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Antisense regulation of the rice *waxy* gene expression using a PCR-amplified fragment of the rice genome reduces the amylose content in grain starch

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Abstract. The *waxy* gene encodes a granule-bound starch synthase. A 1.0-kb portion of the sequence of the rice *waxy* gene, which includes the region between exon 4 and exon 9, was inserted in an antisense orientation between the 35 S promoter and the GUS gene of pBI221. The resultant plasmid, pWXA23, was introduced into rice protoplasts by electroporation. GUS activity was clearly detected in derived callus lines, suggesting that the antisense component of the fusion gene was also expressed. Transgenic rice plants were regenerated from these callus lines and their GUS activity was confirmed. Some of the rice seeds from these transformants showed a significant reduction in the amylose content of grain starch, even though they had become polyploid. These results suggest that even when intron sequences are included, antisense constructs can bring about a reduced level of expression of a target gene. The utility of GUS, included as a reporter gene, for the simple detection of expression of an antisense gene, was apparent from these results.

Key words: Antisense regulation – Transgenic rice – Amylose – *waxy* – Grain starch

Introduction

Rice grain starch consists of amylose and amylopectin, an essentially linear polymer of glucose and a branched polymer of glucose, respectively. The ratio of amylose to amylopectin depends upon the cultivar (the amylose content is 20–30% of the total in cultivars of Indica rice and 17–22% in Japonica rice), and has a major effect on the physical properties of the grain starch. Many mutations affecting the synthesis of starch have been isolated at different loci, suggesting that a combination of several genes is involved in the biosynthesis of starch (Yano et al. 1988).

The ADP-glucose pathway, consisting of the enzymatic reactions catalyzed by ADP-glucose pyrophosphate synthase (EC 2.7.7.27), granule-bound starch synthase (GBSS; EC 2.4.1.21), and starch branching enzyme (SBE; EC 2.4.1.18), is the predominant pathway for the accumulation of storage starch within the amyloplast (Preiss 1992). The gene for GBSS appears to be encoded by the *waxy* gene and the structure of this gene from maize, barley, wheat and rice has already been reported (Klösgen et al. 1986; Okagaki and Wessler 1988; Rohde et al. 1988; Wang et al. 1990; Clark et al. 1991). We previously isolated a fragment that corresponds to the genomic sequence of the *waxy* gene from rice by PCR (Shimada and Tada 1991).

The introduction of the gene for GBSS into a potato *amf* mutant appears to complement the GBSS activity in potato tubers (van der Leij et al. 1991a), showing that GBSS is a key enzyme in the synthesis of amylose in storage starch. In addition, since rice plants with *waxy* mutations have varying amounts of amylose and amylopectin in their endosperms (Sano et al. 1986), it is suggested that the *waxy* gene is a key gene in the control of the amylose content of rice endosperm starch. Therefore, genetic manipulation of this gene may facilitate the control of the level of amylose in grain starch.

Recently, the effects of antisense RNA have been studied and its use has been extended to the control of gene expression (van der Krol et al. 1988). Regulation of the expression of genes that are involved in the

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synthesis of starch has also been examined. When ADP-glucose pyrophosphate synthase in potato was repressed by an antisense RNA that was constructed using the cDNA for this enzyme, inhibition of the enzymatic activity and accumulation of soluble sugars in tubers of transgenic potatoes occurred (Müller-Röber et al. 1992). Inhibition of the expression of the potato gene for GBSS has also been reported. In this case, the antisense RNA prepared from the cDNA of potato GBSS was shown to reduce the amylose content of tuber starch (Visser et al. 1991a). Although the potato gene for GBSS is strongly expressed in tubers (Visser et al. 1991b), structural and genetical similarities are evident between the potato gene for GBSS and the *waxy* gene in monocot plants (van der Leij et al. 1991b). Therefore, antisense regulation of expression of the *waxy* gene may provide a way to reduce the amylose content of the grain starch of cereals.

With the above mentioned possibility in mind, we constructed an antisense *waxy* gene utilizing a PCR-amplified fragment. Here, we describe the features of transgenic rice plants into which the antisense *waxy* gene was introduced. We also show that a fusion gene that contained the antisense *waxy* gene and the GUS gene, as a reporter, allowed the simple selection of transformants in which the antisense gene was expressed.

