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Isolation of an Efficient Actin Promoter for Use in Rice Transformation

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We have characterized the 5′ region of the rice actin 1 gene (*Act1*) and show that it is an efficient promoter for regulating the constitutive expression of a foreign gene in transgenic rice. By constructing plasmids with 5′ regions from the rice *Act1* gene fused to the coding sequence of a gene encoding bacterial β-glucuronidase, we demonstrate 2that a region 1.3 kilobases upstream of the *Act1* translation initiation codon contains all of the 5′-regulatory elements necessary for high-level β-glucuronidase (GUS) expression in transient assays of transformed rice protoplasts. The rice *Act1* primary transcript has a noncoding exon separated by a 5′ intron from the first coding exon. Fusions that lack this *Act1* intron showed no detectable GUS activity in transient assays of transformed rice protoplasts. Deletion analysis of the *Act1* 5′ intron suggests that the intron-mediated stimulation of GUS expression is associated, in part, with an in vivo requirement for efficient intron splicing.

INTRODUCTION

Significant progress has recently been made in the application of gene transfer techniques to previously recalcitrant crop plants, such as rice (Ou-Lee, Turgeon, and Wu, 1986; Uchimiya et al., 1986; Gasser and Fraley, 1989). However, a major limitation in the development of this technology has been the lack of an efficient promoter for high-level constitutive expression of foreign genes in transgenic rice plants. Although the cauliflower mosaic virus *35S* promoter (Guilley et al., 1982) has been widely used for this purpose in a number of dicotyledonous plant transformation systems, previous work in our laboratory has shown that this promoter has low activity in transformed rice cells (D. McElroy, W. Zhang, J. Cao, and R. Wu, unpublished results). Furthermore, a recent report suggests that the pattern of cauliflower mosaic virus *35S* promoter activity in transgenic plants may not be constitutive (Benfey and Chua, 1989). Work in our laboratory has shown that the 5′ region of the maize *Adh1* gene (Dennis et al., 1984) is 10 times to 20 times more active than the CaMV *35S* promoter in transformed rice protoplasts and calli (Zhang and Wu, 1988; W. Zhang, L.-F. Liu, and R. Wu, unpublished results). However, the maize *Adh1* promoter is induced by anaerobic stress in transformed rice protoplasts and is not constitutively active in all transformed rice tissues (Zhang and Wu, 1988).

Plant actin promoters are likely to be active in all tissues because actin is a fundamental component of the plant cell cytoskeleton (Seagull, 1989). In rice, there are at least eight actin-like sequences per haploid genome; four of these have been isolated and shown to differ from each other in the tissue- and stage-specific abundance of their respective transcripts (Reece, 1988; McElroy, Rothenberg, and Wu, 1990; Reece, McElroy, and Wu, 1990). The rice actin 1 gene, *Act1*, was found to encode a transcript that is relatively abundant in all rice tissues and at all developmental stages examined. Therefore, we set out to characterize the 5′-flanking region of the rice *Act1* gene.

In this paper, we describe the isolation and partial sequencing of a genomic clone containing a region 5′ of the rice *Act1* coding sequence. A number of fusions between the *Act1* 5′ region and a bacterial β-glucuronidase (*Gus*) coding region were constructed and used in transient expression assays of transformed rice protoplasts. (Although *Escherichia coli* β-glucuronidase is encoded by the *uidA* gene, we have denoted this gene as *Gus* for the purposes of this publication.) Our results suggest that the regulatory element(s) necessary for maximal *Act1* promoter activity in transformed rice protoplasts are located within a region 1.3 kb upstream of the *Act1* translation initiation codon. The rice *Act1* promoter is approximately 5 times to 10 times more active than the maize *Adh1* promoter in transformed rice cells, suggesting that the rice *Act1* 5′ region contains an efficient promoter for use in rice transformation.

A complete structural analysis of the rice *Act1* gene had previously led to the identification and localization of a 5′-

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noncoding exon, separated by a 5′ intron from the first coding exon of the *Act1* sequence (McElroy et al., 1990). It has been reported that a 5′ intron in the maize *Adh1* gene is essential for the efficient expression of foreign genes from the maize *Adh1* promoter (Callis, Fromm, and Walbot, 1987). To investigate the effect of the rice *Act1* 5′ intron on gene expression, we created a number of *Gus* fusion plasmids containing alterations to the *Act1* 5′ intron. The results of transient assays of GUS activity in rice protoplasts transformed with these various constructs indicate that the 5′ intron of the rice *Act1* gene is essential for efficient foreign gene expression from the *Act1* promoter. Furthermore, our results suggest that the intron-mediated stimulation of gene expression is not a function of any enhancer-like activity within the intron sequence but is associated, in part, with an in vivo requirement for efficient intron splicing.

