Nonsense-Mediated Decay of Mutant *waxy* mRNA in Rice

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Two rice (*Oryza sativa*) *waxy* mutations of the Japonica background were shown to contain approximately 20% of the fully spliced mRNA relative to the wild type. Sequencing analysis of the entire *waxy* genes of the two mutants revealed the presence of premature translation termination codons in exon 2 and exon 7. These results indicated that the lower accumulation of fully spliced RNA in the mutants was caused by nonsense-mediated decay (NMD), which is an RNA surveillance system universally found in eukaryotes. It is interesting that levels of RNA retaining intron 1 were not changed by premature nonsense codons, suggesting that splicing may be linked with NMD in plants, as previously found in mammalian cells. Measurements of the half-lives of *waxy* RNAs in transfected rice protoplasts indicated that the half-life of *waxy* RNA with a premature nonsense codon was 3.3 times shorter than that without a premature nonsense codon. Because the wild-type *waxy* transcripts, which are derived from the Wx^b gene predominantly distributed among Japonica rice, have been shown to be less efficiently spliced and their alternative splicing has been documented, we examined whether these splicing properties influenced the efficiency of NMD. However, no effects were observed. These results established that NMD occurs in rice *waxy* RNA containing a premature nonsense codon.

Nonsense-mediated decay (NMD) is a mechanism in which abnormal mRNAs containing premature translation termination codons are efficiently eliminated so that production of undesirable truncated proteins is avoided (for review, see Maquat, 1995; Culbertson, 1999; Hentze and Kulozik, 1999). This RNA surveillance system is universally present in eukaryotes, and, in particular, it has been extensively studied in yeast and mammals (for review, see Maquat, 1995; Culbertson, 1999; Hentze and Kulozik, 1999). In mammals, several features of NMD have been recently revealed. First, although the majority of mammalian mRNAs are subject to NMD prior to release from their association with nuclei (nucleus-associated NMD), some mRNA are exclusively subject to NMD in the cytoplasm. (Moriarty et al., 1998; Culbertson, 1999; Hentze and Kulozik, 1999; Sun et al., 2000). Second, splicing of at least one intron is required for NMD to occur (Moriarty et al., 1998; Zhang et al., 1998a, 1998b). This observation suggests a link between splicing in the nucleus and actual RNA degradation in the cytoplasm. The current model for NMD in mammalian cells proposes that splicing leaves behind a mark at 3′-most exon-exon junction by proteins stably associated with mRNA after spliceosome dissociation and that positions of premature translation termination codons relative to this mark are important concerning whether or not mRNA is subject to NMD (Thermann et al., 1998; Hentze and Kulozik, 1999; Le Hir et al., 2000; Sun et al., 2000). Third, NMD is dependent on the termination codon position; if translation terminates >50 to 55 nucleotides upstream of the 3′-most exon-exon junction, NMD occurs (Thermann et al., 1998; Zhang et al., 1998a, 1998b; Sun et al., 2000).

In yeast, the presence of a downstream instability element has been found, marking the position of the translation termination codons required for NMD. If translation termination codons are found 3′ from the downstream instability element, the mRNA is subject to NMD (Culbertson, 1999; Hentze and Kulozik, 1999). Similar elements do not appear to be present in mammalian cells, suggesting the existence of major differences in the mechanism of NMD between yeast and mammalian cells. The presence of such elements in plants remains to be studied.

Little is known about NMD in plants. Mutant alleles of a soybean Kunitz trypsin inhibitor gene (*Kti3*) and the phytohemagglutinin gene (*PHA*) from common bean (*Phaseolus vulgaris*), which produce mRNAs containing premature termination codons, have been shown to be more quickly degraded than those of the wild-type genes (Jofuku et al., 1989; Voelker et al., 1990). In the case of the pea ferredoxin gene (*FED1*), its expression is posttranscriptionally regulated by light (Dickey et al., 1994), and insertion of stop codons in the coding region causes a decrease in mRNA stability in the light but not in the dark (Dickey et al., 1994; Petracek et al., 2000). Furthermore, the effect of premature translation termination codons on the stability of *FED1* mRNA depends on their positions, and this mRNA degradation pathway is different from that operating in the dark (Petracek et al., 1998). The position-dependent effect of translation termination codons on mRNA stability has been also demonstrated in bean *PHA* mRNA (van Hoof and Green, 1996). These studies suggest that

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NMD occurs in plant mRNA. However, these three genes, *Kti3*, *PHA*, and *FED1*, which were previously studied for an NMD-like phenomenon, are all intronless genes. Considering the observation that intron splicing is an important step in the NMD pathway in mammalian cells, studies need to be performed with intron-containing plant genes to better understand this phenomenon in plants.

