Molecular Characterization of Wx-mq, a Novel Mutant Gene for Low-amylose Content in Endosperm of Rice (Oryza sativa L.)

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In this paper, we characterized the Wx-mq gene for low amylose content in a rice variety, Milky Queen, at the molecular level. The Wx-mq gene was cloned by RT-PCR, and a nearly full-length cDNA sequence of the gene was determined. Sequence comparison between the Wx-mq gene and the wild type allele (Wx-b), cloned from cv. Koshihikari, revealed that two base changes existed within the coding region; a G to A base change at nucleotide position 497 and a T to C base change at nucleotide position 595. Each nucleotide substitution should generate a missense base change (an Arg-158 to His-158 change in exon4, and a Tyr-191 to His-191 change in exon5). However, it is not known which missense mutation is essential for the activity of the WX protein. To identify rice varieties and lines, which harbored the Wx-mq gene, PCR primers were designed at the gene level. These primers were able to amplify the Wx-mq specific 741 bp band in Milky Queen, and in other rice variety and lines, Milky Princess, Joiku 436 and Etsunan 190, all of which have the same pedigree as that of Milky Queen. On the other hand, no 741 bp band was amplified with the primers in Koshihikari which harbored the wild type allele (Wx-b), and the other low-amylose content variety and line, Snow Pearl and NM391, which do not have the pedigree. Thus, it is possible to detect the Wx-mq gene by PCR.

Key Words: Oryza sativa L., low-amylose content, mutant, Wx-mq.

Introduction

Starch in the rice endosperm contains two types of polysaccharides: amylose and amylopectin. The amylose in grain is synthesized by granule-bound starch synthase, a product of waxy (wx) locus (Nelson and Rines 1962). In nonglutinous rice, two wild type alleles of the wx locus, Wx-a and Wx-b, have been recognized and the expression of the Wx-a gene was 10-fold higher than that of the Wx-b gene at the protein level (Sano 1984). The Wx-a gene is characteristic of indica rice and the Wx-b gene is found mainly in japonica rice (Sano 1984). Judging from the amylose content of the isogenic lines, Kikuchi (1988) suggested that Koshihikari, the most popular japonica rice variety in Japan, also harbours the Wx-b gene. Recently, sequence analysis of the wx locus has revealed that 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 (Cai et al. 1998, Hirano et al. 1998, Ishii et al. 1998, Wang et al. 1995).

Since the amylose content of endosperm starch is negatively correlated with the score of eating quality in Japanese rice varieties (Inatsu 1988, Kunihiro 1989), rice breeders in Japan have focused on the breeding of varieties with a low-amylose content of about 1 to 15% in the last decade. This led to the development of varieties such as Rice Norin-PL13, Aya, Hanabusa, Snow Pearl, Soft 158 and Milky Queen, etc. (Kunihiro et al. 1993, Okuno et al. 1993, Sato et al. 2001). Milky Queen is a N-methyl-N-nitrosourea (MNU)-induced mutant of the variety, Koshihikari (Suto et al. 1996), and the genetic analysis of this mutant showed that the low-amylose content in the endosperm was controlled by a novel mutant gene at the wx locus (Sato et al. 2001). In this report, we have assigned the symbol Wx-mq to the mutant gene, and we report the nucleotide sequence of the Wx-mq gene and a simple detection method of a structural change in the gene by PCR.

Materials and Methods

Plant materials

For RNA extraction, Milky Queen and its wild type Koshihikari were grown at Yawara Experimental Low-land Station, National Agricultural Research Center, Japan, in 1998. Panicles at 14 days after heading were harvested and stored at −80°C until use.

Four varieties and three lines were used for DNA extraction and subjected to PCR (Table 1). Milky Princess, Joiku 436 and Etsunan 190 have the same pedigree as Milky Queen. Joiku 436 is also the progeny strain of a low-amylose content line NM391. NM391 is a useful gene donor for the two varieties with a low-amylose content mentioned above, that is, Aya and Hanabusa. Snow Pearl is a progeny of the
mutant with a low-amylose content induced from Norin 8. Seedlings were hydroponically cultured with tap water for 14 days in a growth chamber with continuous illumination by fluorescent white light at 28°C. Shoots were harvested and stored at −80°C until use.

