Enhancement of foreign gene expression by a dicot intron in rice but not in tobacco is correlated with an increased level of mRNA and an efficient splicing of the intron

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Received October 2, 1990; Accepted November 12, 1990

ABSTRACT

The first intron of castor bean catalase gene, *cat-1* was placed in the N-terminal region of the coding sequence of the β-glucuronidase gene (*gusA*) and the intron-containing *gusA* was fused with the cauliflower mosaic virus (CaMV) 35S promoter. Using this plasmid, pIG221, the effect of the intron on expression of β-glucuronidase (GUS) activity was examined in transgenic rice calli and plants (a monocotyledon), and transgenic tobacco plants (a dicotyledon). The intron-containing plasmid increased the level of GUS enzyme activity 10 to 40-fold and 80 to 90-fold compared with the intronless plasmid, pBI221, in transgenic rice protoplasts and transgenic rice tissues, respectively. In contrast, the presence of the intron hardly influenced the expression of the GUS activity in transgenic tobacco plants. Northern blot analysis showed that the catalase intron was efficiently spliced in rice cells while transgenic tobacco plants contained both spliced and unspliced *gusA* transcripts in equal amounts. Furthermore, the level of the mature *gusA* transcript in transformed rice calli was greatly increased in the presence of the intron. The catalase intron was removed at the same splice junctions in transgenic rice and tobacco plants. These findings indicate that the stimulating effect of the intron on GUS expression is correlated with an efficient splicing of pre-mRNA and an increased level of mature mRNA.

INTRODUCTION

The importance of the presence and processing of intron sequences in gene expression has been studied by applying molecular genetic approaches in both animals and plants. The correct removal of introns not only makes functional mature mRNA but also increases the level of gene expression and introns are now considered to be one of the important elements which control the expression of various foreign genes in eukaryotes(1).

Recent advancement of gene transfer into plant cells and the development of various reporter genes make it possible to quantitatively examine the expression of foreign genes in both monocot and dicot cells(2,3). The expression of maize alcohol dehydrogenase (*Adh-1*) gene is stimulated by the addition of its own intron and chimeric genes consisting of *Adh-1* intron-1 and the chloramphenicol acetyltransferase (CAT) or the luciferase reporter gene were also highly expressed in maize cells(4). In this study with maize cells, an enhanced expression of a reporter gene was correlated with an increased level of mRNA. Similar enhancement of foreign gene expressions has been reported for the maize *Adh-1* intron in wheat(5) and rice(6), the maize *shrunken-1* intron in grass species including maize(7) and the rice actin intron in rice(8). No such stimulation of gene expression by a potato intron was, however, observed in *Arabidopsis thaliana*(9). Therefore, it appears that stimulation of foreign gene expression by introns is a general phenomenon observed in monocotyledonous species or cereal cells. However, the mechanism(s) underlying the stimulating effect of introns on foreign gene expression remains to be elucidated at the molecular level.

As a first step toward understanding the mechanism(s) of stimulating effect of introns on foreign gene expression and possible differentiation in RNA processing machinery between monocot and dicot cells, a CaMV 35S-*gusA* construct containing the first intron of a castor bean catalase gene in the coding region of the *gusA*(10) was introduced into monocot(rice) and dicot(tobacco) cells and its expression was compared to that of an intronless 35S-*gusA* construct. A significant stimulating effect of the intron was observed in rice cells, but not in tobacco cells, and this stimulation on the *gusA* expression was correlated with an efficient splicing of pre-mRNA and an increased level of mRNA. In both rice and tobacco cells, splicing occurred at the same 3'-acceptor site which had been created by *in vitro* mutagenesis at 6 nucleotides upstream of the original 3'-acceptor site of the catalase intron.

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MATERIALS AND METHODS

Plant transformation

Tobacco(*Nicotiana tabacum* cv. BY4) plants were used for *Agrobacterium*-mediated transformation(10). Rice(*Oryza sativa* L.) Oc line or suspension cultures derived from a japonica variety Nipponbare were used for the isolation of protoplasts and electroporation. The methods of rice transformation and assay conditions for GUS enzyme activity were as described previously(6,11).

