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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (15): 2500-2504, 2007
ISSN 1028-8880

Identification of Variant Transcripts of *Waxy* Gene in Non-glutinous Rice (*O. sativa* L.) With Different Amylose Content

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Abstract: The identification of a number of cDNA sequences at the splice sites of *Waxy* (*Wx*) gene in rice endosperm point to the variation of gene expression associated with the G to T mutation at the 5' splice site of intron 1 and the alternative splicing produces variants transcripts by shortening. As consequence, variation of amylose content in this tissue. To determine whether the splicing type of *Wx* genes are also found in rice endosperm of Thai rice cultivars, cDNA of the gene have been isolated by using RT-PCR and sequenced. Analysis of the twenty-five cDNA clones led to the identification of the splicing pattern of the gene, four cryptic splice sites was found in cDNA sequences of low-amylose cultivars (<20%), while two cryptic splice sites of intermediate (20-25%) and high (>25%) amylose cultivars were characterized. The alternatively spliced transcripts were observed in much higher proportion in low amylose cultivars than that in intermediate and high amylose cultivars. These findings raise a possibility explanation that the degree of amylose content in non-glutinous rice cultivars may reflect to the amount of efficiently spliced transcripts.

Key words: Rice, *Waxy* gene, alternative splicing, amylose

INTRODUCTION

It has been a great challenge for biologists to understand the complicated and often myriad mechanisms of gene regulation. The recent success of genome sequencing projects combined with very effective molecular methods have generated abundant gene sequences, leading to much more understanding of gene regulation as of the rice *Waxy* (*Wx*) gene controlling the synthesis of amylose in the endosperm. It has been established that amylose in endosperm is the most important characteristic for predicting cooking and processing behavior of rice (*Oryza sativa*). Rice cultivars with low apparent amylose content have a soft, stickly cooked rice texture. In contrast, those with intermediate to high apparent amylose content typically yielded the cooked rice with separate, firm and drier texture.

Generally, starch composes of two types of glucan polymer, namely, amylose and amylopectin (Smith *et al*., 1997). The amylose content, which is defined as a ratio to the total amount of starch, varies considerably crops and even varieties (Martin and Smith, 1995). The rice *Wx* gene encodes a granule-bound starch synthase (GBSS), the enzyme is responsible for amylose synthesis in rice endosperm (Okagaki and Wessler, 1988). The *Wx* locus of rice (*Oryza sativa*) exhibited two alleles, Wx^a and Wx^b which are classified by level of intensity of the enzyme ADPglucose starch glycosyl transferase or *Waxy* protein (Sano, 1984). It has been shown that rice with the Wx^a produces a higher content of amylose in seeds when compared with the allele Wx^b (Bligh *et al*., 1998; Isshiki *et al*., 1998). However, *du* loci is other loci that control amylose synthesis and the level of *Wx* gene expression (Okuno *et al*., 1983). Larkin and Park (1999) reported that all cultivars with more than 18% amylose had the sequence GGTATA at the leader intron 5' splice site, while all cultivars with a lower proportion of amylose had the sequence GTTATA. However, Thai rice cultivars with lower than 20% amylose had the sequence GTTATA, whereas rice cultivars with more than 20% of amylose content contained the sequence GGTATA at the 5' splice site of the first intron (Prathepha and Baimai, 2004). For the cultivated rice, two wild-type alleles, Wx^a and Wx^b, have the sequence GGTATA and GTTATA at the 5' splice site of the first intron, respectively (Isshiki *et al*., 1998).

Using northern blot analysis, only the completely processed GBSS mRNA (2.3 kb) was detected in the intermediate- and high-amylose cultivars, whereas lower levels of the 2.3 kb GBSS mRNA as well as incompletely spliced 3.3 kb GBSS transcripts containing the first intron was detected in all of the low-amylose cultivars (Bligh *et al*., 1998). In addition, a glutinous cultivar with japonica background (cv. Calmochi 101) accumulated only 3.3 kb GBSS mRNA. Furthermore, cDNA sequences of GBSS transcripts in intermediate-, high-amylose cultivars

show the utilization of the consensus sequence at the 5′ splicing site and cryptic splice sites at 3′ splicing site of the first intron. Meanwhile, all of the cDNA sequences of five clones of low-amylose cultivars containing a single G-to-T mutation show 5′ cryptic splice site, and two of these clones reveal 3′ cryptic splice sites (Bligh *et al*., 1998). The results are consistent with those of Cai *et al*. (1998) that rice cultivars with the allele Wx^b produce the aberrant transcripts. The cDNA sequences of the transcripts show the cryptic sites of both splice donor sites and splice acceptor sites, resulting in the heterogeneous 5′ untranslated region.

