WX1 locus of Hokkai287 and Kirara397 (Fig. 2). The sequence of the WX1 gene of Kirara397 was identical with that of a previously reported rice WX1 gene (Hirano and Sano 1991). However, sequencing for Hokkai287 revealed that the WX1 locus of Hokkai287 has a 37-bp deletion in intron 10. Primer sets to amplify the sequence that includes the deletion were designed by using the online primer design tool Primer 3 (Rozen and Skaletsky 2000; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). The DNA marker Wx-U1L3 was capable of clearly detecting the deletion in the WX1 locus of Hokkai287 (Fig. 3).

The map positions for qAC6 and qAC9, determined by using the 168 SSR and Wx-U1L3 markers, are shown in Figure 4. The presence of qAC6 explained 59.1% of the total phenotypic variation in 2004 and 61.3% in 2005. The Hokkai287 allele decreased the AC at qAC6. The presence of qAC9 explained 9.4% of the total phenotypic variation in 2004 and 7.3% in 2005. The Hokkai-PL9 allele decreased the AC at qAC9 (Table 2). The ACs of progeny with each genotype for qAC6 and qAC9 are shown in Figure 1. In 2004 and 2005, Hokkai287-homozygote genotype for qAC6 with Hokkai-PL9-homozygote genotype for qAC9 showed ACs that were lower by 9.7 and 9.9%, respectively, compared with Hokkai-PL9-homozygote genotype for qAC6 with Hokkai287-homozygote genotype for qAC9. All the progeny with the Hokkai287-homozygote genotype for qAC6 (Wx-U1L3) showed a dull endosperm similar to that of Hokkai287, whereas those with the Hokkai287-heterozygote genotype showed segregation of dull and normal nonglutinous endosperm. All progeny with the Hokkai-PL9-homozygote genotype for qAC6 showed normal nonglutinous endosperm. Wx-U1L3 also clearly differentiated breeding lines with low-AC dull endosperm from others with normal nonglutinous endosperm among the lines derived from other crossings of Hokkai287 (Fig. 5A). The marker was capable of differentiating Oborozuki from other cultivars with
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Inheritance of the low amylose content characteristic

Inheritance of the low AC characteristic was analyzed by segregation of the phenotype of F2 seeds derived from a crossing between Hokkaidi287 and the glutinous rice cultivar Hakuchomochi (Table 3). F2 seeds showed light to heavy dull endosperm and glutinous endosperm. Seeds with dull endosperm were nonglutinous, whereas seeds with normal nonglutinous endosperm were not segregated. All of the seeds with a heavy dull endosperm had a heterozygote genotype for Wx-U1L3. These result demonstrate that the heterozygote has a lower AC than that of Hokkai287-homozygote for qAC6. A ratio of total dull endosperm to glutinous endosperm fitted a 3 : 1 ratio. We have therefore shown that the low-AC characteristic of Hokkai 287 is controlled by a dominant gene for the glutinous characteristic at the WX1 locus.

Immunoblotting analyses of WX1 protein

Immunoblotting analyses using antiserum against WX1 protein showed that the amounts of WX1 protein for Hokkai287 and Oborozuki with qAC6 were lower than that for Kirara397 at both 10 and 13 days after pollination (Fig. 6).