Construction of intron-containing GUS reporter gene

The plasmid pBI221 has been described(2). The details of the construction of pIG221 which contains a castor bean catalase intron within the *gusA* coding region have been described(10). In brief, the first intron of catalase-1 gene located between the 5th and 6th codons was inserted at 15 bp downstream from the initiation codon ATG(Fig. 1). In addition, to eliminate the translation of the *gusA* transcripts initiating from the intron sequences, a termination codon TAG was created at 6 bp upstream of the 3′ splice site in the intron by site-directed mutagenesis. Consequently, the GUS polypeptide produced by pIG221 after the removal of intron is expected to be 8 amino acids longer than that produced by pBI221.

Northern blot analysis

Total or poly(A)$^+$ RNA isolated from tobacco leaves or rice calli by a published method(12) was separated on 1% agarose gel in the presence of formaldehyde and transferred to a nylon membrane Hybond-N. Filters were hybridized to a random-primed probe of the *gusA* gene or the catalase intron by following the protocol of Amersham.

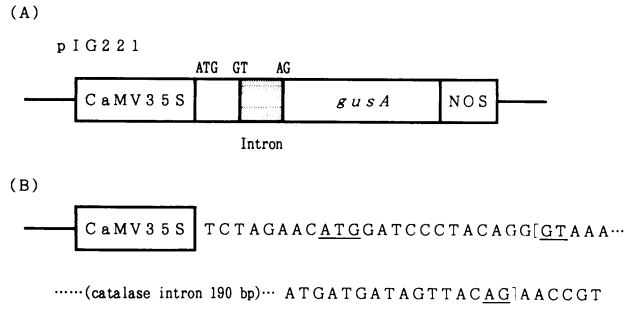

Fig. 1. Structure of intron-containing *gusA* reporter gene. A) The structure of pIG221 which contains the castor bean catalase intron within the *gusA* coding region. Shadowed box shows the catalase intron and 5′ GT and 3′ AG splice sites are indicated. 35S and NOS indicate the CaMV 35S promoter and the nopaline synthase terminator, respectively. B) The nucleotide sequence of the boundary of 5′ and 3′ ends of the catalase intron in pIG221.

Sequence analysis of splice junctions

The complementary DNA(cDNA) was synthesized by the method of Gubler and Hoffman(13) using poly(A)$^+$RNA isolated from transformed rice calli as a template. This cDNA was amplified by the polymerase chain reaction(PCR)(14) using two primers of synthetic oligonucleotides of 17 mers 5′-TCTAGAACATGG-ATCCC3′located just upstream of 5′ end of intron and 18 mers 5′GATTCCGGCATAGTTAAA-3′ which is complementary to the *gusA* coding region. Amplified fragments containing the splice junctions were subcloned in pUC19 and sequenced according to Sanger *et al.*(15).

In the case of tobacco plants, the splice junctions were isolated and sequenced in the cDNA bank constructed from pIG221transformed tobacco leaves.

RESULTS

GUS expression is enhanced by a castor bean intron in rice cells but not in tobacco cells

The GUS reporter gene fused with the CaMV 35S promoter containing the catalase intron (pIG221) or lacking the intron (pBI221) (2) were electroporated into rice protoplasts (6,11). GUS enzyme activity in rice protoplasts was quantitatively measured 48 hours after electroporation (16). The intronless construct, pBI221, showed GUS activity 6 to 20-fold above background. The construct with the intron gave GUS activity 10 to 40 times that of pBI221 (Table 1). This stimulating effect of GUS expression by the catalase intron was more profound in transgenic rice calli and root than that in protoplasts. In these tissues, the presence of the intron stimulated the GUS activity 80 to 90-fold compared with pBI221 (Table 1).

The enhancement of GUS expression by the intron found in rice cells was much greater than that observed in tobacco protoplasts, leaf, root and stem tissues(10). The level of GUS activity in tobacco protoplasts electroporated with pIG221 was approximately 1.7 times higher than that with pBI221. The slight enhancement of GUS activity by the catalase intron was also observed in transformed tobacco tissues in which only 2.2-fold increase of GUS activity was detected(10). These results indicate that a strong enhancement of foreign gene expression by the presence of an intron is species-specific and in our case, a monocotyledonous plant showed a greater stimulation of GUS expression by the presence of the castor bean intron.