Materials and methods

Plant materials

The japonica rice cultivar 'Nipponbare' was used in this work. For the preparation of protoplasts, a suspension culture was established by the method of Fujimura et al. (1985).

Plasmids

Plasmid pWX12 contains 1.0 kb of the rice *waxy* sequence, which was obtained by PCR (Shimada and Tada 1991; GenBank Accession no. M55039). The 1.8-kb cDNA of the rice *waxy* protein, containing most of the coding region, was isolated from a cDNA library which had been constructed from developing seeds using the PCR-amplified *waxy* fragment as a probe. pBI221 (purchased from Clontech Lab., Palo Alto, Calif.) contains the gene for β-glucuronidase (GUS) linked to the CaMV 35 S promoter (see Fig. 1). Plasmid pUC19-HPT contains a hygromycin-resistance gene (Tada et al. 1990).

Construction of plasmids

The manipulation of DNA was performed according to the basic procedures described in Current Protocols in Molecular Biology, (Wiley Interscience 1987).

Electroporation

Rice protoplasts were prepared from suspension-cultured cells and then purified by the method of Akagi et al. (1989). The constructed plasmid and pUC19-HPT were introduced together into purified protoplasts by electroporation as described previously (Tada et al. 1990). The density of protoplast cells and the concentration of plasmid DNA were 2×10^6/ml and 20 μg/ml in electroporation buffer, respectively. The electroporation buffer consisted of 35 mM potassium aspartate, 35 mM potassium glutamate, 5 mM calcium gluconate, 5 mM magnesium aspartate, 5 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 0.4 M mannitol (pH 5.8).

Regeneration of rice plants

The culture of the electroporated protoplasts and regeneration to plants were carried out according to the method of Fujimura et al. (1985). Hygromycin-resistant colonies derived from transformants were selected by the methods described previously (Tada et al. 1990).

Assay of GUS activity

Expression of the GUS gene in callus and plant tissues was detected as a blue coloration after staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), as described by Jefferson et al. (1987).

Determination of the amylose content of rice grains

The amylose content of the starch in rice grains was measured by a colorimetric method with iodine-potassium iodide, as described by Juliano (1968), after grains had been ground and then gelatinized by treatment with alkali.

Southern-blot analysis

Plant DNA was extracted from leaves as described by Rogers and Bendich (1985). The purified DNA was digested with restriction enzymes, subjected to electrophoresis on a 0.9% agarose gel and transferred onto a nylon membrane (Boehringer Mannheim, Mannheim, FRG). Hybridization was carried out by the non-radioactive method using a DIG-ELISA DNA labeling and detection kit (Boehringer Mannheim). The *waxy* sequence contained in pWX12 and the GUS sequence in pBI221 were used as probes. A fragment corresponding to 1.0 kb of the sequence of the rice *waxy* gene was prepared from pWX12 by digestion with *Eco*RI and *Xba*I. Similarly, a fragment corresponding to 2.5 kb of the GUS gene was prepared from pBI221 by treatment with *Eco*RI and *Bam*HI.

Northern-blot analysis

Total RNA was extracted from leaves and developing seeds according to Baba et al. (1991). Total RNA was fractionated on a 1.0% agarose/formaldehyde gel and blotted onto a Hybond-N nylon membrane (Amersham, Buckinghamshire, UK). A fragment corresponding to the GUS gene from pBI221 was used as a probe. This fragment was labeled with ^{32}P using a random-priming kit (Boehringer Mannheim) and hybridization was performed as described in Current Protocols in Molecular Biology (Wiley Interscience 1987). Sequence-specific RNA probes, the antisense *waxy*-specific probe and the sense *waxy*-specific probe, were prepared from *waxy* cDNA using SP6-T7 transcription kit (Boeringer-Mannheim). Positive bands were detected with a BAS2000 Bio-image Analyzer (Fuji Photo Film Co. Ltd., Tokyo).