RESULTS

Characterization of a Clone Containing the Rice *Act1* 5′ Region

To begin our analysis of the rice *Act1* regulatory regions, we carried out restriction mapping of a λEMBL4 phage clone, λ*Act1*, which had previously been shown to contain the rice *Act1* gene (Reece et al., 1990). The resulting restriction map of the 15.1-kb λ*Act1* insert, together with an indication of the position of the *Act1* coding and noncoding exons (McElroy et al., 1990), is shown in Figure 1A. A 5.3-kb HindIII fragment from the λ*Act1* insert, spanning a region from 3.9 kb upstream of the *Act1* coding sequence to a point within its third coding exon, was isolated and cloned into the HindIII site of pBluescriptII-KS (Stratagene) to produce the plasmid p*Act1*-A. A restriction map of the p*Act1*-A insert is shown in Figure 1B, which also indicates the strategy used to sequence an EcoRV fragment that covers a region 2.1 kb upstream of the translation initiation codon of the *Act1* gene. This sequence is shown in Figure 2.

A number of small repetitive elements were identified in the 5′-flanking region of the rice *Act1* gene. A 12-bp direct repeat, GGTTTTTAAGTT (Figure 2, region i), is located between bases −608 to −619 and −559 to −570. A tandem (imperfect) direct repeat of 16 bp, $AA\begin{matrix}G\\C\end{matrix}CCC(T)AAAGT\begin{matrix}G\\C\end{matrix}CTA$ (Figure 2, region ii), is located between bases −316 and −345. Twenty base pairs downstream of this tandem direct repeat are eight tandem copies of an imperfectly repeating pentamer with the consensus sequence CCCAA (Figure 2, region iii). We identified a number of sequences downstream of these repeated elements that have previously been implicated in the control of gene expression. From bases −146 and −186, there is a poly(dA) sequence where 35 out of 40 bases are A (Figure 2, region iv). Between bases −35 and −41, there is a putative TATA box (Figure 2, region v). The sequence and position of the putative *Act1* TATA box are in agreement with that previously determined for a number of other plant genes (Joshi, 1987).

The sequence around the *Act1* transcription initiation site, as previously determined by primer extension analysis (McElroy et al., 1990), CCTACCA, is similar to the consensus sequences for transcription initiation, YYYAYYA (Y = pyrimidine), previously determined for a number of plant genes (Joshi, 1987). The 79-bp noncoding exon located 3′ of the putative *Act1* TATA box is GC-rich (77.5%) and consists of a number of tandemly repeated $\begin{matrix}A\\T\end{matrix}CC$ triplets (Figure 2, region vi). The 5′ intron of the rice *Act1* gene is 313 bp long. Its 5′-acceptor splice site (G/gta), and 3′-donor splice site (ttttttgtag/G), follow the consensus sequence previously determined for a number of plant genes (Shapiro and Senapathy, 1987). Within the *Act1* 5′ intron, between bases +359 and +363 (Figure 2, region vii), we identified a putative branch-point site for mRNA splicing whose sequence, gtgac, and distance from the 3′-donor splice site bear similarity to the location and consensus sequence for animal mRNA branch-point splice sites, YTRAC (R = purine) (Keller and Noon, 1985).

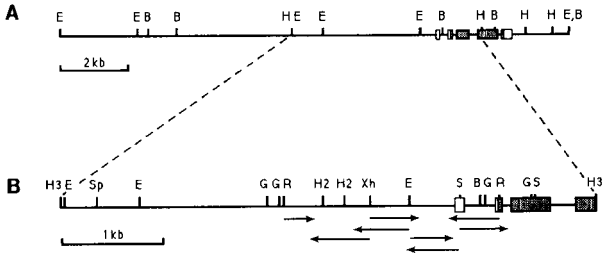

Figure 1. Restriction Maps of the λ*Act1* and p*Act1*-A Inserts.

(A) Restriction maps of the λ*Act1* insert determined by single and double restriction enzyme digestion, as detailed in Methods. The enzyme sites are abbreviated as follows: B, BamHI; E, EcoRI; H, HindIII. Noncoding and coding portions of *Act1* exons are depicted by open and filled boxes, respectively.

(B) Restriction maps of the p*Act1*-A insert determined by single and double restriction enzyme digestion, as detailed in Methods. The enzyme sites are abbreviated as follows: B, BamHI; G, BglII; E, EcoRI; R, EcoRV; H2, HincII; H3, HindIII; Sp, SphI; S, SstI; Xh, XhoI. The position of the p*Act1*-A 5.3-kb HindIII insert within the λ*Act1* restriction map is indicated by dashed lines between **(A)** and **(B)**. Noncoding and coding portions of *Act1* exons are depicted by open and filled boxes, respectively. The strategy used to sequence the 2.1-kb EcoRV fragment within the p*Act1*-A insert is indicated by horizontal arrows. The lengths of the horizontal arrows are indicative of the size of the sequence obtained.