Table 1. GUS enzyme activity in protoplasts and transformed rice tissues.

Plasmid	protoplasts			calli$^+$		root$^+$
DNA	exp.1^{++}	exp.2	exp.3			
None	130	15	41	<10	<10	103
pBI221	1630	390	258	2237	3622	3960
pIG221	18577	14858	6453	388362	129914	306746

Rice protoplasts isolated from Oc or Nipponbare suspensions were used for transient and stable transformation, respectively. Conditions for protoplast isolation and electroporation were described previously(3). GUS enzyme activity is expressed as p mole 4-MU(4-Methylumbelliferone) per minute per mg protein.
$^+$GUS activity of an independent rice callus or root was measured
$^{++}$The results of three independent experiments were shown.

Histochemical analysis of transgenic rice plants showed that GUS activity was highly expressed in roots and around the vascular tissues of leaf (data not shown). In comparison with our previous study showing the expression of the CaMV 35S-*gusA* gene(pBI221) in various tissues of transgenic rice plants(3), we concluded that the presence of the intron did not affect the cell-type specific expression of the CaMV 35S promoter.

Northern blot analysis of GUS transcript

In order to examine whether the enhancement of GUS expression by the intron observed in rice cells is correlated with elevated level of GUS mRNA and/or efficiency of splicing, total RNA and poly(A)$^+$RNA molecules were isolated from the rice calli carrying pIG221 and subjected to Northern blot analysis. RNAs isolated from rice calli transformed with pIG221 and pBI221 showed the signal of 1.8 kilonucleotides hybridizing to the *gusA* coding region(Fig. 2A). Moreover, the rice calli transformed with pIG221 produced approximately 10 times more *gusA* transcripts than that of pBI221. Because no hybridization was detected when the intron was used as a probe, we concluded that the 1.8 kilonucleotide transcript detected was the spliced mRNA of pIG221 in rice cells.

In contrast to the results of Northern blot analysis of RNAs isolated from rice cells, RNA analysis of tobacco cells gave rise to a distinct result. In a transgenic tobacco, one other hybridizing band of 2.0 kilonucleotide was detected in addition to the 1.8 kilonucleotide band, and intensities of the two bands were comparable suggesting that the splicing of the intron was not efficient(Fig. 2B, lane 1 and 2). Furthermore, the intensity of the 1.8 kilonucleotide band which is presumably a spliced mRNA was similar between pBI221- and pIG221-transformed tobacco tissues. That the longer transcript of 2.0 kilonucleotide did contain the intron sequences was confirmed by hybridizing the same blot with the intron as a probe(Fig. 2B lane 3).

These analyses of the *gusA* transcript indicate a strong correlation of the enhanced expression of the GUS reporter gene observed in rice cells with the increased level of spliced mRNA and efficient splicing of the intron. Moreover, it should be noted that a dicot intron is efficiently spliced in rice, a monocot plant, however, not in tobacco. This suggests that the intron whether it is derived from a monocot or a dicot plant may be compatible with a heterologous species depending upon the context in which an intron is placed within a gene. Our results also suggest a possibility that an efficient splicing of the intron leads to an increased level of mRNA resulting in the enhancement of gene expression.

Determination of spliced sites by sequencing the GUS cDNA

In order to examine whether correct splice sites were recognized by heterologous species, we have determined the spliced junctions of the *gusA* transcripts. The poly(A)$^+$ RNA was isolated from the rice calli transformed with pIG221 and cDNA was synthesized