Western-blot analysis

Total protein was extracted from frozen developing seeds ground in buffer containing 125 mM Tris-HCl (pH 6.8), 4%

sodium lauryl sulfate, 4 M urea, 20% glycerol, 5% 2-mercaptoethanol. The resultant crude extract was separated on a SDS-polyacrylamide gel and transferred onto an Immobilon-P PVDF membrane (Millipore). The blotted membrane was treated with the antiserum raised against the maize Wx protein, which was kindly provided by Dr. Baba at the University of Tsukuba, Japan, after blocking with 1% bovine serum albumin in TBS buffer, containing 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl. The immuno-reactive bands were detected using a pico-Blue immunoscreening kit (Stratagene, Calif.).

Results

Construction of the antisense waxy gene

We isolated a 1.0-kb sequence of the *waxy* gene, which includes the region between exon 4 and exon 9, from the rice genome by PCR. In this fragment, the sequence corresponding to introns is approximately 350 bp in length (Shimada and Tada 1991). In order to down-regulate expression of the *waxy* gene, this fragment was inserted in an antisense orientation between the CaMV 35 S promoter and the GUS gene of pBI221 (Fig. 1A). The transcript from the resulting plasmid, pWXA23, was predicted to be a fusion of the antisense *waxy* RNA followed by the mRNA transcribed from the GUS gene. Since the site of initiation of translation remained part of the GUS gene in the fused transcript, β-glucuronidase activity was expected in the transformants (Fig. 1B).

Transformation of rice and screening for transgenic callus lines

The plasmid pWXA23 was introduced into rice protoplasts by electroporation. The plasmid pUC19-HPT, containing a hygromycin-resistance gene, was introduced together with pWXA23 so that resistance of cultured cells to hygromycin could be used for primary selection of transformed cells. Within 4 weeks after electroporation, many hygromycin-resistant cells were obtained and several callus lines were isolated from these cells.