Discussion

By means of analysis using progeny derived from a cross between Hokkai287 and Hokkai-PL9, we identified qAC6 on chromosome 6 and qAC9 on chromosome 9 as producing a low AC. Of these, qAC6 corresponded to the WX1 locus and was dominant to the wx gene. The determination of the sequence of the WX1 gene of Hokkai287 showed the presence of a 37-bp deletion in intron 10. In previous studies for genes on the WX1 locus that control low amylose characteristics, Mikami et al. (1999) reported the existence of Wx-op, which has Wx-a-type nucleotide sequences around the 5′ splice junction of the first intron. Wx-mq in Milky Queen has two base changes in exons 4 and 5 (Sato et al. 2002). Wx-y in Satonoyuki (Yukinomai) has one base change in exon 4. The
The demonstrated that it is possible that a deletion in intron 10 of the gene (Cambien et al. 1992). The present study has shown that the major effect of human ACE gene, which encodes angiotensin-converting enzyme, is associated with an insertion/deletion situated in intron 16 during the maturing period. Sato et al. (2002) reported that the total amount of WX1 protein of Milky Queen with the Wx-b gene results from a single nucleotide substitution at the 5' splice site of the first intron (Hirano et al. 1998). It has been shown that the expression of the Wx-b gene in milky Queen with Wx-mq was not significantly different from that of wild-type Koshihikari with the Wx-b gene. This suggests that the mechanism responsible for the decrease in the AC in Wx1-I is different from that in Wx-mq. As described above, mis-sense base changes in exons were not detected in Wx1-I, although Wx1-I has a deletion in intron 10. The low level of expression of the Wx-b gene results from a single nucleotide substitution at the 5' splice site of the first intron (Hirano et al. 1998). It has been shown that the major effect of human ACE gene, which encodes angiotensin-converting enzyme, is associated with an insertion/deletion situated in intron 16 of the gene (Cambien et al. 1992). The present study has demonstrated that it is possible that a deletion in intron 10 of the Wx1-I gene is associated with the expression of the WX1 protein.

On the other hand, we have detected the presence of qAC9 on chromosome 9 which decreases the AC in the Hokkai-PL9 allele. Wan et al. (2004) also detected two QTLs for AC on chromosome 9: qAC-9-1(t) and qAC-9-2(t). The map position of qAC9 is clearly different from that of qAC-9-2(t). Wada et al. (2006) also detected a QTL that decreases the AC near the SSR marker RM4413 on chromosome 9 in the Koshihikari allele. We are now developing near-isogenic lines containing the Hokkai-PL9 allele of qAC9 with a Koshihikari genetic background to compare the function of the Hokkai-PL9 allele and the Koshihikari allele. Although the allelism of qAC9 and other QTLs previously detected on chromosome 9 is not fully clear, we have assigned the designation qAC9.3 to the qAC9 QTL.

Recently, MAS has been used to develop practical rice cultivars in Japan (Sugiura et al. 2004, Takeuchi et al. 2006, Fukuoka et al. 2009), but few cultivars bred using MAS for good eating quality have been reported so far. Among the genes that control the eating quality of rice, genes for low AC are powerful tools for MAS, because selection by measuring the AC requires considerable labor in rice breeding. Oborozuki has a relatively high AC among the low-amylose-cultivars, so that it is often difficult to differentiate the appearance of its grains from those of cultivars of normal nonglutinous rice in Hokkaido. Thus MAS for Wx1-I would be quite effective for breeding cultivars of Oborozuki-type rice which is receiving a favorable reception in the market. The DNA marker Wx-U1L3, designed on the basis of the characteristic deletion in Wx1-I, clearly differentiate cultivars with Wx1-I from those with other genes on the WX1 locus. Note that the Hokkai-PL9 allele of qAC9.3, which produces a 2.6% decrease in the AC could be useful gene for fine tuning of the AC by MAS. It should contribute to the breeding of Koshihikari-type cultivars with about 16–18% AC without a dull endosperm in Hokkaido. The Wx1-I and Hokkai-PL9 allele of qAC9.3 showed an additive effect in decreasing the AC. This suggests that we could select various phenotypes for AC by MAS by using the two loci in different climatic conditions.

