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Molecular & General Genetics

An International Journal

Continuation of Zeitschrift für Vererbungslehre The First Journal on Genetics Founded in 1908

Volume 231 Number 1 December II 1991

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Subscription information

Volumes 230–234 (3 issues each) will appear in 1992.

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Typesetting

Universitätsdruckerei H. Stürtz AG
W-8700 Würzburg, FRG

Printers

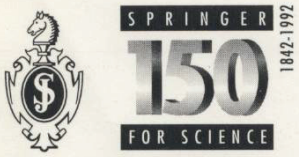
H. Heenemann, W-1000 Berlin 42, FRG

Bookbinding

Lüderitz & Bauer, W-1000 Berlin 11, FRG

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Springer-Verlag GmbH & Co KG
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Printed in Germany



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Mol Gen Genet (1991) 231:150–160

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Construction of expression vectors based on the rice actin 1 (*Act1*) 5′ region for use in monocot transformation

David McElroy[1], Alan D. Blowers[2], Barnabás Jenes[3], and Ray Wu[4]

[1] Field of Botany, Cornell University, Ithaca, NY 14853, USA
[2] Plant Science Center, Biotechnology Building, Cornell University, Ithaca, NY 14853, USA
[3] Institute for Plant Sciences, Agricultural Biotechnology Center, Gödöllö, 2101 Szent-Györgyi Albert út 4, P.O. Box 170, Hungary
[4] Section of Biochemistry, Molecular and Cell Biology, Biotechnology Building, Cornell University, Ithaca, NY 14853, USA

Received March 1, 1991

Summary. It has been previously reported that the 5′ region of the rice actin 1 gene (*Act1*) promoted high-level expression of a β-glucuronidase reporter gene (*Gus*) in transformed rice cells. In this paper we describe the construction of *Act1*-based expression vectors for use in monocot transformation. As part of the development of these vectors, we have evaluated the influence of the *Act1* first intron, the *Act1-Gus* junction-encoded N-terminal amino acids, and the sequence context surrounding the *Act1* and *Gus* translation initiation site on *Act1-Gus* gene expression in rice and maize cells. We have found that addition of *Act1* intron 1 to the transcription unit of a *Gus* reporter gene under control of the cauliflower mosaic virus (CaMV) 35S promoter stimulated GUS activity more than 10-fold in transformed rice cells. Optimization of the sequence context around the *Gus* translation initiation site resulted in a 4-fold stimulation of *Gus* expression in transformed rice cells. By utilizing both the *Act1* intron 1 and optimized *Gus* translation initiation site, a 40-fold stimulation in *Gus* expression from the CaMV 35S promoter has been achieved in transformed rice cells; very similar results were obtained in transformed maize cells. Taken together these results suggest that the *Act1*-based expression vectors described here should promote the expression of foreign genes in most, if not all, transformed monocot cells to levels that have not previoulsy been attainable with alternative expression vectors.

Key words: Actin gene – Monocot transformation – Expression vectors – Intron – Translation initiation

Introduction

One of the major limitations in the application of gene transfer technology to cereals has been the lack of efficient promoters for the expression of foreign genes in transgenic monocots. Although promoters from CaMV 35S RNA (Guilley et al. 1982) and the maize alcohol dehydrogenase 1 gene (*Adh1*) (Dennis et al. 1984) have been widely used in monocot transformation (Fromm et al. 1990; Gordon-Kamm et al. 1990; Kyozuka et al. 1990; Spencer et al. 1990; Tereda and Shimamoto 1990), both have been reported to show relatively low activity in transformed rice cells (Peterhans et al. 1990; McElroy et al. 1990c). Furthermore, in transgenic rice plants the CaMV 35S promoter is not active in all cell types (Tereda and Shimamoto 1990) and the maize *Adh1* promoter is more highly active in root tissue than in leaf tissue (Zhang and Wu 1988; Kyozuka et al. 1990, 1991). With a view towards overcoming the limitations of the CaMV 35S and maize *Adh1* promoters in monocot transformation, we have previously reported that the 5′ region of the rice *Act1* gene directed high levels of *Gus* reporter gene expression in transformed rice protoplasts (McElroy et al. 1990c). (Although *Escherichia coli* β-glucuronidase is encoded by the *uidA* gene, we have denoted this gene as *Gus* for the purposes of this publication.) In rice plants, the endogenous *Act1* transcript is abundant in all tissues and development stages of the plant examined (McElroy et al. 1990b). Likewise, relatively high levels of GUS activity were observed in virtually all tissues of transgenic rice plants expressing an *Act1*-Gus chimeric gene (Zhang et al. 1991). We have now developed a set of *Act1*-based expression vectors for foreign gene expression in transformed monocot cells.