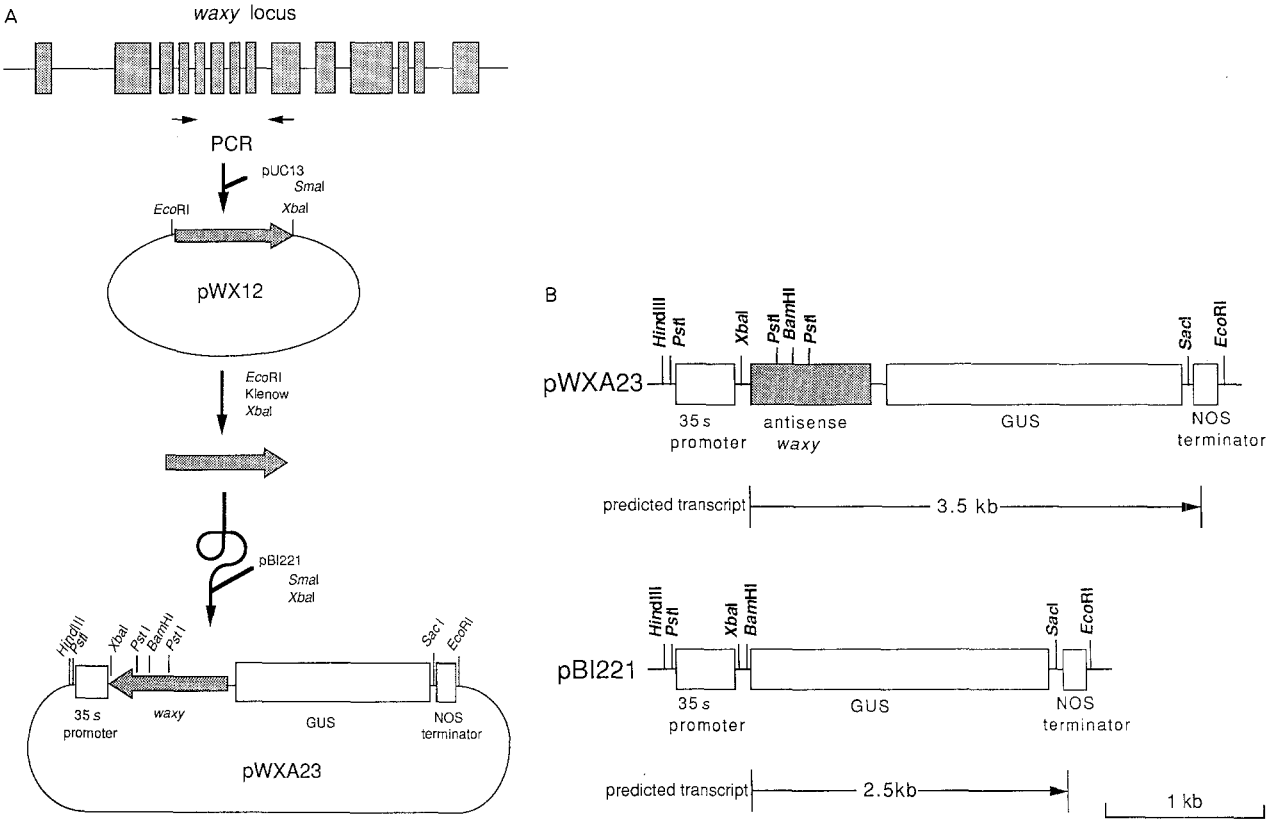

Fig. 1. Strategy for introducing the *waxy* sequence into pBI221 and for the construction of a plasmid (pWXA23) that contains the antisense gene (**A**), and physical maps and predicted transcript from the constructed plasmid (**B**). **A** A sequence was obtained from the *waxy* locus of the rice genome by PCR (Shimada and Tada 1992). *Shaded arrows* indicate the rice *waxy* sequence, with the direction of the gene. 35 S, GUS and NOS in the figure indicate the CaMV 35 S promoter, the GUS gene and the NOS terminator, which are derived from pBI221, respectively. **B** The antisense *waxy* sequence is indicated by a *shaded box*. Cleavage sites by restriction enzymes are indicated above the map. Predicted transcripts and their sizes are shown below the physical maps

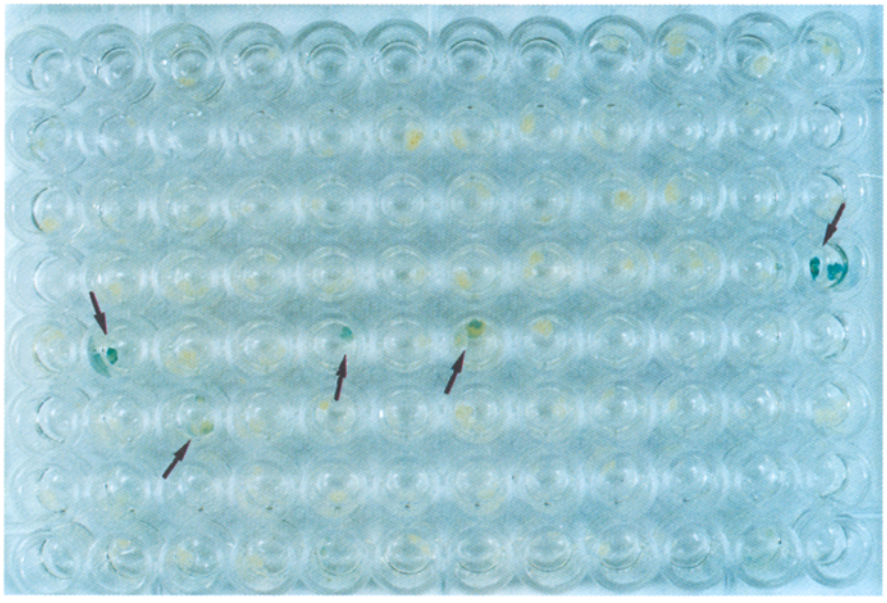

Fig. 2. GUS activity in callus tissues. Cells from each callus line were sampled and stained with X-Gluc for the detection of GUS activity. Cells that were stained blue are indicated by *arrows*

In order to screen recombinants, these callus lines were monitored for their GUS activity by staining with X-Gluc. As shown in Fig. 2, cells from 19 callus lines stained positive, an indication that the introduced fusion gene was actually expressed in these cells. This result indicates that the antisense *waxy* RNA was also expressed in these cells. However, levels of GUS activity in such callus lines were lower than that in a transformant that carried pBI221 (with the GUS gene linked to the 35 S promoter). The activity of β-glucuronidase in these cells was determined to be ten-times lower than that in cells carrying pBI221 (data not shown).

