

SHORT COMMUNICATION

A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the 5' splice site of the first intron

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Summary

In cultivated rice two wild-type alleles, *Wx^a* and *Wx^b*, predominate at the waxy locus, which encodes granule-bound starch synthase. The activity of *Wx^a* is 10-fold higher than that of *Wx^b* at the level of both protein and mRNA. *Wx^b* has a +1G to T mutation at the 5' splice site of the first intron. Sequence analysis of *Wx^b* transcripts revealed that splicing occurs at the mutant AG/UU site and at two cryptic sites: the first is A/GUU, one base upstream of the original site and the second is AG/GU found approximately 100 bases upstream of the mutant splice site. We introduced single base mutations to the 5' splice sites of both *Wx^a* and *Wx^b*, fused with the *gus* reporter gene and introduced them into rice protoplasts. Analysis of GUS activities and transcripts indicated that a G to T mutation in *Wx^a* reduced GUS activity and the level of spliced RNA. Conversely, a T to G mutation of *Wx^b* restored GUS activity and the level of spliced RNA to that of wild-type *Wx^a*. These results demonstrated that the low level expression of *Wx^b* results from a single base mutation at the 5' splice site of the first intron. It is of interest that the *Wx^b* allele of rice carrying the G to T mutation of intron 1 has been conserved in the history of rice cultivation because there is a low amylose content of the seed caused by this mutation.

Introduction

The waxy locus encodes granule-bound starch synthase (GBSS) which is a key enzyme in amylose synthesis of plants (reviewed by Nelson and Pan, 1995; Preiss, 1991). This gene is ubiquitously present in plants, and in cereals it is expressed in endosperm, pollen and embryo sac. The coding region of the rice *Waxy* gene is interrupted by 12 introns (Hirano and Sano, 1991; Umeda *et al.*, 1991; Wang *et al.*, 1995) and a 1.1 kb intron is present in the untranslated leader (Wang *et al.*, 1995; M. Nakajima *et al.*, unpublished results). Two wild-type alleles, *Wx^a* and *Wx^b*, predominate at the waxy locus in cultivated rice. Expression levels of *Wx^a* is 10-fold higher than that of *Wx^b* at the RNA and protein. There is only one amino acid difference between *Wx^a* and *Wx^b* gene products and it is likely that their specific activities are similar (Hirano and Sano, 1991; Okagaki, 1992; Wang *et al.*, 1995). *Wx^a* is characteristic of indica rice, and *Wx^b* is found mainly in japonica rice (Sano *et al.*, 1985; Sano, 1984). High expression of *Wx^a* results in high amylose content, which makes cooked rice discrete; the lower expression of *Wx^b* results in stickier japonica rice. The geographical distribution of these two alleles reflects local preference for stickiness.

Previously, Wang *et al.* (1995) examined WAXY protein and mRNA levels of 31 rice cultivars and found that low amylose cultivars accumulate substantial amounts of unspliced *Wx* transcripts that still contain intron 1; high amylose cultivars do not contain unspliced transcripts. These findings suggest that the low amylose cultivars with the *Wx^b* allele may have defects in splicing, however, the molecular basis of the difference in the expression levels of the two *Wx* alleles remains to be determined.

Pre-mRNA splicing is carried out in two steps within the spliceosome, a large RNA-protein complex which contains four small ribonucleoprotein particles (U1, U2, U4/U6 and U5) and numerous protein factors. In the first step of splicing, cleavage occurs at the intron 5' border with the formation of a 2'-5' phosphodiester bond between the first nucleotide of the intron (+1G) and a branch point located 10–50 nucleotides upstream of the 3' splice site. In the second step, cleavage at the 3' splice site releases the intron lariat and the exons are ligated together. This process is considered to be conserved in mammals, yeast, and plants although biochemical documentation in plants is

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limited because there is no *in vitro* assay (reviewed by Brown, 1996; Filipowicz *et al.*, 1995; Luehrsen *et al.*, 1994; Moore *et al.*, 1993; Simpson and Filipowicz, 1996). Recently, a minor class of introns with AT-AC splice sites have been found in mammals (Hall and Padgett, 1996; Tarn and Steiz, 1996).