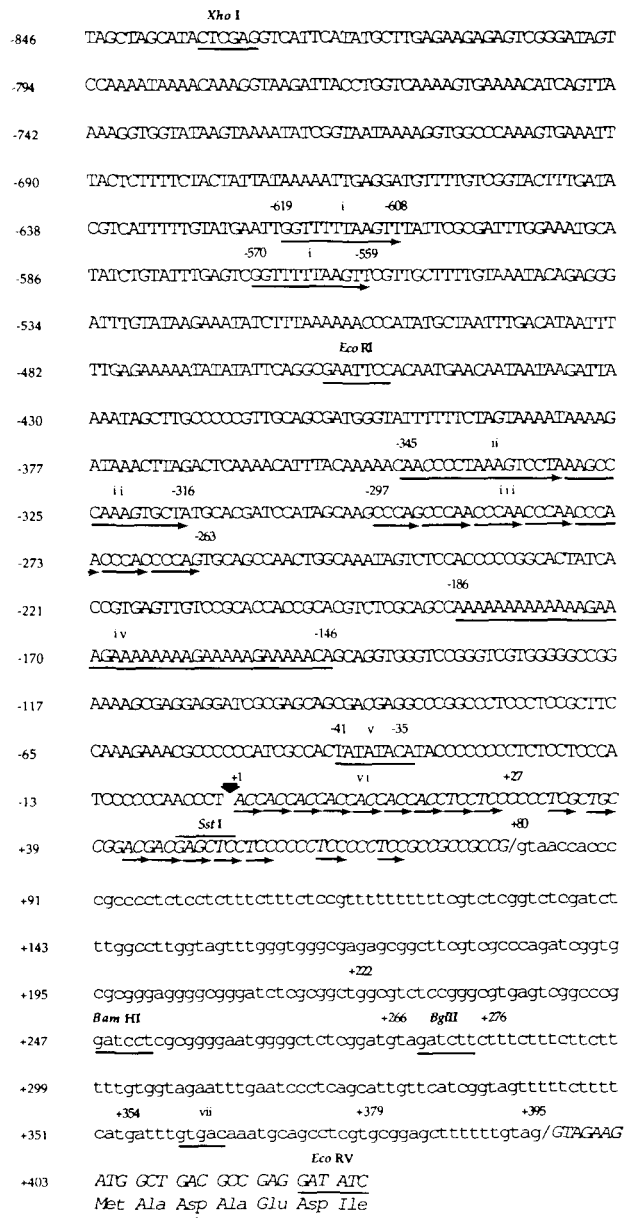

Figure 2. Nucleotide Sequence of the 5′ Region of the Rice *Act1* Gene.

Nucleotides are numbered with the A of the rice *Act1* transcription initiation site designated as +1. Restriction sites used in the subsequent construction of the various *Act1-Gus* fusion plasmids are indicated in italics and underlined. The specific regions described in the text are designated by lower-case Roman numerals and are underlined with arrows (for direct repeats) or lines. Upper-case capital letters represent 5′-flanking sequences, upper-case italic letters represent exon sequences, and lower-case letters represent the intron sequence. The codons of the *Act1* first coding exon have their translation product indicated below them. The vertical arrow indicates the position of the transcription initiation point, and the slashed lines indicate exon-intron boundaries.

Visualization of GUS Activity in Rice Protoplast and Suspension Culture Cells Transformed with *Act1-Gus* Fusion Constructs

We constructed four *Act1-Gus* fusion plasmids, p*Act1*-D, p*Act1*-F, p*Act1*-G, and p*Act1*-H, to determine the minimum amount of rice *Act1* 5′-flanking sequence required for maximal GUS activity. The features of these plasmids are described in the legend to Figure 3. p*Act1*-D has the 2.1-kb EcoRV restriction fragment from p*Act1*-A fused to the *Gus* coding region and 3′-noncoding transcript terminator region of the nopaline synthase (*nos*) gene (Jefferson, 1987). p*Act1*-D encodes a transcript containing two in-frame translation start codons, one each from the *Act1* and *Gus* genes, adding 15 amino acids to the N-terminal end of the wild-type GUS protein. The plasmids p*Act1*-F and p*Act1*-G (with truncated *Act1* 5′ regions) and p*Act1*-H (with a 1.2-kb inversion in the 5′-most region of the *Act1* sequence) were derived from p*Act1*-D.

We constructed the plasmids p*Act1*-J, p*Act1*-K, p*Act1*-L, p*Act1*-M, and p*Act1*-R (Figure 3) containing various deletions to their respective *Act1* 5′ introns to investigate the importance of this intron sequence on *Act1*-promoter-mediated gene expression (Figure 3). The plasmids p*Act1*-S and p*Act1*-T were constructed to test for any potential enhancer-like activity within the *Act1* 5′ intron. In p*Act1*-S, the intron sequence was cloned in a forward orientation, 5′ of the p*Act1*-R transcription unit. In p*Act1*-T, the intron sequence was introduced in a reverse orientation, 3′ of the p*Act1*-R transcription unit.

The plasmid p*Act1*-E was constructed as a negative control. It contains the 2.1-kb EcoRV fragment from p*Act1*-A cloned in the opposite orientation to that in p*Act1*-D. As a positive control, we used the plasmid pAl$_1$*Gus*N (Klein *et al.*, 1988), which contains the promoter, first exon, and first intron of the maize *Adh1* gene fused to a *Gus-nos* sequence.