It was very recently shown in *Caenorhabditis elegans* that NMD is linked with RNA interference (RNAi) caused by dsRNA (Fire et al., 1998) by the analysis of the effects of *smg* mutations originally isolated as mutations affecting NMD on the initiation and maintenance of RNAi (Domeier et al., 2000). Because RNAi and posttranscriptional gene silencing (PTGS), which are widely observed in plants, are thought to share some molecular mechanisms (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Morel and Vaucheret, 2000; Zamore et al., 2000), it is becoming increasingly important to study the NMD phenomenon in plants to better understand the epigenetic silencing caused at the level of RNA.

In this study, we show that two mutant alleles of the rice (*Oryza sativa*) *waxy* gene cause decreased accumulation of the fully spliced RNA but not of the RNA retaining intron 1. The sequencing of these alleles showed that, in each of the two mutant genes, a premature termination codon is created by mutations. Measurements of the half-lives of unspliced and spliced RNAs containing the identical premature termination codon indicated that the fully spliced RNA is degraded much faster than the partially spliced RNA. These results indicate that NMD does occur in plant mRNA and that splicing may be linked with NMD, as has been observed in mammalian RNA.

RESULTS

Decreased Accumulation of Spliced mRNA But Not of Partially Spliced mRNA Retaining Intron 1 in Two Mutant *waxy* Genes of Rice

To elucidate the molecular basis of the two *waxy* mutations of rice in which no amylose synthesis occurs in the endosperm, we initially analyzed the *waxy* transcripts of the mutant endosperms by RNA gel-blot analysis. Two wild-type alleles are known at the *waxy* locus of cultivated rice, and the Wx^b allele is present in all Japonica cultivars (Sano, 1984). The two mutant *waxy* lines used for this study are both in the Japonica background. The Wx^b allele has a single-base mutation at the 5′-splice site of intron 1, and it causes accumulation of transcripts of 3.4 kb in size in which intron 1 is unspliced but the other 12 introns are fully spliced (Bligh et al., 1998; Cai et al., 1998; Hirano et al., 1998; Isshiki et al., 1998). As in our previous reports (Isshiki et al., 1998, 2000) the partially spliced 3.4-kb transcript retaining the 1.1-kb intron 1 is called unspliced RNA in this study. As shown in Figure 1, results of RNA gel-blot analysis showed that levels of spliced transcripts for both mutants, EM21 and cv Musashimochi, were approximately 20% of those in the wild type, whereas the levels of transcripts still retaining the 1.1-kb intron 1 were comparable between the wild type and the mutants. The decreased accumulation of spliced transcripts, but not of unspliced transcripts, suggested that mutant *waxy* transcripts are spliced either less efficiently than those of the wild type or that spliced transcripts are less stable than those of the wild type.

Sequence Analysis of the Mutant *waxy* Genes Revealed the Presence of Premature Translation Termination Codons in the Coding Region

To understand the basis of the decreased RNA accumulation of the spliced mRNA in the *waxy* mutants, sequences of the *waxy* genes from −630 bp relative to the transcription start site through the 3′-untranslated region were determined for the two mutant *waxy* genes. Results of the sequence analysis indicated that, in the EM21 mutant, a G to A mutation in a TGG codon created a TGA termination codon in exon 7 (Fig. 2, A and B). In cv Musashimochi, duplication of the 23-bp sequence was found in exon 2, which generated a TGA stop codon within this exon (Fig. 2, A and C). No other base changes were found between the two mutants and the corresponding wild type (data not shown). Reduced accumulation of spliced transcripts in the mutant endosperms might be caused by the NMD of *waxy* mRNAs, since premature stop codons were found in the second and seventh exon for cv Musashimochi and EM21 *waxy* mutations, respectively. Studies of mammalian NMD clearly established that all prema-

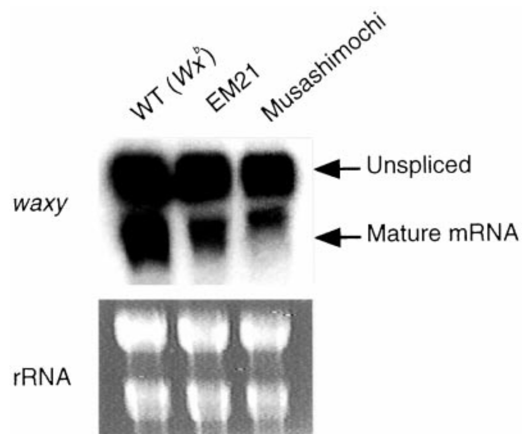

Figure 1. RNA gel-blot analysis of Wx^b RNAs in mutant *waxy* endosperms. Two different transcripts, the unspliced 3.4-kb transcript retaining the 1.1-kb intron 1 and the spliced 2.3-kb transcript, were detected in the wild type carrying the Wx^b allele and two *waxy* mutants originated from the Wx^b allele. As in our previous reports (Isshiki et al., 1998, 2000), the partially spliced 3.4-kb transcript retaining the 1.1-kb intron 1 is called unspliced RNA in this study.