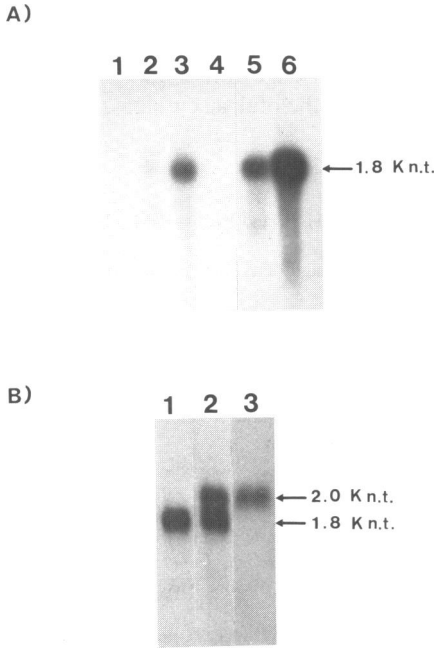

Fig. 2. Northern blot analysis of RNAs isolated from transformed rice calli and tobacco plants. A) Northern analysis of transformed rice calli. Total RNAs (10 μg each, lane 1−3) or poly(A)$^+$RNAs (1.5 μg each, lane 4−6) were isolated from transformed or untransformed rice calli and separated in agarose gel. Blotted membrane was hybridized to the ^{32}P-labelled *Xba*I-*Sac*I fragment of pBI221 which contains the GUS coding region(2). (lane 1,4) untransformed rice calli, (lane 2,5) pBI221- transformed rice calli, (lane 3,6) pIG221-transformed rice calli. B) Northern analysis of transformed tobacco leaves. Poly (A)$^+$ RNAs (1.5 μg each) isolated from transformed tobacco leaves were analyzed using *gusA* DNA (lane 1 and 2) or catalase intron (lane 3) as a probe. (lane 1) pBI221-transformed tobacco leaves, (lane 2,3) pIG221-transformed tobacco leaves.

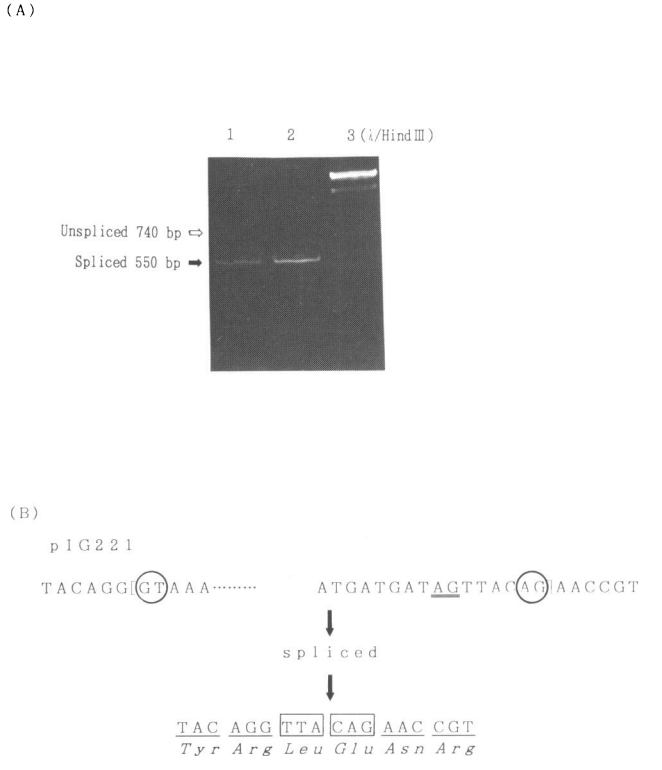

Fig. 3. Characterization of splice junctions in transgenic rice. A) Analysis of the DNA molecules with splice junctions amplified by PCR. An amplified DNA fragment using different amounts of the template cDNA (lane 1, 10 ng, lane 2, 30 ng)were analyzed in 5% polyacrylamide gel. Lane 3; molecular size marker λ-*Hind*III. B) The nucleotide sequence of the boundary of 5′ and 3′ splice sites in pIG221 spliced in rice or tobacco plants. The native 5′ and 3′ splice sites in the catalase intron are indicated by circles. Another 3′ splice site AG was created by site-directed mutagenesis shown by double underline. This new AG was exclusively used as a 3′ splice site both in rice and tobacco plants. Two amino acids newly added to GUS polypeptides are indicated by boxes.

by the method of Gubler and Hoffman(13). Then fragments flanking the expected spliced junctions were amplified by polymerase chain reaction(14). The PCR products were expected to be 550 bp and 740 bp for spliced and unspliced mRNAs, respectively. In accordance with the results of Northern blot analysis, only the band of 550 bp was amplified indicating that the majority of the GUS mRNA was correctly spliced in rice cells(Fig. 3A).