As a rapid method of assaying GUS expression from the various *Act1-Gus* fusion plasmids, we transformed rice suspension culture cells by the biolistic method (Wang et al., 1988; Cao et al., 1990) and assayed for high-level GUS activity by visual inspection (Jefferson, Kavanagh, and Bevan, 1987) 10 days after transformation. The results of this rapid assay are shown in the final column of Table 1. Suspension cultures transformed with the plasmids p*Act1*-D, p*Act1*-F, p*Act1*-G, p*Act1*-H, and pAl$_1$*Gus*N all displayed positive GUS activity. Untransformed cultures or those transformed with the negative control plasmid p*Act1*-E, the intronless plasmid p*Act1*-R, or the plasmids p*Act1*-S and p*Act1*-T, containing the *Act1* intron 5′ or 3′ of their p*Act1*-R-derived transcription units, displayed no visible GUS expression.

The third column of Table 1 shows the results of GUS expression efficiencies, defined as the percent of intact cells displaying visible GUS activity 10 days after PEG-mediated transformation of rice protoplasts with the *Act1*-

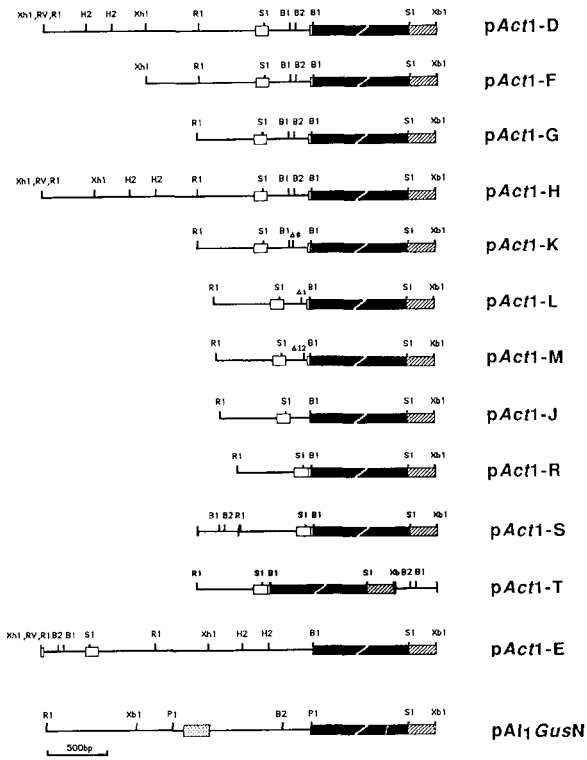

Figure 3. *Act1-Gus* Fusion Constructs.

Maps of constructs containing various portions of the 5′-flanking and 5′-transcribed sequence of the rice *Act1* gene fused, in-frame, to a sequence containing the *Gus* coding region and 3′ *nos* transcription terminator are shown next to their respective names. Open boxes represent noncoding exons of the *Act1* gene, filled boxes represent the *Gus* coding region (not to scale), and striped boxes represent the *nos* terminator sequence. The stippled box in the construct pAI$_1$*Gus*N represents the first exon of the maize *Adh1* gene. The "Δ" symbol indicates the deletion point in the various *Act1* first intron deletion constructs. The indicated restriction enzyme sites are abbreviated as follows: B1, BamHI; B2, BglII; R1, EcoRI; RV, EcoRV; H2, HincII; H3, HindIII; S1, SstI; Xh1, XhoI; Xb1, XbaI.

The characteristics of the rice *Act1* 5′ region in each construct are as follows: p*Act1*-D has a 2.1-kb EcoRV fragment from p*Act1*-A, containing the *Act1* translation initiation codon, 5′ intron, and 5′-noncoding exon; p*Act1*-F and p*Act1*-G were created by deletion of 0.8 kb and 1.2 kb of sequence, respectively, from the 5′ end of p*Act1*-D; p*Act1*-H has the 5′ 1.2-kb EcoRI fragment from p*Act1*-D cloned in the opposite direction; p*Act1*-K contains a deletion of 9 bp around its *Act1* 5′ intron BglII site; p*Act1*-L contains a deletion of 133 bp, between bases +222 and +354 (Figure 2) of its *Act1* 5′ intron; p*Act1*-M has a deletion of 157 bp, between bases +222 and +379 (Figure 2), removing the putative mRNA branch site from its p*Act1* 5′ intron; p*Act1*-J was created by deletion of a 170-bp BamHI site from p*Act1*-G, removing the putative mRNA branch site and 3′-donor splice site from its *Act1* 5′ intron; p*Act1*-R is identical to p*Act1*-G except that it has had the *Act1* 5′ intron deleted; cloning of the *Act1* intron in a 5′ "forward" orientation and a 3′ "reverse" orientation of the p*Act1*-R transcription unit created the plasmids p*Act1*-S and p*Act1*-T, respectively; p*Act1*-E contains the 2.1-kb EcoRV fragment from p*Act1*-A cloned in the opposite orientation to that in p*Act1*-D.