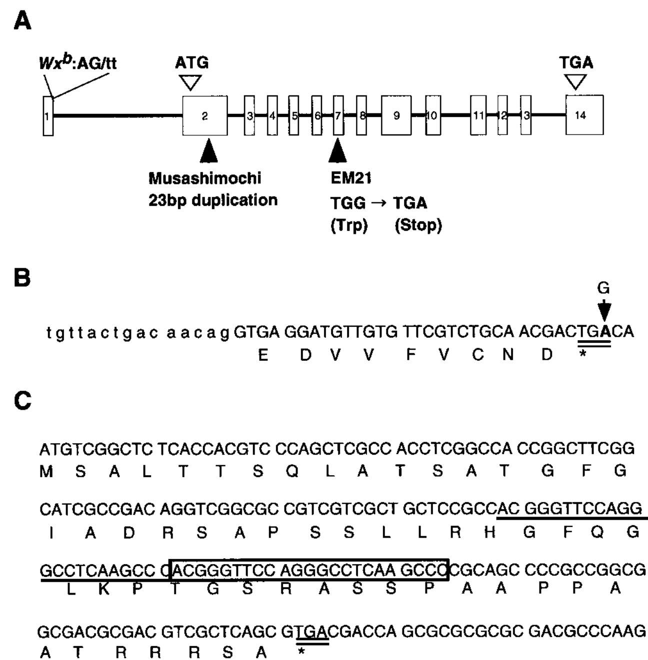

Figure 2. Sequence changes in the *waxy* genes of the two mutants. A, The structure of the Wx^b gene and locations of sequence alterations in the two mutants. Exons are shown by boxes and numbered 1 through 14. △, The initiation and the stop codons; ▲, the locations of mutations in cv Musashimochi and EM21. AG/tt indicates the sequences of the 5′-splice site of intron 1. B, The sequence of the 3′ end of intron 6 and the 5′ end of exon 7 in the EM21 mutant. C, The sequence from the region of the mutation in exon 2 of cv Musashimochi. The white box indicates duplication of the 23-bp sequence. Introns are indicated in lowercase letters, and exons are shown in capital letters. A premature nonsense codon generated by the mutation is double underlined.

ture translation termination codons that are present more than 55 nucleotides upstream of the 3′-most exon-exon cause NMD (Thermann et al., 1998; Zhang et al., 1998a, 1998b; Sun et al., 2000).

The observation that there were no differences in levels of unspliced mRNA between the wild type and the mutants suggests that the NMD of *waxy* mRNA might be linked with splicing although the actual intracellular site of RNA degradation remains to be studied. This finding is consistent with previous observations in mammalian cells, in which splicing is required for appropriate NMD irrespective of the site of RNA degradation (Moriarty et al., 1998; Zhang et al., 1998a, 1998b; Culbertson, 1999; Hentze and Kulozik, 1999).

Alternatively Spliced mRNAs Are Subject to NMD with Similar Efficiencies

The *waxy* mutants used in this study were in the Japonica background, and our sequence analysis showed that they have a splice site mutation in intron 1 as does their progenitor Wx^b allele (Fig. 2A). Because there are several lines of evidence indicating that the efficiency of splicing and alternative splicing is related to NMD (Dietz et al., 1993; Lozano et al., 1994) we tested whether certain aspects of intron splicing influenced the efficiency of NMD in *waxy* mRNA. The rice *waxy* genes provide a unique in vivo situation in which NMD occurs with mRNA that has undergone alternative splicing and the efficiency of those splicing reactions is low.

First, we examined whether alternatively spliced mRNAs were subject to NMD with similar efficiencies. Alternative splicing of intron 1 in Wx^b RNAs produce three different mRNAs in the endosperms (Fig. 3A). Reverse transcriptase (RT)-PCR analysis and sequencing revealed two different mRNAs that are spliced at site 1 and one class of mRNA spliced at site 2 (Fig. 3A; Isshiki et al., 1998, 2000). Because the two mRNAs spliced at site 1 have only one nucleotide difference, they are not distinguished by RT-PCR analysis. Therefore, we examined whether mRNA spliced at site 1 or site 2 was differentially degraded by using a competitive RT-PCR analysis. Results shown in Figure 3, B and D, clearly indicated that alternatively spliced mRNAs were subject to NMD with similar efficiencies. By using the same RT-PCR method, we quantified the levels of unspliced RNAs in the wild type and the two mutants. Results indicated that the levels of unspliced *waxy* RNAs were similar between the wild type and the two mutants (Fig. 3, C and D) and that they were consistent with the results of the RNA-blot analysis (Fig. 1). These results suggest that alternatively spliced mRNAs are subject to NMD with similar efficiencies.