The sensory test is currently the most effective and practical method in rice breeding for evaluating the overall eating quality, but it can only be used with breeding materials at

Table 2. Putative QTLs controlling the amylose content

<table>
<thead>
<tr>
<th>QTL</th>
<th>Year</th>
<th>Nearest marker</th>
<th>Chr.</th>
<th>a</th>
<th>r</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>qAC6</td>
<td>2004</td>
<td>Wx-U1L3</td>
<td>6</td>
<td>1.6</td>
<td>59.1</td>
<td>31.3</td>
</tr>
<tr>
<td>qAC9</td>
<td>2004</td>
<td>RM23804</td>
<td>9</td>
<td>−0.6</td>
<td>9.4</td>
<td>14.7</td>
</tr>
<tr>
<td>qAC6</td>
<td>2005</td>
<td>Wx-U1L3</td>
<td>6</td>
<td>1.7</td>
<td>61.3</td>
<td>31.5</td>
</tr>
<tr>
<td>qAC9</td>
<td>2005</td>
<td>RM23804</td>
<td>9</td>
<td>−0.6</td>
<td>7.3</td>
<td>12.4</td>
</tr>
</tbody>
</table>

a Additive effect of the Hokkai-PL9 allele.
b Percentage of total phenotypic variance explained by the QTL.

WX1 gene of Hokkai287 detected in our studies did not correspond with any of the genes previously identified. We have therefore designated the novel low-amylose gene for WX1 locus in Hokkai287 (Accession No. AB535524) as gene WX1-1.

The results of immunoblotting analyses suggested that low ACs in Hokkai287 and Oborozuki with WX1-I are induced by the small amount of WX1 protein that is present during the maturing period. Sato et al. (2002) reported that the total amount of WX1 protein of Milky Queen with WX1-mq was not significantly different from that of wild-type Koshihikari with the Wx-b gene. This suggests that the mechanism responsible for the decrease in the AC in WX1-I is different from that in WX-mq. As described above, mis-sense base changes in exons were not detected in WX1-I, although WX1-I has a deletion in intron 10. The low level of expression of the WX-b gene results from a single nucleotide substitution at the 5' splice site of the first intron (Hirano et al. 1998). It has been shown that the major effect of human ACE gene, which encodes angiotensin-converting enzyme, is associated with an insertion/deletion situated in intron 16 of the gene (Cambien et al. 1992). The present study has demonstrated that it is possible that a deletion in intron 10 of the WX1-I gene is associated with the expression of the WX1 protein.

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The sensory test is currently the most effective and practical method in rice breeding for evaluating the overall eating quality, but it can only be used with breeding materials at

Table 3. Brown rice phenotype of F2 seeds from the cross between Hakuchomechi and Hokkai287

<table>
<thead>
<tr>
<th>Cross and Parenta</th>
<th>Genotype of F2 seeds for Wx-U1L3b</th>
<th>Brown rice phenotype of F2 seeds or self-seeds of parentsc</th>
<th>ratio</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>normal</td>
<td>dull-</td>
<td>dull++</td>
<td>nonglutinous to glutinous</td>
</tr>
<tr>
<td>Hakuchomechi</td>
<td>H287/H287</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>× H287/Haku</td>
<td></td>
<td>0</td>
<td>62</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>Hokkai287</td>
<td>Haku/Haku</td>
<td>0</td>
<td>0</td>
<td>44</td>
<td>1 : 2 : 1</td>
</tr>
</tbody>
</table>

a Number of parent phenotype shows the average of 3 plants.
b 'H287/Haku' means the Hakukai287/Hakuchomechi heterozygote genotype for Wx-U1L3.
c Brown rice phenotype were divided into normal nonglutinous, dull+ (light), dull++ (heavy) and glutinous grain.
advanced generations because the sensory tests always require large amounts of grains, time, and labor. QTLs for sensory evaluation of good eating quality of Koshihikari (Takeuchi et al. 2007, Takeuchi et al. 2008, Wada et al. 2008) and Sakihikari (Kobayashi and Tomita 2008) have been reported. Among these, a QTL on the short arm of chromosome 3 was detected consistently. The QTL has a major effect on sensory evaluation but is not related to the AC. Pyramiding the QTL with Wx1-1 or qAC9.3 is a practical strategy for breeding rice cultivars with excellent eating quality by means of MAS.

Acknowledgment

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