Gus fusion constructs. The plasmids p*Act1*-D and p-*Act1*-F displayed the highest GUS expression efficiencies at 8.4% and 7.9%, respectively. The plasmids p*Act1*-G and p*Act1*-H showed GUS expression efficiencies of 5.7% and 6.1%, respectively. All four plasmids displayed significantly higher GUS expression efficiencies than was found for pAI$_1$*Gus*N. Partial deletion of *Act1* 5′ intron sequences resulted in a significant reduction in the number of cells displaying visible GUS activity, as is shown for the plasmids p*Act1*-K (2.8%), containing a 9-bp deletion around its *Act1* 5′ intron BglII site, and p*Act1*-L (1.4%), containing a deletion of 133 bp between the branch point and 5′-acceptor splice sites of its *Act1* 5′ intron. Removal of the branch point for mRNA splicing in the plasmid p*Act1*-M and the additional removal of the 3′-donor splice site in the plasmid p*Act1*-J led to a 96% reduction in GUS expression efficiencies relative to p*Act1*-G. Untransformed protoplasts or protoplasts transformed with either the negative control

Table 1. Activity of GUS in Rice Protoplasts Transformed with *Act1-Gus* Fusion Constructs

	Protoplasts			
Names	Mean Specific Activity (nmol/hr/mg)	SE	Mean GUS Expression Efficiency (%)	Cell Suspension: Visible GUS Activity
p*Act1*-D	211.4[a]	65.4	8.4	+
p*Act1*-F	195.0[a]	58.4	7.9	+
p*Act1*-G	94.5[b]	29.8	5.7	+
p*Act1*-H	92.5[b]	11.1	6.1	+
p*Act1*-K	95.4[b]	26.2	2.8	NA
p*Act1*-L	53.4[c]	9.2	1.4	NA
p*Act1*-M	17.0[d]	5.5	0.2	NA
p*Act1*-J	8.8[e]	7.6	0.2	NA
p*Act1*-R	4.7[e]	6.9	0.0	–
p*Act1*-S	NA	NA	0.0	–
p*Act1*-T	NA	NA	0.0	–
p*Act1*-E	0.7[e]	5.8	0.0	–
Untransformed	0.0[e]	7.8	0.0	–
pAI$_1$*Gus*N	31.9[f]	15.9	4.2	+

The results of four independent transformation experiments, assayed 20 days after transformation, were used to estimate the mean specific activity of GUS in transformed rice protoplasts after correction for background. Mean specific activity results with identical superscripted letters are not statistically different from each other. Two independent protoplast transformation experiments were carried out to estimate the mean GUS expression efficiency where the number of blue cells (indicative of visible GUS expression) were expressed as a percent of the total number of intact viable cells assayed 10 days after transformation. Suspension cultures were visually examined for GUS expression 10 days after transformation. + indicates that visible GUS activity was observed; – indicates that visible GUS activity was not observed; NA means that these constructs were not assayed.

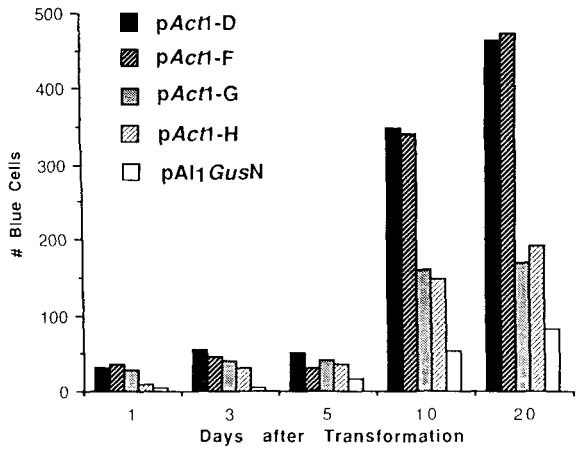

Figure 4. Histogram of the Temporal Pattern of GUS Expression after Transformation of Rice Protoplasts with *Act1-Gus* Fusion Plasmids.

Rice protoplasts were assayed visually for GUS expression by counting the number of blue cells among 10^5 protoplasts 1, 3, 5, 10, and 20 days after transformation with the plasmids p*Act1*-D, p*Act1*-F, p*Act1*-G, p*Act1*-H, and pAI$_1$*Gus*N. The histogram represents the mean of three independent experiments for each time point.

plasmid p*Act1*-E, the intronless plasmid p*Act1*-R, or the plasmids p*Act1*-S and p*Act1*-T gave no blue cells.

To determine the optimal time, subsequent to protoplast transformation, for quantifying GUS activity, rice protoplasts were transformed with the various constructs that had shown positive GUS expression in transformed rice suspension culture cells. The number of blue cells, indicative of high-level GUS activity, was then determined at various time intervals after transformation. A histogram analysis of the results is shown in Figure 4. For the constructs tested, there was an initial lag period of 5 days in which relatively few cells showed any visible GUS expression. The number of cells displaying visible GUS activity increased rapidly for all constructs between 5 and 20 days after protoplast transformation. Beyond 20 days after protoplast transformation, we found no significant increase in the number of cells displaying GUS activity (results not shown). Based upon these results, we decided to quantitatively assay GUS activity in rice protoplasts 20 days after transformation with the various *Act1-Gus* fusion plasmids.

Quantitative Analysis of GUS Expression in Transformed Protoplasts

To determine the quantitative differences in GUS expression from the various *Act1-Gus* fusion plasmids, the constructs were introduced into rice protoplasts by PEG-mediated transformation. The results of this analysis of GUS expression are presented in the first two columns of Table 1.