Efficiency of Intron Splicing Does Not Influence the NMD of the *waxy* mRNA

It is known that *waxy* mutations are rarely found in Indica varieties that carry the nonmutant Wx^a allele (Y. Sato, unpublished data), in which no transcripts retaining intron 1 are produced. These observations made us wonder whether or not there is a link between the inefficient splicing of intron 1 usually observed for Wx^b RNAs and NMD. To address this question, we constructed four *waxy* genes (Fig. 4A). Furthermore, to perform transient expression in rice protoplasts, we used the 35S promoter for these constructs. 35S Wx^b is a wild-type Wx^b having the mutant AG/*TT* 5′-splice site of intron 1 and no premature nonsense codon. In contrast, 35S Wx^b Stop has a premature nonsense codon in exon 7 as does the EM21 mutant. The nonmutant AG/GT was introduced to these two constructs to make the 35S Wx^a and 35S Wx^a Stop. These constructs were transfected into rice protoplasts by electroporation, and RNAs isolated from the protoplasts were subjected to a competitive RT-PCR analysis.

First, we compared 35S Wx^b Stop with 35S Wx^b. In rice protoplasts, site 2 was preferentially used for splicing, and transcripts spliced at site 1 were not detectable. Results indicate that spliced RNA was

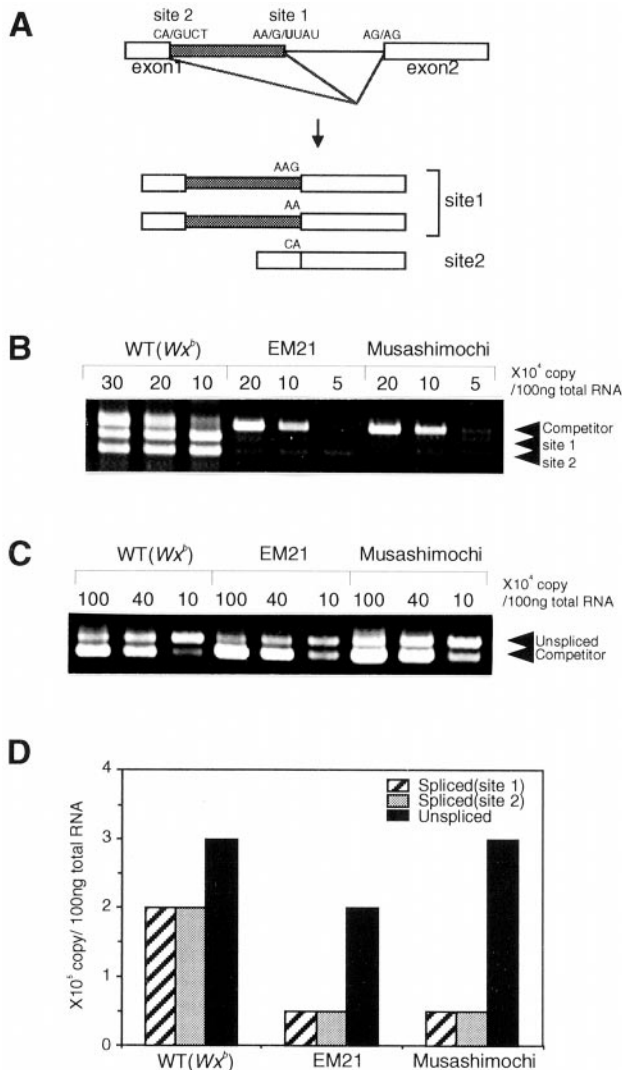

Figure 3. Alternatively spliced *Wx* mRNAs are subject to NMD with similar efficiencies in the endosperm. A, The splicing pattern of rice Wx^b pre-mRNAs. Wx^b has a G-to-T mutation at the 5′-splice site of intron 1 and generates two kinds of mRNAs spliced at two locations at site 1 (Isshiki et al., 1998; 2000). They are not distinguishable by RT-PCR analysis. In addition, mRNA spliced at a cryptic site (site 2), 100 nucleotides upstream of site 1, is produced. White bars indicate exons, and black bars indicate the distal part of exon 1 flanked by the site-2 splice site and the authentic 5′-splice site (site 1). When site 2 is used for splicing, transcripts become approximately 100 nucleotides shorter than those spliced at site 1.Thin bars indicate the first intron. B, Competitive RT-PCR analysis of spliced *waxy* transcripts in the wild type (WT) and the two mutants. C, Competitive RT-PCR analysis of unspliced *waxy* transcripts in the wild type (WT) and the two mutants. D, Quantitative representations of the spliced and unspliced *waxy* transcripts shown in B and C.