Furthermore, Hirano *et al*. (1998) and Isshiki *et al*. (1998) reported that a mutation (G to T) in the 5′ splicing site in the first intron of the *Wx* gene associated with low level expression of the *Wx* gene. Moreover, rice with allele Wx^b could be effected by cool temperature during seed development, resulting in higher contents of amylose in seeds (Hirano *et al*., 1998; Larkin and Park, 1999). An attractive explanation that low temperature enhances of the *Wx* gene expression is that at lower temperature may increase the transcription rate as well as transcript stability and/or translational efficiency of the *Wx* gene (Larkin and Park, 1999). However, the mutation at the splice junction may not effect the transcription level, because the transient assay indicates that the promoter activity of the two allele of rice *Wx* gene are similar. It is also suggested that the inefficient splicing of the first intron may be responsible for the reduction in the level of mature transcript (Hirano *et al*., 1998). The abnormal transcripts containing the first intron is unstable and quickly degraded, thus the level of the mature transcript may be reduced. In addition, the accumulation of the abnormal transcript may suppress the transcription of its own gene. As a consequence, the total amount of translatable *Wx* transcripts are reduced.

In previous papers demonstrated the variation of amylose content in rice cultivars that carry the mutation (G to T) at the 5' splice site of intron 1, which leads to incomplete post-transcriptional processing of the *Wx* pre-mRNA. This study here report the isolation of a cDNA clone that represent an alternatively spliced variant of the *Wx* gene in Thai rice cultivars. The result would lead to much more understanding that alternative splicing may be of biological significance in the regulation of variation in amylose content of rice.

MATERIALS AND METHODS

Plant material: In 2004, indica rice (*Oryza sativa* L.) representing the low-amylose variety (cv. KDML 105, 14.5%; RD 15, 14.1%), intermediate (cv. Leung 11, 22.1%) and high-amylose variety (cv. Chainart 1, 28.9%) were grown in the greenhouse under natural condition at Mahasarakham University of Thailand during May-December. Seeds were collected at the immature endosperm stage of development, 18 days after flowering.

Reverse transcription and Polymerase Chain Reaction (PCR) amplification: Total RNA was isolated from immature rice endosperm, 18 days after flowering by using TRIZOL® reagent (Life Technologies, Gaithersburg, MD), as described by the manufacturer's instructions. Subsequently, 1 μg poly (A)+ RNA was reverse transcribed into cDNA using the SUPERSCRIPT™ One-Step RT-PCR with PLATINUM® *Taq* from Life Technologies (Gaithersburg, MD). The cDNA synthesis was at 50°C for 30 min, followed by 94°C for 2 min. PCR amplification of *Wx* cDNA was performed using GBSS-specific primers 484 (5′-CTTTGTCTA TCT CAAGACAC-3′) and 466 (5′-AGCCGGTGGC CGAGGTGGCG-3′) (Bligh *et al*., 1998). The reaction mixture containing 10 picograms each primer, 1 μL (2 units) enzyme mix, 1X buffer (which includes 0.2 mM of each dNTP and 1.2 mM $MgSO_4$). The amplification conditions were 35 cycles of 94°C for 15, 50°C for 30 sec, and 72°C for 1 min. A final extension step at 72°C for 5 min was performed after the 35 cycles. PCR products were resolved using 3.5% agarose gel electrophoresis.

Sequencing of *Wx* cDNA: The PCR products of the expected size (ca~120 and 210 bp) was purified with GeneClean and cloned into the pGEM-T vector (Promega, Madison, WI). The positive clones were sequenced using the M 13 forward or reverse primer with a BigDye Terminator kit (PE Biosystem) and ABIPRISM DNA sequencing system. The cDNA sequences obtained derived from these rice varieties were compared with the prediction of splice sites of exon 1 to exon 3 sequences of rice varieties for the allele Wx^a of *Wx* gene sequences (Genbank Accession No. AF031162).

RESULTS

Isolation of cDNA clones and cDNA sequences of rice *Wx* gene: Using the GBSS-specific primer GBSS484 and GBSS466, RT-PCR analysis of GBSS transcripts of low-amylose cultivars, KDML105 and RD 15 gave a predominant one of ca. 120 bp and addition RT-PCR product of ca. 210 bp, which was predominant in intermediate- and high-amylose cultivars (Leung 11, Chainart 1) (Fig. 1 A, B).