The highest GUS specific activities were recorded for the plasmids p*Act1*-D and p*Act1*-F, which have the longest *Act1* 5′ regions. The plasmid pAI$_1$*Gus*N, which has the *Gus* gene expressed from the maize *Adh1* promoter, displayed less than 17% of the GUS specific activity shown by p*Act1*-D and p*Act1*-F. The plasmids p*Act1*-G, with a truncated *Act1* 5′ region, and p*Act1*-H, with an inverted *Act1* 5′ end, were found to have GUS specific activities approximately 48% of that for p*Act1*-D. Thus, the inversion of the *Act1* 5′ end in p*Act1*-H did not enhance GUS expression relative to the truncated p*Act1*-G construct.

The effects of the various rice *Act1* intron deletions could be seen when their GUS specific activities were compared with that of their progenitor plasmid, p*Act1*-G. The small 9-bp deletion in the *Act1* 5′ intron of plasmid p*Act1*-K had no effect on the quantitative level of GUS activity. The plasmid p*Act1*-L, containing a deletion of 133 bp between the 5′-acceptor splice site and the putative mRNA branch-point splice site, displayed a GUS specific activity that was less than 57% of that observed for p*Act1*-G. The plasmid p*Act1*-M, which had the putative mRNA branch-point site removed, showed a further decrease in GUS specific activity to 17% of that observed for p*Act1*-G. The plasmid p*Act1*-J, which lacks the putative mRNA splicing branch site and 3′-splicing donor site of the *Act1* 5′ intron, and p*Act1*-R, which lacks the entire *Act1* 5′ intron, displayed no significant GUS specific activities over that observed for untransformed samples.

DISCUSSION

We have characterized the 5′ regions of the rice *Act1* gene and demonstrated that it contains an efficient promoter (relative to the maize *Adh1* 5′-region) for regulating foreign gene expression in transgenic rice. The construction of a number of *Act1-Gus* fusion plasmids allowed us to determine that the plasmid p*Act1*-F, containing 0.83 kb of 5′-flanking sequence, and the noncoding exon and 5′ intron of the rice *Act1* gene, has the minimal amount of *Act1* sequence necessary for maximal GUS expression in transient assays of transformed rice protoplasts. Although the *Gus* mRNAs and protein produced from the rice *Act1* and the maize *Adh1* 5′ regions are not identical, we observed that the rice *Act1* 5′-flanking region was at least 6 times more active than that of the maize *Adh1* gene in stimulating GUS expression in transformed rice protoplasts.

Deletion of a 0.4-kb region from the 5′ end of p*Act1*-F, in the construction of p*Act1*-G, resulted in a 52% reduction in GUS activity. When this 0.4-kb region was inverted and displaced 0.8 kb further upstream (in p*Act1*-H), high-level GUS activity was not restored. This suggests that the sequence element(s) in the 5′ end of the p*Act1*-F insert

that are responsible for high-level GUS expression do not display any enhancer-like activity.

Within the *Act1* 5′ sequence, we noted the occurrence of a long poly(dA) stretch located between bases −146 and −186. Such poly(dA) regions have been shown to prevent nucleosome formation in vitro (Prunell, 1982), and it has also been found that naturally occurring poly(dA) tracts act as 5′-promoter elements for the constitutive expression of three different yeast genes (Struhl, 1985). It is believed that poly(dA) stretches change chromatin structure, enabling general transcription factors to access the DNA template and activate constitutive transcription in the absence of more specific transcription factors, which may play some role in the constitutive activation of *Act1* gene expression.

By constructing *Act1*-intron-deletion-*Gus* fusion plasmids, we were able to show that GUS expression in transformed rice protoplasts was dependent on the presence of an intact rice *Act1* 5′ intron. The effect of the rice *Act1* intron on gene expression is similar to the previously described dependence on introns of mouse *dihydrofolate reductase* (Gasser et al., 1982) and maize *Adh1-S* (Callis et al., 1987) gene expression. We do not believe that the *Act1* 5′ intron increases expression as a result of some enhancer-like element within its sequence. We obtained no stimulation of visible GUS activity from the intronless p*Act1*-R sequence when the *Act1* intron was introduced either in a forward orientation, 5′ of its transcription unit (in p*Act1*-S), or in the opposite orientation, 3′ of its transcription unit (in p*Act1*-T). A similar finding was also reported in the case of the maize *Adh1* intron (Callis et al., 1987). There is no indication that the *Act1* first intron codes for any functionally active transcript or protein product because previous RNA gel blot hybridization with a double-stranded probe that spans the *Act1* first intron failed to show any binding to total RNA from 7-day-old rice shoots, other than to the 1.7-kb *Act1* transcript itself (McElroy et al., 1990). Furthermore, the *Act1* 5′ intron contains no open reading frames of greater than 25 codons.