considerably reduced in 35S Wx^b Stop, whereas the level of unspliced RNA was comparable between 35S Wx^b and 35S Wx^b Stop (Fig. 4B). These results were essentially the same as those obtained in the endosperm, suggesting that a similar mechanism of NMD operates in the endosperm and cultured cells of rice. Next, we tested whether NMD occurs on mRNA produced from 35S Wx^a Stop by comparing mRNA produced from 35S Wx^a Stop with that from 35S Wx^a (Fig. 4C). It was noted that levels of RNAs were more than two orders of magnitude higher than those derived from 35S Wx^b as demonstrated previously (Isshiki et al., 1998). The level of mRNA obtained from 35S Wx^a Stop was approximately 25% of that of 35S Wx^a. These results clearly indicate that NMD also occurs with Wx^a mRNA, which has no mutation at the intron 1 splice site.

Decreased Half-Life of *waxy* mRNA Containing a Premature Nonsense Codon

To test if a premature nonsense codon decreases the stability of mRNA, the half-lives of spliced and unspliced *waxy* RNAs were measured by incubating protoplasts transformed with 35S Wx^b and 35S Wx^b Stop with actinomycin D, an inhibitor of transcription, for various times and quantifying their amounts by RT-PCR (Fig. 5). The half-life of spliced *waxy* mRNA with a premature stop codon was 5.3 min, whereas that of the spliced mRNA without a premature stop codon was 17.5 min. These results clearly indicated that the presence of premature translation termination codons decreases the stability of *waxy* mRNA approximately 3-fold. This value is in good agreement with the decrease in the half-life of mRNA derived from a mutant frame-shift allele of the bean *PHA* gene having a premature nonsense codon (van Hoof and Green, 1996). Another important finding was that unspliced RNA derived either from Wx^b or Wx^b Stop was equally stable and that no sign of degradation was detected even 20 min after actinomycin D treatment (Fig. 5). These results suggest that the NMD of the rice *waxy* gene operates only with those RNAs that contain premature nonsense codons and are fully spliced.

DISCUSSION

Rice *waxy* Mutations Provide an Opportunity to Study NMD of Plant mRNA

Rice *waxy* mutations provided an opportunity to study NMDof both spliced and partially spliced RNAs, since the progenitor wild-type allele of the mutants is the Wx^b allele, which generates both unspliced and spliced RNAs due to a single-base mutation at the 5′-splice site of intron 1 (Bligh et al., 1998; Cai et al., 1998; Hirano et al., 1998; Isshiki et al., 1998). Because of this unique situation, we could clearly demonstrate that fully spliced mRNA containing a premature nonsense codon was subject to NMD, whereas the partially spliced RNAs were not degraded although they contained a premature nonsense codon at an identical position.

One interesting observation is that unspliced mRNA is stable although it carries a number of non-