Regeneration of transgenic rice plants

Five plants were regenerated from the independent callus lines that showed GUS activity within 2 months after induction of regeneration. GUS activity was consistently detected in the leaves of these transformants by staining with X-Gluc. This result indicates that the introduced fusion gene was constitutively expressed in these plants. As in callus cells, levels of GUS activity were relatively low (approximately 10%) as compared with the activity in cells that carried pBI221.

Features of transgenic plants

The five transgenic rice plants obtained here all had peculiar features. For example, mature plants were larger than wild-type plants; they also had fewer tillers, thicker shoots and larger glumes than the wild-type plants. These characteristics resembled those of tetraploid plants generated by cell fusion. The control plant, carrying pBI221, had similar features. It appears that all transformants may become polyploid, probably by duplication of their genomes.

Southern-blot analysis of the transformants

We monitored the presence of the introduced fragments within the genomic DNA of the transgenic rice plants by Southern-blot analysis. Genomic DNA prepared from each plant was digested by *Eco*RI and *Hin*dIII and then analyzed. The control plant showed that only the 6.0-kb fragment that corresponds to the genomic *waxy* fragment could hybridize when the 1.0-kb *waxy* fragment was used as probe (Fig. 3A). The genomic sequence at the rice *waxy* locus generates a 6.0-kb fragment upon digestion with *Eco*RI and *Hin*dIII. In the case of WX-2, WX-8 and WX-13, a 4.4-kb fragment hybridized to the *waxy* sequence, as well as the 6.0-kb fragment of the genomic *waxy* sequence. However, in the other transformants, WX-17 and WX-18, a 5.0-kb fragment was detected instead of the 4.4-kb fragment. In addition, a 6.6-kb fragment hybridized to the *waxy* sequence in all transformants (Fig. 3A).

The GUS sequence hybridized to the 4.4-kb fragment in the genomes of WX-2, WX-8 and WX-13 (Fig. 3B). In addition, 6.6-kb, 6.0-kb and 3.0-kb fragments were detected in digests of these genomes. The 6.6-kb fragment also appeared in the case of WX-17 and WX-18 but the 6.0-kb, 4.4-kb and 3.0-kb fragments were not detected. In both these transformants, another fragment, of 9.0 kb, was detected. The wild-type plant gave no distinct bands that hybridized to the GUS sequence (Fig. 3B).

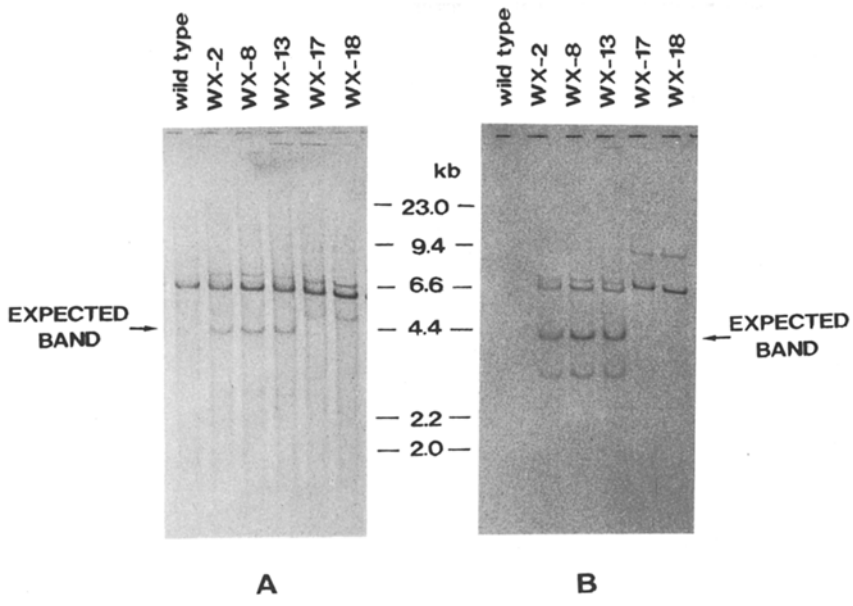

Fig. 3A, B. Southern-blot analysis of genomic DNA from transformants. A *waxy* sequence from pWX12 (**A**) and a GUS sequence from pBI221 (**B**) were used as probes. Bands corresponding to the expected fragments from the introduced plasmid are shown by *arrows*. Genomic DNA from the Nipponbare cultivar was used as the wild-type