This amplified DNA fragment of 550 bp was subcloned and sequenced. The sequence of the cDNA revealed that the catalase intron was correctly removed at the original 5′ splice site GT. However, a newly created dinucleotide AG at 6 basepairs upstream of the native splice site was used as a 3′ splice site(Fig. 3B). This AG site was created by inserting A residue by *in vitro* mutagenesis to produce a TAG stop codon in the intron. To further examine whether this newly created AG site is exclusively used as the 3′ splice site, we have sequenced eight independent clones derived from the PCR products and found that all the clones had the same sequences. Similar sequence analysis of nine independent cDNA clones containing the spliced *gusA* mRNA from transformed tobacco cells showed that the same 3′ AG dinucleotide instead of the native 3′ splice site was exclusively used for splicing. As a result of this change in the 3′ splite site, two amino acids, leucine and glutamine were added to the native GUS protein in both rice and tobacco. These sequence analyses of *gusA* mRNAs isolated from rice and tobacco cells indicate that both species use the same 5′ and 3′ splice sites. Interestingly, the newly created 3′ splice site at 6 bp upstream of the native 3′ splice site was exclusively used by these two heterologous species.

DISCUSSION

We describe here that the presence of the first intron of the castor bean catalase gene greatly enhances the GUS expression in transformed rice cells but not in tobacco cells. The difference in the effect of the intron on foreign gene expression in these two species is correlated with the difference in the amount of mature *gusA* transcript produced in transgenic plants and the efficiency of the intron-splicing.

The stimulating effect of plant introns on foreign gene expression has been previously reported. Insertion of maize *Adh1* introns in untranslated leader region of chimeric constructs increases expression of maize *Adh* gene or CAT reporter gene 50 to 100-fold in transformed maize cells(4). In this case, a construct containing the *Adh-1* intron located between the *Adh* promoter and the *Adh-1* gene produced 40-fold more mRNA than a construct lacking the intron. The mechanism of the stimulating effect of *Adh* introns was very similar to that of the catalase intron though the castor bean catalase intron was located within the *gusA* coding region. The effect of maize *Adh-1* intron placed in the untranslated leader sequence of the *gusA* transcript has been also investigated in rice cells(6). The expression of the *gusA* reporter gene driven by the 35S promoter was increased only 4 to 6-fold in rice protoplasts. The analysis of the *gusA* mRNA revealed the incomplete splicing of maize *Adh-1* intron in rice cells(Kyozuka *et al.* unpublished results). These results also support the notion that the stimulating effect of introns is correlated with the efficient splicing of the intron and the increased level of mature mRNA.

Our results indicated that an intron derived from a dicotyledonous species is efficiently processed in a monocot species. This agrees with a recent report(17) showing that the third intron of a french bean phaseolin gene which is inserted into the coding sequence of the *nptII* gene is efficiently spliced in transformed rice cells. However, it has been also shown that a wheat gene for small subunit of Rubisco is not efficiently processed in transgenic tobacco plants(18). Similarly, the maize *Adh-1* intron in 35S-*gusA* construct is not efficiently spliced in transgenic rice plants. These studies suggest that the machinery used for the processing of pre-mRNAs may be different in each plant species and that efficiency with which a foreign intron is spliced is influenced by several factors such as sequence of the intron(19), sequence of the exon flanking the intron and location of the intron within a gene(20).

Sequence analysis of the *gusA* mRNAs from pIG221-transformed plants showed that both rice and tobacco plants used the same splice sites. More interestingly, the AG dinucleotide newly created at 6 bp upstream of the native 3′ splice site of the catalase intron was exclusively used for splicing in both species. This is the first finding that an artificial splice site was selected for intron-splicing in plant cells. In mammalian cells, 3′ splice site is determined by a scanning process of spliceosome which recognizes the first AG located at 3′ of the branch point/polypyrimidine tract(21). The same mechanism of intron-splicing may be applicable to the plant system because the first AG dinucleotide which is found at downstream of the branch point was exclusicely used as the 3′ splice site in rice and tobacco.

So far, it is not clear why a large amount of mRNA is produced in relation to the intron-splicing. Further analysis of the mechanism of splicing and mRNA accumulation will make clear the stimulating effect of plant intron on foreign gene expression.

ACKNOWLEDGEMENTS

We gratefully acknowledge all of the members of Plantech Research Institute for helpful discussions.

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