The 5' splice site of the plant introns is similar to mammals and yeast and the consensus is AG/GTAAGT (Brown, 1996; Filipowicz *et al.*, 1995; Luehrsen *et al.*, 1994; Simpson and Filipowicz, 1996). The +1G is highly conserved as in other organisms and +2T is almost invariant, suggesting that mechanisms of recognition of the plant 5' splice site in plants is similar to those in mammals and yeast. A growing number of mutations in 5' and 3' splice sites have been identified in plants (Brown, 1996). For instance, a G to A mutation at the 5' splice site of intron 3 of *Arabidopsis* rubisco activase gene was shown to have defects in splicing (Orozco *et al.*, 1993). The detailed study of this mutation gave the first evidence for the requirement of the branch site in splicing of plant introns (Liu and Filipowicz, 1996). The +1G to A mutation produced by *in vitro* mutagenesis was also shown to activate cryptic splice sites in *in vivo* transient assays using plant cells (Carle-Urioste *et al.*, 1994; McCullough *et al.*, 1993).

In this study we demonstrate that the rice *Wx^b* allele, which is one of the wild-type alleles of this locus, has a GT to TT mutation at the 5' splice site of intron 1. We further show that splicing occurs at the mutated +1U position in addition to two cryptic splice sites. These and other results show that the low level expression of *Wx^b* occurs because of the mutation at the +1G of intron 1.

Results and discussion

WAXY proteins encoded by *Wx^a* and *Wx^b* were first examined in different genetic backgrounds. The protein level of *Wx^a* was approximately 10 times higher than that of *Wx^b* (Figure 1a). Next, the relative amounts of *Wx* transcripts were analyzed in total RNA prepared from developing endosperm. A strong 2.3 kb signal, corresponding to the size of mature *Wx* mRNA, was detected in *Wx^a* when *Wx* cDNA was used as a probe (Figure 1b). A faint signal was detected in *Wx^b* at this position in addition to a signal of 3.4 kb, which corresponds to the size of *Wx* transcript with intron 1. Confirmation that this transcript retained intron 1 was obtained by demonstrating that a 3.4 kb transcript also hybridized with an intron 1 probe (Figure 1c). These results confirmed previous studies (Wang *et al.*, 1995) and suggested that the low level expression of *Wx^b* reflects inefficient splicing of intron 1.

To see if inefficient splicing of *Wx^b* results from mutations present in intron 1 or flanking regions, sequences of the promoter, exon 1, intron 1 and part of exon 2 were compared to *Wx^a* (Figure 2a). Previous studies had identi-

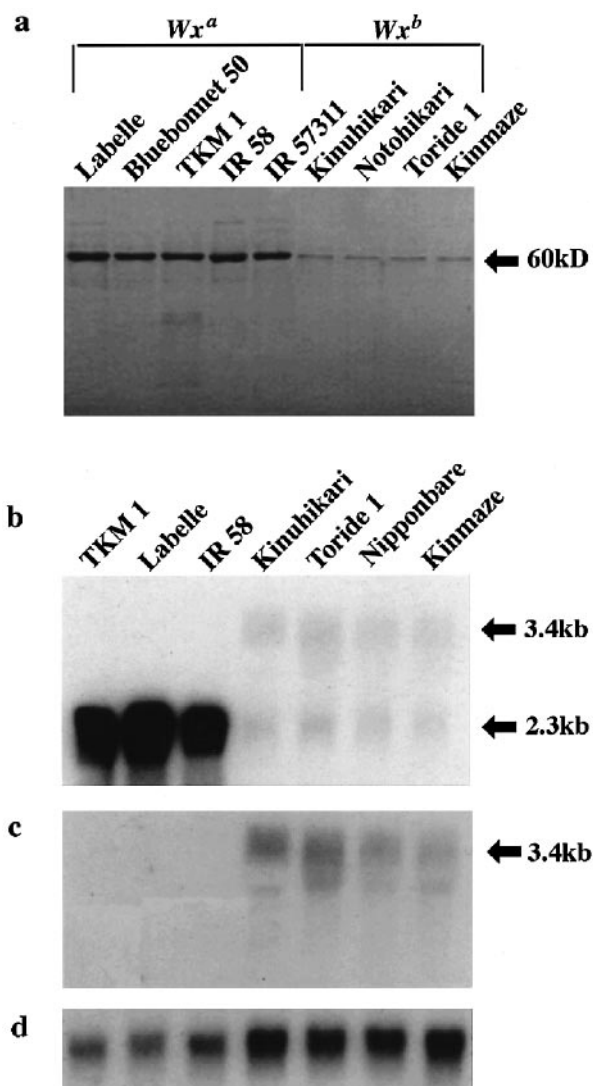


Figure 1. Expression of *Wx^a* and *Wx^b* at the protein and mRNA levels in developing endosperm.

(a) WAXY proteins extracted from various cultivars carrying *Wx^a* and *Wx^b*. WAXY proteins were extracted from mature seeds and analyzed as described previously (Echt and Schwartz, 1981).