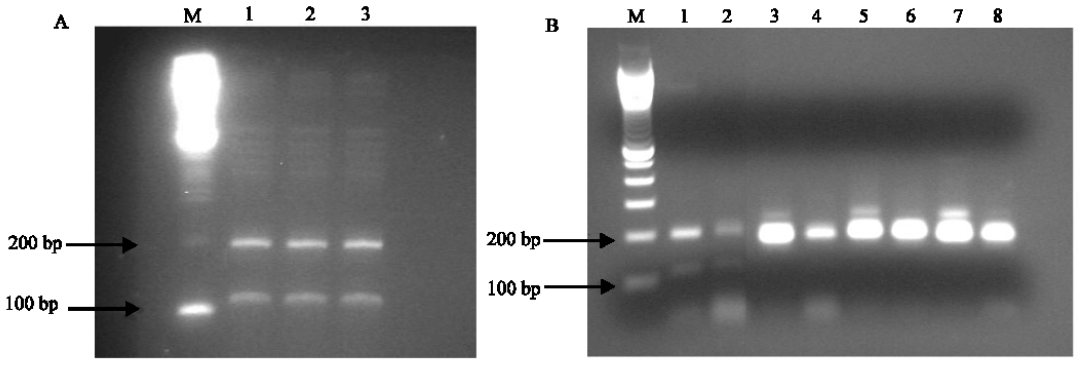

Fig. 1: RT-PCR products of GBSS mRNA in low-amylose varieties (cv. KDML 105) with three separated experiments (A), and in intermediate and high amylose varieties. Lanes: 1-3, Leung 11; 4-8, Chainart 1 (B). M = molecular weight marker (bp)

(A) Intermediate and high amylose content rice

clone L11-A10 (normally spliced transcript)

Exon 1 _ Exon 2 _

CTTTGTCTATCTCAAGACACAAATAACTGCAGT $(CT)_{11}$

GCTTCACTTCTCTGCTTGTGTTGTTCTGTTGTTCATCAGGAAGAACATCTGCAAGTGCAGAGATCTTCCACAGCAACAGCTAGACAACCACCATGTCGGCTCTCACCACGTCCCAGCTCGCCA
CCTCGGCCACCGGCT

I. AST1 (alternatively spliced transcript type 1)

clone CN1-10B2

CTTTGTCTATCTCAAGACACAAATAACTGCAGT $(CT)_{11}$ GCTTCACTTCTCTGCTTGTGTTGTTCTGTTGTTCATCAGGAAGAACATCTGCAAG-----
AGATCTTCCACAGCAACAGCTAGACAACCACCATGTCGGCTCTCACCACGTCCCAGCTCGCCACCTCGGCCACCGGCT

II.AST2

clone CN1-5A

CTTTGTCTATCTCAAGACACAAATAACTGCA//TGCAGAGATCTTCCACAGCAACAGCTAGA
CAACCACCATGTCGGCTCTCACCACGTCCCAGCCCGCCACCTCGGCCACCGGCT

(B) Low amylose content rice

clone MLKC-5A2 (normally spliced transcript)

Exon 1 Exon 2

CTTTGTCTATCTCAAGACACAAATAACTGCAGT$(CT)_{11}$GCTTCACTTCTCTGCTTGTGTTGTTCTGTTGTTCATCAGGAAGAACATCTGCAAGTGCAGAGATCTTCCACAGCAACAGCTAGACA
ACCACCATGTCGGCTCTCACCACGTCCCAGCTCGCCACCTCGGCCACCGGCT

III.AST3

clone ML28-15A

CTTTGTCTATCTCAAGACACAAATAACTGCA//-----
AGATCTTCCACAGCAACAGCTAGACAACCACCATGTCGGCTCTCACCACGTCCCAGCTCGCCACCTCGGCCACCGGCT

IV.AST4

clone ML28-20A

CTTTGTCTATCTCAAGACACAAATAACTGCA//TGCAGAGATCTTCCACAGCAACAGCTAGA
CAACCACCATGTCGGCTCTCACCACGTCCCAGCTCGCCACCTCGGCCACCGGCT

V. AST5

clone RD15-20B

CTTTGTCTATCTCAAGACACAAATAACTGCAGT$(CT)_{11}$GCTTCACTTCTCTGCTTGTGTTGTTCTGTTGTTCATCAGGAAGAACATCTGCAA/
TGCAGAGATCTTCCACAGCAACAGCTAGACAACCACCATGTCGGCTCTCACCACGTCCCAGCTCGCCACCTCGGCCACCGGCT