Our results suggest that the primary effect of the *Act1* 5′ intron is associated with an in vivo requirement for efficient mRNA splicing. A 9-bp deletion in the *Act1* intron of the plasmid p*Act1*-K caused no significant reduction in GUS specific activity relative to that of its progenitor plasmid p*Act1*-G. However, a 133-bp deletion, in the plasmid p*Act1*-L, although not removing any of the sequences previously implicated in intron splicing (Green, 1986), did cause a reduction in the distance between the 5′-acceptor and putative mRNA branch-point splice sites with an associated 44% reduction in GUS specific activity. Similar effects have been reported in animal systems (Wieringa, Hoffer, and Weissmann, 1984; Ruskin, Greene, and Greene, 1985) and probably reflect a requirement for multiple splicing factors to interact with specific intron regions. The in vivo requirement for efficient intron splicing was further supported by the observation that a deletion that removed the putative 5′ intron mRNA branch-point splice site (p*Act1*-M) or removed both the branch-point and 3′-donor splice sites (p*Act1*-J) led to GUS specific activities that were 18% and 9% of that observed for p*Act1*-G. It is known that deletion of the normal branch-point and 3′-donor splice sites of animal genes does not abolish splicing but rather leads to the use of cryptic branch-point and 3′-donor splice sites, with associated reductions in splicing efficiency and gene expression (Fukamaki et al., 1982; Ruskin et al., 1984; Hornig, Aebi, and Weissmann, 1986).

Our results demonstrate that the rice *Act1* 5′-flanking sequence contains a very efficient promoter for rice transformation. Experiments are under way to investigate the activity of the *Act1* 5′-flanking sequences in regenerated transgenic plants of rice and other monocot crop plants. Our results support the general conclusion of Callis et al. (1987) and Oard, Paige, and Dvorak (1989) that the expression of a foreign gene in transformed plant cells can be dependent upon, but not necessarily an absolute function of, the presence of an intact intron sequence. It has previously been noted that the occurrence of 5′-noncoding exons, such as that of the rice *Act1* gene, is extremely rare for plant genes (Hawkins, 1988). We suggest that a functional requirement for the presence of the 5′ intron may be correlated with the conservation of the 5′-noncoding exon during the evolution of this rice actin gene. The dependence on an intact 5′ intron for *Act1*-regulated gene expression cannot be related to intron splicing alone because the plasmid p*Act1*-R (which showed no GUS activity above background levels) was effectively spliced in vitro prior to its introduction into rice protoplasts. Rather, the intron dependence may be related to a splicing-associated function, such as transport to or across the nuclear membrane. It may be noted that the maize 5′ intron is located between the first and second coding exons of the *Adh1* gene, whereas the rice 5′ intron is located between a 5′-noncoding exon and the first coding exon of the *Act1* gene. This suggests that there may be a common positional component to the intron-mediated stimulation of gene expression observed for the maize *Adh1* and rice *Act1* 5′ introns.

In conclusion, we agree with Callis et al. (1987) that an investigation of the role of 5′ introns should be an integral part of any experiments aimed at optimizing foreign gene expression in transformed plant cells.

METHODS

Genomic Clone Characterization

A genomic restriction map of the 15.1-kb insert from λ*Act1* (Reece, 1988; Reece et al., 1990) was prepared by analyzing all possible single and double digests with the enzymes BamHI,

EcoRI, HindIII, and SalI (Boehringer Mannheim, Indianapolis, IN). A 5.3-kb HindIII-HindIII restriction fragment from the λ*Act1* clone was subcloned into pBluescriptII-KS (Stratagene, La Jolla, CA) to generate the plasmid p*Act1*-A. A restriction map of p*Act1*-A was prepared by analyzing all possible single and double digests with the enzymes BamHI, BglII, EcoRI, EcoRV, HincII, HindIII, KpnI, PstI, SmaI, SphI, SstI, XbaI, and XhoI (Boehringer Mannheim, Indianapolis, IN).

A 2.1-kb EcoRV-EcoRV region within the p*Act1*-A insert was further subcloned into pBluescriptII-KS and its DNA sequence determined according to the sequencing strategy outlined in Figure 1B. Double-stranded DNA sequencing reactions were run using the dideoxynucleotide chain termination method following the T7 polymerase protocol (Pharmacia LKB Biotechnology, Piscataway, NJ). Analysis of the DNA sequence data was performed using the Microgenie computer program of Queen and Korn (1984).

Construction of Plasmids

The 2.1-kb EcoRV-EcoRV fragment from p*Act1*-A was subcloned into the SmaI site of pBluescriptII-KS (Stratagene) in both possible orientations to produce the plasmids p*Act1*-B and p*Act1*-C. A promoterless β-glucuronidase (*Gus*) gene, containing the 3′-noncoding region of the nopaline synthase (*nos*) gene (Jefferson et al., 1987), was excised by BamHI-XbaI digestion of pEXAG3 (a gift from Stanley Goldman), and cloned between the BamHI and XbaI sites of p*Act1*-B and p*Act1*-C to produce the plasmids p*Act1*-D and p*Act1*-E. Deletion of the 0.8-kb XhoI-XhoI and 1.2-kb EcoRI-EcoRI fragments from p*Act1*-D generated the plasmids p*Act1*-F and p*Act1*-G, respectively. Introduction of the 1.2-kb EcoRI-EcoRI fragment into the EcoRI site of p*Act1*-G, in the reverse orientation, produced the plasmid p*Act1*-H.