Since the 6.6-kb and 4.4-kb fragments hybridized to both probes, it appears that these fragments contain both *waxy* and GUS sequences. Other detected fragments were probably the result of recombination during integration into the genome. From these results, transformants can be divided into two groups: one consisting of WX-2, WX-8 and WX-13 in which the fusion gene is of the expected size; and the other, consisting of WX-17 and WX-18, in which some recombination may have occurred. Transformants from each group may well be derived from the same transgenic cell, since the patterns of hybridization coincided with each other. Thus, we obtained two different kinds of transgenic rice clones carrying the antisense *waxy* gene.

Expression of the fusion gene

Expression of the fusion gene was determined by Northern-blot analysis of leaves of the transgenic plants. A transcript of approximately 3.5 kb was detected in leaves of all transformants and its size coincided precisely with that of the predicted transcript consisting of 1.0 kb of the *waxy* sequence and 2.5 kb of the GUS gene (Figs. 1B, 4). When the antisense *waxy* sequence-specific probe was used, the 3.5-kb transcript, which may be identical to the transcript hybridizing to the GUS probe, was also detected (data not shown). Therefore, it appears that the antisense *waxy* sequence can be co-transcribed with the GUS sequence. However, amounts of the mRNA were lower in WX-2, WX-8 and WX-13 than in NG-14 which carried pBI221. Rates of transcription in these transformants were estimated to be approximately three-times lower

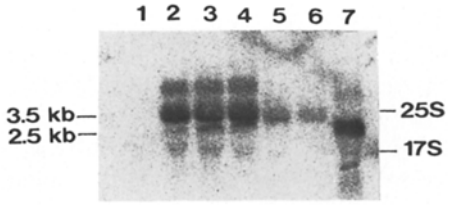

Fig. 4. Northern-blot analysis of the transformants. The GUS sequence from pBI221 was used as a probe. RNA from leaves of the Nipponbare cultivar was used as the wild-type. *Lanes 1–7* contain 10 μg of total RNA from leaves of Nipponbare, WX-2, WX-8, WX-13, WX-17, WX-18 and NG-14, respectively. Internal rRNAs, 25 S, and 17 S rRNA, were used as size markers

than that in NG-14, while in WX-17 and WX-18 plants the rates were far lower (Fig. 4).

Fertility of transgenic plants

It is generally the case that the fertility of tetraploid rice plants is relatively low (unpublished data). Among our five transformants, the fertility of three plants, WX-2, WX-8 and WX-13, was very low and only small numbers of seeds were obtained from these plants. Only five grains from WX-2, one grain from WX-8 and one grain from WX-13 were obtained. The fertility of the other transformants was relatively higher but was still less than 5% of normal. Yields of grains from WX-17 and WX-18 were 30 and 50, respectively, while 100 grains were obtained from NG-14. Transformants containing this antisense construct might be reduced in their fertilities, although the reason for the lower fertility is

unclear. The shapes and sizes of the seeds were similar to those of seeds obtained from tetraploid plants.

Amylose content of seed grains from transformants

The amylose contents of grain starch were assayed by the colorization method using an iodine solution. The amylose content of seeds from NG-14, which contained pBI221, was 17% on average, with a standard deviation estimated as 1.5. It seems that amylose contents in such tetraploid plants are slightly lower than the wild-type ones, as compared with Nipponbare (19% on average; Table 1).

Grains from WX-2 and WX-13 showed obviously lower amylose contents (between 6% and 14%), although few seeds were produced. On the other hand, four out of eight grains from WX-17 and four out of ten grains from WX-18 showed a significantly low amylose content (less than 14%) whereas the amylose content of the others was similar to that of the control (Table 1). Probably, The antisense *waxy* gene was heterozygous in WX-17 and WX-18, and so partitioned into seeds with or without this gene.