(b-d) Northern blot analysis of RNA isolated from various cultivars carrying *Wx^a* and *Wx^b*. Total RNA was prepared from developing endosperms by the method described previously (Nagy *et al.*, 1988). Ten mg of total RNA was used for each lane and probes were labelled with ³²P-dCTP by a random priming kit (Amersham). Probes were a 1.3 kb *Bgl*II fragment of *Wx* cDNA, a 770 bp *Bgl*II fragment of intron 1, rice *actin 1*, for b, c and d, respectively.

fied the location of the transcription start site and intron 1 by primer extension and PCR analysis (M. Nakajima *et al.*, unpublished results). In addition, promoter analysis using transgenic rice indicated that the -640 bp promoter is sufficient for high level expression of the *Wx* gene in endosperm and pollen (M. Nakajima *et al.*, unpublished results). Sequence comparison of *Wx^a* (cv. Labelle) and *Wx^b* (cv. Kinuhikari) revealed that *Wx^b* carries a G to T change at the 5' splice site of intron 1, suggesting that this

may be the primary cause for the inefficient splicing of intron 1.

Lack of differences in the promoter sequence suggests that the reduction of the total amount of Wx^b transcripts (Figure 1b) is caused by the inefficient splicing of intron 1. This could reflect instability of unspliced Wx^b transcripts since many non-sense codons are present in intron 1, and this may cause increased instability of unspliced transcripts (Abler and Green, 1996).

To examine whether the G to T mutation at the 5' splice site of intron 1 of Wx^b is a general feature of Wx^b derived from different cultivars, we sequenced exon 1 and part of intron 1 of four more Wx^a and Wx^b alleles. The results indicate that all five Wx^b alleles sequenced contain the G to T mutation at +1 position of intron 1, whereas all five Wx^a alleles examined have G at this position (Figure 2b) although some may have the same origin.

Despite the presence of a mutation at +1G, some Wx^b transcripts must be spliced in some manner that permits gene function. To analyze splicing of Wx^b transcripts, a quantitative RT-PCR gel blot analysis (McCullough and Schuler, 1993; McCullough *et al.*, 1996) was employed. Wx^a transcripts were spliced at the original site (site I in Figure 3a) with an apparent efficiency of 100%. For Wx^b transcripts two spliced products, one at site I and the other approximately 100 nucleotides upstream (site II), were found in addition to unspliced transcripts. The amounts of these products relative to that of spliced Wx^a transcripts were 9.0% and 8.5% for site I and site II, respectively. The amount of unspliced transcripts was 8.4%. These results indicate that Wx^b transcripts were spliced with an efficiency of 17.5% compared with Wx^a transcripts, and that 74.1% of Wx^b transcripts appeared to be degraded.

To determine the splice sites of Wx^b transcripts, 12 independent cDNAs were sequenced (Figure 3b). Of these, six represented transcripts spliced at site I; three were spliced at the mutant +1U and the other three at G, one nucleotide upstream of the original site. The use of UU as a 5' splice site is unusual although a recent study showed that a cryptic UU site was used when a +1G to A mutation was introduced into intron 4 of β -conglycinin expressed in transfected tobacco plants (McCullough *et al.*, 1996). The use of the -1G conforms to a new consensus GU (CAAGU-UUAU) created one base upstream of the original UU by the +1G to T mutation. A similar case was previously found in a mutated rabbit globin intron in which AGGT was mutated to AGTT. When this mutated intron was analyzed in *in vitro* assays, the 5' cleavage site was shifted one base upstream (A/GU) (Aebi *et al.*, 1987). In addition to the two spliced products cleaved at site I, one cryptic site (site II) was identified; 6 of 12 cDNAs sequenced were spliced at AG/GU present in exon 1 approximately 90 nucleotides upstream of the original site; expands the intron from 1.1 to 1.2 kb. Together, these results show that

Wx^b transcripts are spliced at the mutant T and two cryptic sites with low efficiencies. Because intron 1 is in the 5' UTR all of the spliced transcript should yield translatable message.