VI. AST6

clone MlKC-20B2

CTTTGTCTATCTCAAGACACAAATAACTGCAGT$(CT)_{11}$GCTTCACTTCTCTGCTTGTGTTGTTCTGTTGTTCATCAGGAAGAACATCTGCAA/-----
AGAGATCTTCCACAGCAACAGCTAGACAACCACCATGTCGGCTCTCACCACGTCCCAGCTCGCCACCTCGGCCACCGGCT

Fig. 2: Comparison of cDNA sequences of *Wx* transcripts between normal splicing and aberrant splicing pattern from a representative clones of (A) intermediate and high amylose rice abd (B) low amylose rice. Among the 25 clones examined, two aberrant splicing patterns (I and II) were observed in intermediate and high amylose rice, whereas four splicing patterns, III, IV, V and VI were found in low amylose rice./indicates nucleotide deletion for exon 1, ----- indicates 5 bp of the 5' end of exon 2 deletion

Twenty-five *Wx* cDNA clones from the endosperm of these rice samples were isolated and sequenced. Based on cDNA sequence alignment, the result showed that 14 clones of them were completely spliced transcripts and the rest one were alternative spliced transcripts. Among the 13 *Wx* transcripts of the two low-amylose cultivars, there were two types of classification of the transcripts, four clones exhibited completely spliced transcript (4 out of 13, 31%) and the rest one showed alternative spliced transcript (9 out of 13, 69%). Among 12 clones of intermediate and high amylose rice cultivars, two clones (2 out of 12, 17%) were alternative spliced transcripts. Four types of the alternative spliced transcript was found in low amylose rice cultivars (Fig. 2). Based on the splicing types of eukaryotes that reviewed by Roos and Simmons (2005), the four splicing patterns were classified as alternative 5' splice site and alternative 3' splice site. This alternative splicing produced variant transcripts by shortening exon 1 and exon 2 of the *Wx* cDNA of these rice cultivars.

For intermediate and high amylose cultivars, two alternative splicing pattern were observed in 12 cDNA clones. Such splicing patterns were the deletion of 5 bp of the upstream end of exon 2 and the deletion of 79 bp of exon 1. These splicing types were similar type to low amylose cultivars.

DISCUSSION

This study reports on the identification and characterization of an alternative splice variant of rice *Wx* gene. Previous investigation of alternative splicing in rice *Wx* gene have been reported (Bligh *et al*., 1998; Larkin and Park, 1999; Cai *et al*., 1998). The type of alternative splicing were observed in the *Wx* gene of rice involves the alternative use of both donor and acceptor sites of the intron 1. Such splicing, which one is classified as 'retained intron 1' sequence, has been found in rice cultivars that reported by Cai *et al.* (1998) and Bligh *et al.* (1998). Whereas, the second one is classified as 'without intron 1'sequence, which is observed in this study. Alternative splicing occurred in the *Wx* gene of rice may caused by effects of single-base substitutions (G to T) at the 5′ splice site of the intron 1 of *Wx* gene (Cai *et al*., 1998).

The consensus sequence of the splice donor site and the acceptor site of intron of eukaryotic gene is "GT…AG" (Breathnach and Chambon, 1981). In addition, the 5' splice site of plant intron and the consensus sequence is AG/GTAAGT (Brown, 1996). The +G is highly conserved as in other organisms and +2T is almost invariant. The 5' splice site of intron 1 of the two cultivars with intermediate or high amylose rice used in this study was AG/GTATA. These results are consistent with a previous report (Ayres *et al*., 1997). Whereas, the two low amylose cultivars (KDML 105 and RD 15) showed the sequence AG/TTATA, which is similar to low amylose rice cultivars in the same report. Furthermore, Isshiki *et al.* (1998) demonstrated that the low level expression of Wx^b occurred because of the mutation at the GT to TT at the 5' splice site of intron 1. As consequence, the lower level of amylose content in group of low amylose cultivar thus depends on the availability of normal transcripts, which have correctly spliced from *Wx* pre-mRNA. However, less amounts of alternative spliced transcripts was found in rice cultivars with intermediate and high amylose content used in this study. Thus alternative splicing was not occurred in only low amylose rice cultivar, but also in rice cultivars with intermediate and high amylose content. Thus, the occurrence of the cryptic splice site is characteristic of non-glutinous cultivars. However, it is rare case for alternative splicing in rice cultivar with high amylose content.

ACKNOWLEDGMENTS

This study was partly supported by BIOTEC and the TRF/BIOTEC Special Program for Biodiversity Research and Training.

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