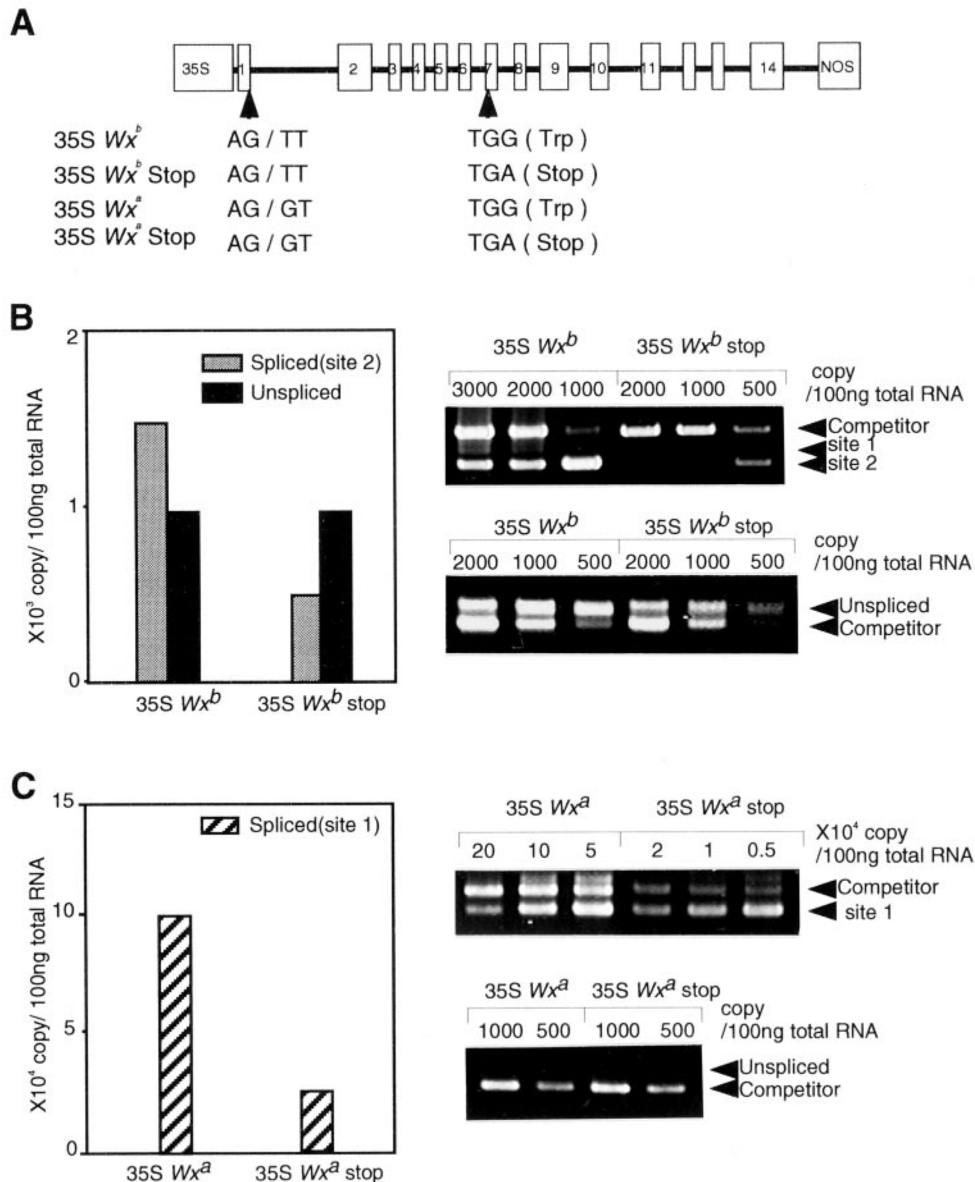

Figure 4. Relationship between efficiencies of intron 1 splicing and NMD of *waxy* mRNA. A, Constructs used for assays. White boxes represent exons, and solid lines represent introns. 35S Wx^b has a TT mutation at the 5′-splice site of intron 1, and 35S Wx^b Stop has a premature stop codon in exon 7. The TT at the 5′-splice site of intron 1 present in 35S Wx^b and 35S Wx^b Stop was mutated to GT in 35S Wx^a and 35S Wx^a Stop. B, Competitive RT-PCR analysis of RNAs from 35S Wx^b and 35S Wx^b Stop in transfected rice protoplasts. In rice protoplasts, site 2 was preferentially used for splicing, and RNAs spliced at site 1 were not detectable. C, Competitive RT-PCR analysis of RNA from 35S Wx^a and 35S Wx^a Stop in transfected protoplasts. Unspliced *waxy* RNA was not detected because Wx^a RNA was completely spliced at site 1 (Isshiki et al., 1998; 2000).

sense codons within intron 1 (Isshiki et al., 1998). This implies that nonsense codons present in intron 1 were not recognized by the NMD system and only those nonsense codons present downstream within the exons are recognized by the NMD system in rice. These findings raise a possibility that not only splicing but other processing steps may be involved in NMD of rice *waxy* mRNA.

Although we found a linkage between splicing and NMD in rice *waxy* mRNA, one major difference between our findings and mammalian NMD is clear. Normally splicing of an intron 3′ to the nonsense codon is important for mammalian NMD, whereas in the case of rice *waxy* NMD, splicing of the first intron present upstream of the premature nonsense codon was important. This difference may reflect differences in the mechanisms of NMD between mammals and plants. Future studies will clarify this important issue.