The plasmid p*Act1*-J was produced by deletion of a 0.17-kb BamHI-BamHI fragment from p*Act1*-G. The plasmids p*Act1*-K, p*Act1*-L, and p*Act1*-M were generated by cleavage of p*Act1*-G at its BglII site, followed by exonuclease III deletion and S1 nuclease treatment (Guo and Wu, 1983) to remove different amounts of the *Act1* 5′ intron. To construct the plasmid p*Act1*-R, the 0.9-kb EcoRI-EcoRV fragment from p*Act1*-A, containing the 5′ intron of the rice *Act1* gene, was cloned between the EcoRI and EcoRV sites of pBR322 (Bethesda Research Laboratories, Gaithersburg, MD) to produce the plasmid p*Act1*-N. The 0.4-kb SstI-EcoRV fragment from p*Act1*-N, containing the *Act1* intron, was excised and replaced with an intronless 0.1-kb SstI-EcoRV fragment from the insert of an *Act1* cDNA clone, pcRAc1.3 (McElroy et al., 1990) to produce the plasmid p*Act1*-P. The intronless 0.6-kb EcoRI-EcoRV fragment from p*Act1*-P was excised and cloned between the EcoRI and SmaI sites of pBluescriptII-KS to yield the plasmid p*Act1*-Q. Cloning of the *Gus-nos*-containing BamHI-XbaI fragment from pEXAG3 between the BamHI and XbaI site of p*Act1*-Q produced the plasmid p*Act1*-R. To construct p*Act1*-S and p*Act1*-T, the plasmid p*Act1*-E was digested with BstEII (which has a single site in this vector between nucleotides 79 and 85) and XbaI, subjected to S1 nuclease treatment, and self-ligated to produce the plasmid p*Act1*-U. The *Act1-Gus-nos* fragment from p*Act1*-R was isolated by Xho1-XbaI digestion and cloned into the XhoI site of p*Act1*-U, in both orientations, to produce the plasmids p*Act1*-S (containing the *Act1* intron in a "forward" orientation, 5′ of the *Act1-Gus* transcription unit) and p*Act1*-T (containing the *Act1* intron in a "reverse" orientation, 3′ of the *Act1-Gus* transcription unit). The structure of these *Act1-Gus* fusion constructs was confirmed by double-stranded DNA sequencing.

Culture, Transformation, and Visualization of GUS Activity in Rice Cell Suspension Cultures and Protoplasts

Cell suspension cultures were generated from rice (*Oryza sativa* cv Lemont) calli, cultured in liquid R2 media (Ohira, Ojima, and Fujiwara, 1973) containing 3% (w/v) sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid, and 2 mg/L vitamin B5 (Sigma, St. Louis, MO) with weekly subculturing and incubation in the dark at 26°C. Cell suspensions were filtered through a 700-μm mesh (Medical Instruments, Inc., Los Angeles, CA) before particle gun bombardment with 1.2-μm diameter tungsten particles mixed with a solution containing 10 μg of plasmid DNA (Wang et al., 1988; Cao et al., 1990). GUS activity in intact cells and developing calli was determined by the appearance of blue spots 2 and 10 days after bombardment with plasmid DNA following the GUS assay procedure of Jefferson et al. (1987).

Protoplasts were isolated from rice (*O. sativa* cv Nipponbare) cell suspension cultures (Abdullah, Cocking, and Thompson, 1986) and resuspended in MaMg medium (Zhang and Wu, 1988) to a final density of 10^6 protoplasts/mL. For transformation, 1 mL of protoplast suspension was incubated with 10 μg of circular plasmid DNA, 50 μg/mL calf thymus carrier DNA, and an equal volume of 30% polyethylene glycol 4000 (Sigma, St. Louis, MO). The mixture was incubated for 30 min, diluted with CPW13 medium (Abdullah et al., 1986), washed 3 times in CPW13 media, with centrifugation between each wash, before being resuspended in simplified KPR liquid media (Zhang and Wu, 1988) to a final density of 10^6 protoplasts/mL. Aliquots (0.1 mL) of this final suspension were incubated by thin-layer culture in 96-well plates (Krackeler Scientific, Inc., Albany, NY) before subsequent assays for GUS activity (Jefferson, 1987; Zhang and Wu, 1988).

Quantitative Analysis of GUS Activity in Transformed Protoplasts

Cells were collected 20 days after transformation by low-speed centrifugation in a bench-top microcentrifuge. Total soluble protein was isolated in a GUS extraction buffer (Jefferson et al., 1987). Protein extracts were incubated with 1 mM methylumbelliferyl-β-D-glucuronide (Sigma, St. Louis, MO) in a standard assay at 37°C for 3 hr to 6 hr. The liberation of 4-methylumbelliferone was followed by measuring fluorescence with excitation at 365 nM and emission at 455 nM in a spectrofluorometer (American Instrument Co., Silver Spring, MD). Protein concentrations of plant extracts were determined by the dye-binding method of Bradford (1976) with a Bio-Rad Laboratories kit (Richmond, CA). Statistical analysis of the quantitative GUS activity results was done using a paired *t* test on the StatView SE+ program (Abacus Concepts, Inc., Berkeley, CA).

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