**RNA extraction and RT-PCR**

Total RNA was extracted from the panicles using the RNeasy plant mini-kit (Qiagen). Five hundred ng of RNA was subjected to RT-PCR (High-fidelity RNA PCR kit, Takara). Two oligonucleotide primers C1 and C2 (Table 2) were designed from the sequence of rice Wx cDNA (Okagaki 1992) and used for RT-PCR with the following cycling conditions: 30 cycles of 0.5 min at 94°C, 0.5 min at 60°C and 3 min at 72°C.

**Cloning and sequencing**

Approximately 1.9 kb cDNA products from Milky Queen and Koshihikari were cloned into the pT7 Blue T-Vector (Novagen). The nucleotide sequence of the cDNAs was determined by the dye terminator method (Applied Biosystems) with 8 sequence primers designed from the previously determined wx cDNA sequence (Okagaki 1992).

**DNA extraction and PCR**

Total DNA was extracted from the shoots by the CTAB method (Murray and Thompson 1980). Four primers (W1, W2, W3 and W4) were designed from the sequences of rice Wx genomic DNA (Frances et al. 1998) and Wx-mq cDNA (Table 1 and Fig. 1). PCR amplification with 2.5 U Ampli-Taq (Perkin Elmer) was conducted in 50 µl of the manufacturer’s reaction buffer containing 0.2 mM dNTP, 50 ng DNA and 20 pmol of each primer. Cycling conditions were 28 cycles of 1 min at 94°C, 1 min at 69°C and 2 min at 72°C followed by 4 min at 72°C for final extension.

**Results**

**Sequencing analysis of Wx-mq gene**

Comparison of cDNA and amino acid sequences between Milky Queen and the wild type, Koshihikari is shown in Fig. 1. The deduced amino acid sequence in Koshihikari was identical with that of the previously reported rice gene Wx (Okagaki 1992). However, sequencing of the Wx-mq gene from Milky Queen revealed that two base changes existed within the coding region, that is, a G to A base change at nucleotide position 497 and a T to C base change at nucleotide position 595 (Fig. 1). Each nucleotide substitution should generate a missense base change: a CGT (Arg) to CAT (His) at codon 158 in exon4, and a TAC (Tyr) to CAG (His) at codon 191 in exon5 (Fig. 1). Thus, the Wx-mq gene was characterized at the molecular level. Since the level of expression of the WX protein in Milky Queen was almost the same as that in the wild type (Sato et al. 2001), it is reasonable to assume that both or either of the missense mutations led to the decrease of the amylose content in the endosperm.

**Table 1.** List of varieties and lines used for varietal identification by PCR

<table>
<thead>
<tr>
<th>Varieties and lines</th>
<th>Amylose content</th>
<th>Cross combination or origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koshihikari</td>
<td>Medium(^1)</td>
<td>Norin 22/Norin 1</td>
</tr>
<tr>
<td>Snow Pearl</td>
<td>Low(^2)</td>
<td>74wx2N1/Reimei</td>
</tr>
<tr>
<td>NM391 (Pedigree of Milky Queen)</td>
<td>Low(^2)</td>
<td>Mutant induced from Nihonmasari</td>
</tr>
<tr>
<td>Milky Queen</td>
<td>Low(^2)</td>
<td>Mutant induced from Koshihikari</td>
</tr>
<tr>
<td>Milky Princess</td>
<td>Low(^2)</td>
<td>Kanto 163/Ko 272(^3)</td>
</tr>
<tr>
<td>Joiku 436</td>
<td>Low(^2)</td>
<td>Kanto 168 (Milky Queen)/2*AC90306(^4)</td>
</tr>
<tr>
<td>Etsun 190</td>
<td>Low(^2)</td>
<td>Etsun 148/Kanto 168 (Milky Queen)</td>
</tr>
</tbody>
</table>

\(^1\) Medium: amylose content of 15 to 20%
\(^2\) Low: amylose content of 1 to 15%
\(^3\) Ko 272: Sibling of Milky Queen
\(^4\) AC90300: Progeny strain of NM391