We determined GUS activity, as well as the amylose content, in seven seeds from each of NG-14 and WX-18. Although all tested seeds from NG-14 showed GUS activity, some of seeds from WX-18 lacked such activity. The presence of GUS activity was tightly correlated with a reduction in amylose content (Table 1). Therefore, these low values may reflect a decrease in the amylose content in grain starch.

Regeneration of normal diploid plants was performed by the anther-culture procedure (Mercy and Zapata 1987). Several plants were obtained from anthers of each strains. Since seeds from some of these plants also showed a decrease in the amylose content of the grain starch (11–14%) and in GUS activity (data not shown), the antisense gene, as well as the GUS gene, were clearly transmitted to these plants.

Analysis of expression of the antisense waxy gene in developing seeds

We determined the level of expression of the *waxy* gene in developing seeds by Northern-blot analysis. As shown in Fig. 5A, when the expression of the *waxy* gene was analyzed using the sense *waxy* sequence-specific probe, the level of the *waxy* mRNA was significantly reduced, while a very low level of the 3.5-kb transcript corresponding to the antisense transcript was detected (data not shown). The level of expression of *sbe1*, encoding a starch branching enzyme (Kawasaki et al. 1993), was similar between NG-14 and WX-18 (data not shown). These results suggest that the antisense transcript may function to decrease the amount of sense mRNA, resulting in a reduction in the level of expression of the *waxy* gene.

Accumulation of the Wx protein (the granule-bound starch synthase, the gene product of the *waxy* gene) was also determined by Western-blot analysis. The antiserum that we used shows an unexpected immuno-reaction to several proteins as well as to the target Wx protein since it has been created using the partially purified Wx protein. As shown in Fig. 5B, the amount of the 60-kDa Wx protein was specifically reduced, while no difference in the intensity of the immuno-reaction to the other proteins was found. This result presumably reflects a decrease of *waxy* mRNA. Thus, it appears that expression of the antisense *waxy* gene may result in a decrease of the Wx protein and a low level of amylose production in seeds.

Table 1. Amylose content of seeds of transgenic plants

Strains	WX-2	WX-8	WX-13	WX-17	WX-18	NG-14	Nipponbare
Introduced plasmid	pWXA23	pWXA23	pWXA23	pWXA23	pWXA23	pBI221	None
GUS activity	+	+	+	+	+	+++	−
Amylose content in seed grain (%)	12	nd	9	9	18	15	18
	14			19	9	18	20
	6			18	19	19	20
	14			14	13(+)	16(+)	19
	10			14	18(−)	16(+)	18
				11	19(−)	18(+)	
				18	13(+)	17(+)	
					18(−)	15(+)	
					17(−)	15(+)	
					15(+)	19(+)	

GUS activity of the mature plants is indicated by "+" for moderate, and "+++" for strong activity, respectively. GUS activity in the tested seeds is shown by (+) and no activity by (−). The amylose content of each grain was measured. Field-grown rice (Nipponbare) was used as a control for the non-tranformed plant; nd, not determined

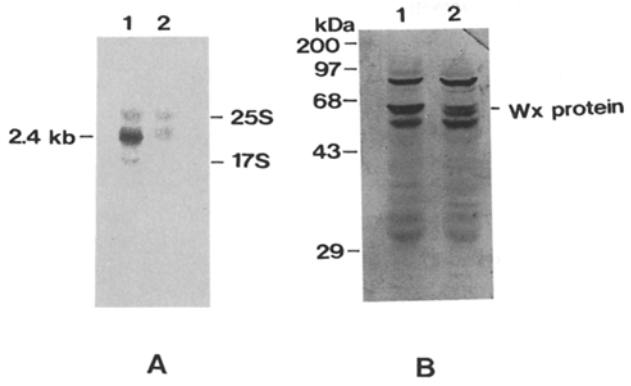

Fig. 5. Expression of the *waxy* gene (**A**) and accumulation of the Wx protein (**B**) in the grains. In **A**, each lane contains 10 µg of total RNA prepared from each of a mixture of a five developing seeds of NG-14 (*lane 1*) and WX-18 (*lane 2*). The sense *waxy* sequence-specific probe was used for Northern analysis. Each lane in **B** contains crude extract from each of a mixture of five grains of NG-14 (*lane 1*) and WX-18 (*lane 2*). The expected bands corresponding to the *waxy* mRNA and the Wx protein, respectively, are indicated by *arrows*

Discussion

Our experiment demonstrated that an antisense gene, constructed from a portion of the genomic sequence of the corresponding gene, can be used for down-regulation of the expression of the gene. It was also apparent that the GUS gene which followed the antisense sequence was translated and functioned as a reporter, which may be useful for the simple selection of recombinant plants.