To demonstrate unequivocally that the low expression level of Wx^b can be ascribed to the single base mutation at the 5' splice site of intron 1, we assayed reporter constructs containing mutations in the 5' splice sites of both Wx^a and Wx^b : To this end, the 5' regulatory region including the 640 bp promoter, exon 1 and intron 1 of Wx^a and Wx^b or Wx^a (G to T) or Wx^b (T to G) were translationally fused with the *gus* reporter gene and activities of these chimeric genes were measured after transfection into rice suspension protoplasts (Figure 4a,b). If the mutation at the 5' splice site of intron 1 is the major cause for the low level expression of Wx^b , then mutating the TT of Wx^b intron 1 should restore Wx^b activity to the level of Wx^a . Conversely, when the GT of Wx^a is changed to TT, the Wx^a activity is expected to be greatly reduced. As shown in Figure 4(b) the results of the experiments demonstrated that this was the case. To confirm that the differences in GUS activity paralleled differences in intron 1 splicing efficiency, RNA isolated from transfected protoplasts was subjected to RT-PCR gel blot analysis. Results indicated that intron 1 of the non-mutated Wx^a and the mutated Wx^b were spliced efficiently. Furthermore, mutated Wx^a and non-mutated Wx^b accumulated both unspliced and two classes of spliced transcripts (Figure 4c), as was found for natural Wx^b transcripts isolated from endosperm. Based on these results, we conclude that the G to T mutation in intron 1 of Wx^b is the major reason for the low expression of Wx^b .

Experimental procedures

RT-PCR gel blot analysis

Total RNA was isolated by a published protocol (Chomczynski and Sacchi, 1987). Two hundred ng of RNA was subjected to RT-PCR (RNA PCR Kit, TaKaRa). To determine the optimal conditions for quantitative RT-PCR (McCullough and Schuler, 1993; McCullough *et al.*, 1996), 9, 11, 13 and 15 cycles of PCR were initially examined. Because a linear relationship between the amount of input RNA and the PCR products was obtained over the entire range, 13 cycle was chosen for the analysis. An upstream primer in exon 1, 5'-ACCATTCCTTCAGTCTTTG-3', and a downstream primer in exon 5, 5'-TCGCTAGATCTTCTCACC GG-3', were used. After PCR reactions, amplified DNA was electrophoresed in an agarose gel, transferred to a nylon membrane and the membrane was hybridized with radiolabelled *waxy* cDNA. Hybridization signals were quantified in a Bio Imaging Analyzer BAS 2000 (Fuji). Values for splicing efficiencies are the average of three independent experiments.

Construction of plasmids with mutated introns

Promoter-exon 1 regions of Wx^a and Wx^b were amplified by PCR using primers, 5'-GCCCAAGCTTAGAAACCACACACCAC-3' and 5'-

a

[illegible]**b**

Gene	Labelle	Bluebonnet 50	TKM1	IR58	IR57311	Kinuhikari	Nipponbare	Kinmaze	Notohikari	Toride 1
<i>Wx^a</i>	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						
	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						
	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						
	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						
	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						
<i>Wx^b</i>	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						
	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						
	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						
	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						
	AAGAACATCT	GCAAGgtata	catatatggtt	tataa						

Figure 2. The nucleotide sequences of 5' regulatory regions of *Wx^a* and *Wx^b*. (a) Sequence comparison of the promoter-exon 1-intron 1 regions of *Wx^a* and *Wx^b*. A 2.3 kb *Pst*I fragment of the *Wx^a* derived from cv. Labelle (Okagaki and Weisler, 1988) was subcloned into pUC19 and sequenced. To sequence *Wx^b*, leaf DNA was isolated and the promoter, exon 1 and intron 1 regions were separately amplified by PCR by using primers designed according to the sequence of *Wx^a*. (b) Sequence comparison of part of exon 1 and intron 1 of 5 cultivars each carrying *Wx^a* and *Wx^b*. The corresponding region was amplified by PCR and sequenced by an automated sequencer (ABI 373, ABI).

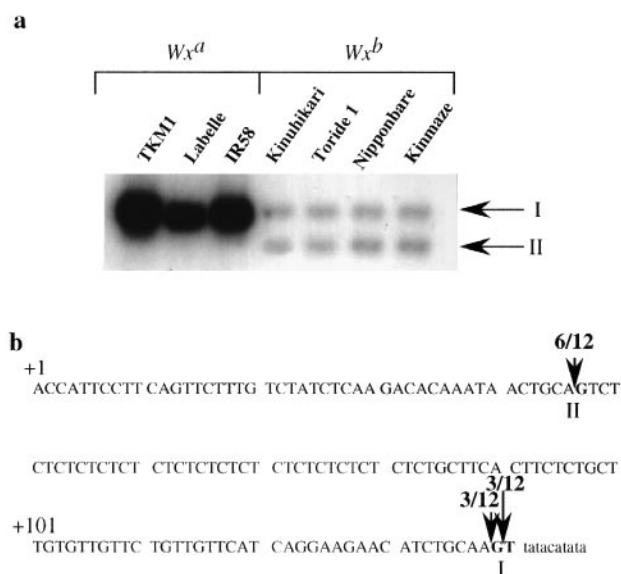


Figure 3. Splicing of Wx^b transcripts. (a) RT-PCR analysis of spliced Wx^a and Wx^b transcripts. Fragments at site II contained two species of cDNAs originated from Wx^b transcripts spliced at either G or U. (b) Summarized results of splice sites identified by sequence analysis of Wx^b cDNA. The denominator of the fraction shows the total number of cDNAs sequenced and the numerator shows the number of cDNAs derived from transcripts spliced at the sites indicated by arrows.