In mammals, the majority of NMD occurs while mRNA is still associated with the nucleus, whereas some mRNAs are subject to NMD exclusively in the cytoplasm (Maquat, 1995; Moriarty et al., 1998; Hentze

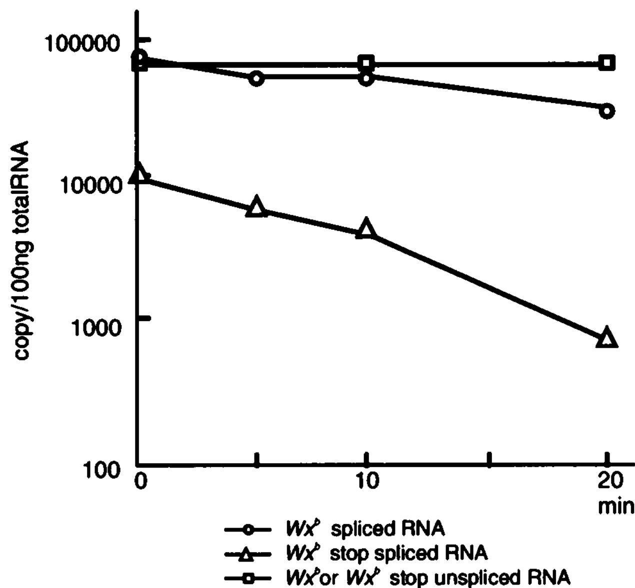

Figure 5. Faster decay of the spliced *waxy* mRNA containing a premature stop codon in transfected rice protoplasts. 35S Wx^b and 35S Wx^a Stop were transfected into rice protoplasts by electroporation, and levels of spliced and unspliced *waxy* RNAs were quantified by competitive RT-PCR analysis at various times after actinomycin D addition.

and Kulozik, 1999; Sun et al., 2000). In the case of rice *waxy* mRNA, NMD correlates with splicing of intron 1 although the site of NMD remains to be studied. NMD may only occur with properly processed RNAs and partially spliced RNA might be physically separated from other RNAs in the nucleus. Alternatively, if NMD of *waxy* mRNA exclusively occurs in the cytoplasm, lack of splicing may prevent the export of unspliced RNA to the cytoplasm for RNA decay. In this latter case splicing per se would not be an important factor for NMD.

All three plant genes whose transcripts were previously shown to be degraded due to the presence of premature nonsense codons are intronless (Jofuku et al., 1989; Voelker et al., 1990; Dickey et al., 1994; van Hoof and Green, 1996; Petracek et al., 2000). If positions of premature stop codons are recognized by proteins involved in splicing reactions and stably associated with mRNA at exon-exon junctions even after spliceosome dissociation, as postulated for mammalian NMD (Thermann et al., 1998; Hentze and Kulozik, 1999; Le Hir et al., 2000; Sun et al., 2000), a question arises asto whether degradation of RNAs containing premature stop codons derived from intronless genes occurs through the same mechanism observed in mammalian NMD. This remains to be studied.

NMD and Other Types of Posttranscriptional Regulation of Rice *Wx* Gene Expression

We have previously shown the other types of posttranscriptional regulation of rice *waxy* gene expression (Itoh et al., 1997; Terada et al., 2000). In transgenic Japonica rice carrying the antisense *Wx* gene, reduction of sense RNA by the antisense RNA was only observed with the spliced RNA, and no changes were found with unspliced RNA retaining intron 1 (Terada et al., 2000). This observation is very similar to observations regarding the *waxy* mutants described in this paper. This similarity may suggest that RNA degradation caused by antisense RNA is mechanistically related to NMD. The molecular mechanism of gene suppression by antisense genes has not been well understood (Bourque, 1995), and it is now considered that some effects are caused by RNA degradation mediated by dsRNA (for review, see Kooter et al., 1999; Meins, 2000; Morel and Vaucheret, 2000).

Introduction of the rice wild-type *waxy* gene into a Japonica rice causes silencing of both the endogenous *waxy* and the transgenes in pollen with high frequency (Itoh et al., 1997). Although this study did not examine experimentally whether the observed gene silencing in transgenic rice was post-transcriptional, the observed cosuppression of the endogenous *waxy* and the transgenes is consistent with a posttranscriptional mechanism. Future experiments examining both NMD and PTGS of *Wx* gene expression in transgenic *waxy* mutants may give insights into common elements in the molecular mechanisms of NMD, PTGS, and RNAi in plants.

NMD Is One of Many RNA Degradation Pathways in Plants

Regulation of mRNA stability plays a key role in the control of gene expression in plants and other eukaryotes. Various mRNA decay pathways are thought to exist in plants (Gutierrez et al., 1999). For instance, a DST (downstream element) present in the 3′UTR was shown to be an mRNA instability element of the Arabidopsis SAUR gene (Gil and Green, 1996), and adenylate/uridylate-rich elements present in some mammalian genes confer instability to reporter transcripts in transformed tobacco cells (Ohme-Takagi et al., 1993). Premature nonsense codons may be categorized into such instability elements. Therefore, the elucidation of various mRNA decay pathways and mechanistic relationships among multiple pathways will become critical in future studies. The molecular mechanisms of RNA degradation in PTGS may also be related to the above-mentioned RNA decay pathways. Rice *waxy* genes will be a useful experimental tool for studies in the regulation of RNA stability in plants.