**Table 2.** Primers used for this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Position</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>5’-CACAGCAAACAGCTAGACAACCACC-3’</td>
<td>429-452(^2)</td>
<td>Forward</td>
</tr>
<tr>
<td>C2</td>
<td>5’-CCACTGGTTCTCATTGCTTCTCCATCC-3’</td>
<td>2355-2331(^2)</td>
<td>Reverse</td>
</tr>
<tr>
<td>W1</td>
<td>5’-GGTGAGGTTTTTCCATTTGCTACAGCA-3’</td>
<td>471-497(^3)</td>
<td>Forward</td>
</tr>
<tr>
<td>W2</td>
<td>5’-GCCCCGTGGAGAGATGTGTGGAT-3’</td>
<td>2710-2687(^2)</td>
<td>Reverse</td>
</tr>
<tr>
<td>W3</td>
<td>5’-CAAGTACGACGCAACCACCGTGA-3’</td>
<td>2279-2302(^2)</td>
<td>Forward</td>
</tr>
<tr>
<td>W4</td>
<td>5’-GGTGGACTAGAGAGATCTGGGTCCAA-3’</td>
<td>2747-2723(^2)</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

\(^1,2,3\) Nucleotide numbers correspond to those of Okagaki (1992), Frances et al. (1998) and Wx-mq (DDBJ Acc. No. AB066093), respectively.
Simple detection of the Wx-mq gene by PCR

In order to detect the Wx-mq gene simply and rapidly, we have tried to develop a PCR assay. Four PCR primers, W1, W2, W3 and W4, were designed at the gene level (Table 2). As the 3′-end of the W1 primer was designed at one of the base substitution points (nucleotide 497) (Fig. 1), the W1 and W2 primers were able to amplify a 741 bp band of Wx-mq genomic DNA in Milky Queen (Fig. 2A). However, in Koshihikari, no band was amplified with these primers, because the nucleotide at position 497 was G (Fig. 2A). On the other hand, control primers (W3 and W4) could amplify a 469 bp band in both varieties (Fig. 2A). Thus, it was demonstrated that the W1 and W2 primers were able to amplify the Wx-mq specific band.

Finally, we attempted to apply the PCR assay to the identification of the varieties and lines, which harbored the Wx-mq gene (Fig. 2B). In this experiment, we used the four primers at a time. In Milky Queen, Milky Princess, Joiku 436 and Etsunan 190, all of which have the same pedigree, two major bands including a 741 bp Wx-mq specific band appeared. On the other hand, only one control band was amplified in Koshihikari and in the other low-amylose variety and line, that is, Snow Pearl and NM391. Therefore, we were able to identify Milky Queen and its pedigree at the molecular level.

**Discussion**

It is well known that ‘the dull endosperm mutation’ leads to a reduction of the amylose content in the rice endosperm. Okuno et al. (1983) firstly characterized a low-amylose content mutant with dull endosperm, of which grain appearance could be readily distinguished from that of the waxy and nonglutinous endosperms. This mutant harbored the du gene, which was nonallelic to the wx locus (Okuno et al. 1983). Up to now, at least six du genes (du1, 2, 3, 4, 5 and 6(t)) have been identified in rice (Yano et al. 1988, Tomita and Nakagahara 1990). Recently, it has been suggested that the du1 and du2 genes encode the tissue-specific splicing factors affecting the splicing of Wx-b transcripts (Isshiki et al. 2000). Low-amylose content lines bearing the du1 and du2 genes have been used for rice breeding as Norin-PL 13 and Norin-PL 14, respectively (Okuno et al. 1993). In 1991, the first variety with a low-amylose content, Aya, was released (Kunihiro et al. 1993). Gene donor of the variety, NM391 is likely to carry a single recessive du(t) gene, because the mode of inheritance of the low-amylose content is independent of the wx gene (Kikuchi 1988, Kunihiro et al. 1993). However, allelism test between the du(t) and the known six du genes has not been reported yet. Nevertheless, the du genes have contributed to the progress of the rice breeding program for low-amylose content in Japan.
the gene from Milky Queen, and then we compared the nucleotide sequence of $Wx$-$mq$ cDNA with that of $Wx$-$b$ cDNA from the wild type, Koshihikari. The sequence data revealed that two missense mutations occurred in a $Wx$-$mq$ coding region, that is, an Arg-158 to His-158 in exon4 and a Tyr-191 to His-191 in exon5 (Fig. 1). Both amino acid residues were conserved among the six waxy protein sequences derived from wheat, barley, maize, potato, pea and rice (Ainsworth et al. 1993). Inukai et al. (2000) characterized the M8F (74-8) mutant, which is one of the four $wx$ leaky mutants screened by Amano (1985), at the molecular level and one amino acid substitution (Ile-182 to Asn-182 change) was also found in exon 5. This residue is conserved between the five plant species except for pea (Ainsworth et al. 1993).