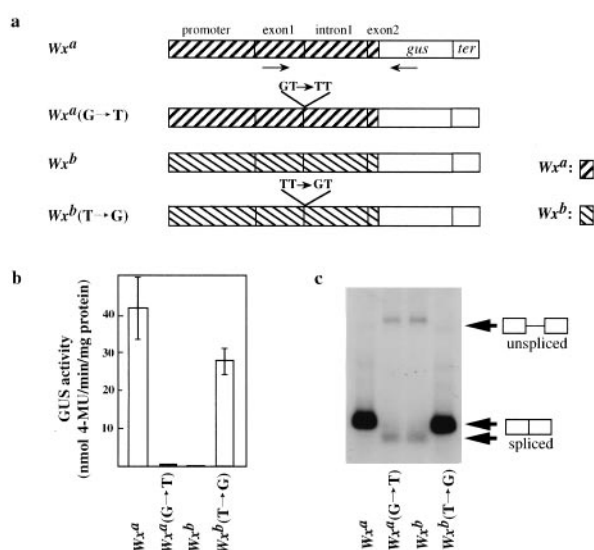


Figure 4. Transfection assays of Wx^a and Wx^b carrying mutated introns. (a) Constructs used for assays. The promoter regions of Wx^a and Wx^b were translationally fused to the gus reporter gene. The GT at the 5' splice site of Wx^a intron 1 was mutated to TT in the Wx^a (G→T) construct and the TT of Wx^b was mutated to GT in the Wx^b (T→G) construct. (b) GUS activity in transfected protoplasts. For GUS assays, the mean and standard error for four independent experiments is shown. Mean GUS activities of Wx^a (G→T) and Wx^b were 0.37 and 0.12 nmol min⁻¹ mg⁻¹ protein, respectively. The background activity was normally 0.01 nmol min⁻¹ mg⁻¹ protein. (c) RT-PCR analysis of RNA isolated from transfected protoplasts. The 1.5 kb products were derived from unspliced transcripts and 0.4 and 0.5 kb products were derived from spliced transcripts.

GATGTTCTTCCTGATGAACAACAG-3'. The upstream primer contains base substitutions to create a *Hind*III site. Intron 1-exon 2 region was amplified using primers, 5'-CATCAGGAAGAACATCTGCAAG/TTTATAC-3' and 5'-TGGGATCCGGTGAGAGCCGACATG-3'. This upstream primer contains a point mutation to create mutated 5' splice site and the downstream primer has base substitutions to create a *Bam*HI site. PCR products having overlapping junctions were mixed and fused by using the *Hind*III and the *Bam*HI primers (Higuchi, 1990). Mutations were verified by partial sequencing. The mutated promoter-intron 1 sequence was fused to this gus reporter gene by using appropriate restriction sites.

Transfection of rice protoplasts

Protoplasts (7×10^6) were prepared from the rice Oc suspension cultures, mixed with 20 µg of test plasmid DNA and electroporated using a Gene Pulser (Bio-Rad). After overnight incubation at 30°C, cells were harvested and assayed for GUS activity. For RT-PCR analysis of RNA isolated from transfected protoplasts, 50 ng of RNA was used. To detect amplified products of Wx^b and mutated Wx^a constructs two rounds of amplification was performed. The first amplification was 25 cycles with primers, 5'-ACCATTCCTTCAGTTC-3' and 5'-ACGCGTGGTTACAGCTTGC-3' and the second cycle was 25 cycles with primers, 5'-ACCATTCCTTCAGTTCCTTTG-3' and 5'-TCTGCCAGTTCAGTTCGTTG-3'. PCR products were analyzed as described above.

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Note added in proof

While this manuscript was in press, Cai *et al.* (*Plant J.* **14**, 459–465) published a paper describing aberrant splicing of intron 1 of the *Wx^b*-type alleles in several rice cultivars and suggested that the aberrant splicing causes the low expression of these alleles.