MATERIALS AND METHODS

Plant Material

A Japonica rice (*Oryza sativa*) cultivar, cv Kinmaze, a *waxy* mutant EM21 with the Kinmaze background (Satoh, 1986), and a glutinous rice cultivar, cv Musashimochi, were all grown in a greenhouse under a light-dark cycle consisting of 14 h of light and 10 h of dark.

RNA Gel-Blot Analysis

Total RNA was prepared from immature seeds 15 d after pollination by the published method (Chomczynski and Sacchi, 1987). RNA (10 μg) was separated by electrophoresis in 1% (w/v) agarose gels containing formaldehyde, blotted, and hybridized with a *waxy* cDNA probe that had been labeled with [α-^{32}P]dCTP using the Multiprime DNA labeling system (Amersham, Buckinghamshire, UK). Probes used were a 1.3-kb *Bgl*II fragment of *waxy* cDNA (exon 5–exon 14). After the membrane was washed, an autoradiography was obtained by exposing the membrane to an x-ray film.

DNA Sequencing

The exon, intron, and promoter (−630 bp relative to the transcription initiation site) sequences of cv Kinmaze, EM21, and cv Musashimochi were amplified by PCR from genomic DNA using pairs of *Wx*-specific primers. PCR products were cloned into the pGEM-T vector (Promega, Madison, WI). Sequencing of cloned products was performed with a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE-Applied Biosystems, Foster City, CA).

Construction of Plasmids

35S Wx^b carries the coding region of Wx^b isolated from a genomic library of cv Kinmaze under the control of the cauliflower mosaic virus 35S promoter. 5′UTR, open reading frame, and 3′UTR regions of Wx^b were separately cloned after PCR amplification and subsequently ligated together on a pSN221 vector. 35S Wx^b had the following structure with restriction sites used for ligation: 35S promoter-*Sal*I-5′UTR-*Nhe*I-*waxy* ORF-*Hpa*I-3′UTR-*Not*I-NOS terminator. To produce 35S Wx^a, 35S Wx^b was mutagenized using one of two mutagenic antisense primers, each of which creates a point mutation of T to G at the 5′-splice site of intron 1. A 526-bp *Sal*I-*Nhe*I fragment of the PCR product was used to replace the corresponding fragment of 35S Wx^b. 35S Wx^b Stop and 35S Wx^a Stop were produced from 35S Wx^b and 35S Wx^a, respectively, by PCR, with one of two mutagenic antisense primers, each of which creates a nonsense mutation (TG*G* to TG*A* in exon 7). The 800-bp *Bam*HI fragments of the PCR products were used to replace the corresponding fragments of 35S Wx^b and 35S Wx^a.

Transformation of Rice Protoplasts

Protoplasts (5×10^6) were prepared from the rice Oc suspension cultures, mixed with 20 μg of plasmid DNA, and electroporated using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA). After overnight incubation at 30°C, RNA was isolated from protoplasts for competitive RT-PCR or RNA decay measurements.

Competitive RT-PCR Analysis

The method for quantifying spliced *waxy* transcripts was as described previously (Isshiki et al., 2000). A competitor DNA for quantifying unspliced *waxy* transcripts was constructed by deleting a 61-bp DNA fragment from two *Dra*I sites in intron 1 of Wx^a genomic DNA (exon 1–2) cloned into a pGEM-T vector. Serial dilutions of the competitor RNA transcribed in vitro were co-amplified with 500 ng of total RNA using an mRNA Selective PCR Kit (Takara, Kyoto). PCR cycle conditions were followed by 25 cycles (for endosperm RNA) or 40 cycles (for transformed Oc protoplast RNA) of 85°C for 1 min, 55°C for 1 min, and 72°C for 1 min with primers 5′-AACGGCCAGGATATTTATTGTG-3′ and 5′-GAGAGCCG-ACATGGTGGTTG-3′. After PCR reactions, amplified DNA was electrophoresed in 2% (w/v) agarose gels and visualized by ethidium bromide staining. Quantitation of the amount of amplified fragments was performed as described previously (Gilliland et al., 1990).

RNA Decay Measurement

RNA decay measurements were performed with transformed Oc protoplasts. Actinomycin D (Sigma, St. Louis) was added to the cultures to a final concentration of 100 μg mL^{-1}, and protoplasts were removed from the culture for 5, 10, and 20 min after actinomycin D addition. Each sample was immediately sedimented at 18,500*g* for 30 s and frozen in liquid nitrogen. Samples were analyzed by quantitative RT-PCR as described above.

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