We reported that the expression level of the Wx protein in Milky Queen was almost the same as that in Koshihikari (Sato et al. 2001). Therefore, it is reasonable to assume that the Arg-158 and/or Tyr-191 residues were critical for the activity of the Wx protein. However, in the current study, it was not possible to identify accurately which amino acid residue was essential for the activity. Site-directed mutagenesis will be employed and these residues will be substituted for other amino acids to elucidate this aspect.

In potato, cyst nematode resistance was simply identified by the PCR marker designed at the point of nucleotide base change (Tanaka 2001). Also in rice, nitrate reductase (NR)-deficiency mutation was detected by the PCR primer designed at the nucleotide deletion point in the NR gene (Sato et al. 1997). On the other hand, the PCR-RFLP method was useful for the detection of the single-nucleotide polymorphism between $Wx$-$a$ and $Wx$-$b$ (Ayres et al. 1997). In the present study, we attempted to detect the mutation located at the $Wx$-$mq$ gene by the PCR assay (Fig. 2A). Both the W1 primer designed at one of the mutation points, and its reverse primer W2 were able to amplify the $Wx$-$mq$ specific 741 bp band. However, the design of the W1 primer was based only on one nucleotide substitution particularly at nucleotide 497, and in rare cases, an ‘artifact’ band was slightly amplified in Koshihikari (data not shown). Therefore, the control primers (W3 and W4) were employed and the density of the 469 bp band was compared to that of the 741 bp band. In this method, two strong bands appeared in Milky Queen, while only one in Koshihikari (Fig. 2A).

Finally, we attempted to apply the PCR assay to the identification of the varieties and lines, which harbored the $Wx$-$mq$ gene (Fig. 2B). In all of the varieties and lines showing the same pedigree as that of Milky Queen, amplification of the 741 bp band was revealed. On the other hand, the other low-amylose content variety and line, Snow Pearl and NM391, lacked band amplification. Thus, it was possible to identify the rice varieties and lines, which harbored the $Wx$-$mq$ gene, at the molecular level. Because Joiku 436 is also the progeny strain of NM391 (Table 1), it is reasonable to assume that the low-amylose content line may harbor the $du(t)$ gene in addition to the $Wx$-$mq$ gene. In further studies, this assumption will have to be verified.

On the other hand, some low-amylose content mutations at the $wx$ gene were detected in both the spontaneous and the induced mutants. The former is an opaque endosperm mutation identified in cultivars from Nepal, India, etc. (Mikami et al. 1999, Heu and Kim 1989). The characteristics of the opaque endosperm are that amylose is produced in the endosperm (about 10%) in spite of the waxy-like appearance of the endosperm and that the trait is controlled by $Wx$-$op$, an allele of the $wx$ gene (Mikami et al. 1999, Heu and Kim 1989). The latter mutation was reported by Amano (1985), that is, the four leaky mutants among the twelve induced $wx$ mutants showed an intermediate amylose content between that of waxy and nonglutinous rice. Thus, it is possible to utilize the $wx$ mutations for rice breeding of commercial varieties with a low-amylose content, in Japan.

A rice mutant variety, Milky Queen has a dull endosperm in which the amylose content was about 10%, corresponding to two-thirds of the content in the wild type endosperm (Suto et al. 1996). Allelism test showed that the low-amylose content in Milky Queen was not controlled by $du$ genes, but by a single gene at the $wx$ locus (Sato et al. 2001). When Milky Queen was crossed with waxy rice, the low-amylose content was inherited as a dominant character (Sato et al. 2001). Here, we assigned the symbol $Wx$-$mq$ to
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Literature cited