A significant reduction in the amylose content of rice grain starch was observed. This result also indicates that the rice Wx protein is intimately involved in the synthesis of amylose in grain starch. A rice Wx mutant has no amylose in the grain starch. If our antisense RNA completely repressed the expression of the gene at the *waxy* locus, the transformants might resemble *wx* mutants. In our experiments, grains having an apparently lower level of amylose were obtained but no complete absence of amylose was observed. This result may be due to only partial repression of *waxy* gene expression. The antisense gene used here was constructed from a portion of the *waxy* gene which was isolated by PCR. Since it has been reported that antisense genes constructed from a partial sequence of cDNA for chalcone synthase (CHS) gave various levels of antisense regulation (van der Krol et al. 1990) there appears to be a precedent for our results.

Several intron sequences accounted for approximately 35% of the 1.0-kb region. These introns were not removed by splicing in the antisense transcript (Fig. 4). Nevertheless, an effect of the antisense RNA was observed. When does the antisense RNA interact with the target RNA? The pre-mRNA, which is still unspliced, might be the target of the antisense RNA. Low-level repression by the antisense RNA might be caused by inefficient interaction between the spliced transcript and the antisense RNA that contains the additional intron sequences.

An antisense gene consisting of a partial sequence and even one containing several intron sequences can be functional in the regulation of gene expression. Perhaps such structures can be modified to enable us to control the level of antisense activity. Since improvements in rice grain starch require fine-tuned regulation of gene expression, a partial sequence or intron sequences may be of use in the construction of appropriate antisense genes.

During the transformation process, unexpected recombination often occurs. Our transformants may be polyploid, probably tetraploid, plants since their phenotypic features showed a similarity to those of tetraploid rice plants. Even in our case, recombination may have occurred and multiple copies of the introduced gene may have been integrated to generate variations in the inserted fragments. When numbers of foreign genes are introduced, many mutations may be induced within the genome by insertion and many genes in the genome will be interrupted by such insertion. In order to rescue lethal damage, duplication of the genome may occur, probably by self-duplication or by cell fusion, enabling mature plants to regenerate. Somatic mutation may occur during regeneration of the transformants, since similar events have been frequently observed (unpublished data). In our case, unidentified selective factors might be operatative under our experimental conditions. It seems that the level of fertility may be related to the frequency of appearance of low-amylose seeds, though a position effect of the insertion can not be excluded. The fertility of the transformants might also be involved in the strength of the effect of the antisense *waxy* gene.

The transcript from the antisense *waxy* gene constructed here consist of two portions, a sequence which is expected to be functional as the antisense *waxy* gene and a region for the GUS gene (Fig. 1B). GUS activity was expressed from this fusion even in the callus. Since a correlation between the level of reduction of amylose content in the grain starch and the expression of GUS activity was evident, GUS activity can be used as an efficient reporter for the expression of the antisense gene. However, levels of GUS activity were lower than that in control plants. The efficiency of translation might be depressed by the antisense gene since the transcript contained such a long sequence preceding the site of initiation of translation.

Expression of the antisense *waxy* gene resulted in a reduction of the level of *waxy* mRNA and an accumulation of the Wx protein (Fig. 5). It was also shown that

the *waxy* gene encodes the major amylose-synthesizing enzyme of the rice grain, though it has been reported that in pea the "waxy" gene product may not be involved in major amylose synthesis (Smith 1990).

Since seeds from the transgenic plants correspond to the T2 generation, the antisense gene could be inherited in accordance with Mendel's rule. The variation of amylose content in the T2 generation may depend on this rule. It is clear that antisense regulation can be applied to molecular breeding for down-regulation of gene expression in rice grains though it is necessary that this antisense effect be maintained through several generations for it to be of commercial value.

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