Previously, the *Act1* 5′ region was described as a promoter (McElroy et al. 1990c). However, this region contains a number of *Act1* transcribed elements, including the first exon (non-coding), an intron contained within the mRNA 5′ untranslated leader (intron 1) and a portion of the second, ATG-containing exon of the *Act1* gene (McElroy et al. 1990a). From previous studies, the contribution that each of these elements may have made in promoting high levels of *Gus* expression in transformed rice was not evaluated. As part of the development of the *Act1*-based expression vectors described in this report, we set out to investigate the influence on

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Gus expression of (i) the *Act1* first intron; (ii) the sequence context surrounding the *Act1* and *Gus* translation initiation sites; and (iii) the *Act1-Gus* junction-encoded N-terminal amino acids. Earlier studies demonstrated that deletion of the *Act1* first intron from an *Act1-Gus* chimeric gene resulted in nearly complete loss of GUS activity in transformed rice cells (McElroy et al. 1990c). This suggested that insertion of *Act1* intron 1 into a transcription unit originating from a heterologous plant promoter might stimulate gene expression in transformed monocot cells, as has been observed for other monocot introns (Callis et al. 1987; Oard et al. 1989; Vasil et al. 1989; Kyozuka et al. 1990; Mascarenhas et al. 1990; Tanaka et al. 1990; Luehrsen and Walbot 1991; Maas et al. 1991). Finally, it is well-established that the sequence context surrounding the initiation codon in eukaryotes (Kozak 1984, 1986, 1987) and the identity of the N-terminal amino acids of a protein (Bachmair et al. 1986; Gonda et al. 1989) can have dramatic effects on transcript translatability and protein stability, respectively.

In this report we show that (1) addition of rice *Act1* intron 1 to the transcriptional unit of a 35S-*Gus* chimeric gene can stimulate *Gus* expression more than 10- and 7-fold in transformed rice and maize cells, respectively; (2) optimization of the sequence context around the *Gus* initiation codon leads to a 4- and 10-fold enhancement in GUS activity in transformed rice and maize cells, respectively; (3) a chimeric 35S-*Act1* intron 1-*Gus* gene (with an optimized translation initiation region) produced 40- and 65-fold more GUS activity in transformed rice and maize cells, respectively, than a 35S-*Gus* chimeric gene; and (4) the intron-mediated stimulation of *Gus* expression was limited to rice and maize cells, as tobacco cells showed reduced GUS activity after transformation with intron 1-containing test constructs (compared to intronless *Gus* genes). Based on these results we have constructed a set of *Act1* 5′-based expression vectors for use in monocot transformation. Both translational, containing either the efficient *Act1* translation initiation codon region or a modified initiation codon region, and transcriptional expression vectors are described. The vectors contain a wide range of polylinker sites to simplify cloning and use of these vectors results in (relatively) high levels of foreign gene expression in transformed monocots.

Materials and methods

General methods. All enzymatic DNA manipulations, including subcloning strategies, exonuclease III and mung bean nuclease digestions, Klenow DNA polymerase fill-in reactions, and double-stranded sequencing of construct junction regions were performed following standard protocols (Maniatis et al. 1982) using modifications recommended by the enzyme manufacturers.

Construction of Act1-Gus *fusion constructs*. Construction of the *Act1-Gus* translational fusion plasmid p*Act1*-F has been described previously (McElroy et al. 1990c). A 317 bp *Bam*HI restriction fragment from pCOR103, containing the modified *Act1* translation initiation codon region (ACCATGG) replaced the corresponding *Act1* region of p*Act1*-F, thereby creating the translational fusion plasmid p*Act1*-F2.

Plasmid p*Act1*-F2 was linearized by digestion with *Nco*I at the modified *Act1* translation initiation codon (C/CATGG), the ATG codon removed by digestion with exonuclease III, the ends blunted with mung bean nuclease, and ligated to produce plasmid p*Act1*-F3. Plasmid p*Act1*-F3 now contains an out-of-frame translation initiation codon (relative to the *Gus* ATG) located 29 bp upstream of the *Gus* coding region, and could potentially encode a polypeptide of 14 amino acids. Plasmid p*Act1*-F2 was digested with *Nco*I and *Sna*BI to remove the *Act1-Gus* junction region and associated *Gus* coding sequence and replaced with an *Nco*I – *Sna*BI restriction fragment from pRAJ275 (Sleat et al. 1987). This new plasmid, p*Act1*-F4, now contains an *Act1-Gus* gene that possesses the eukaryotic consensus translation initiation sequence (ACCATGG), rather than the bacterial sequence (CTTATGT), around the *Gus* initiation ATG codon. Plasmid p*Act1*-F2 was digested with *Acc*I and *Sma*I, which removes a 32 bp fragment containing the modified *Act1* translation initiation codon region, five adjacent codons, and a part of the polylinker sequence. The plasmid was ligated after repair synthesis of the *Acc*I site with Klenow enzyme to produce the *Act1-Gus* transcriptional fusion plasmid p*Act1*-F5.

The *Act1-Gus* sequence from plasmid p*Act1*-F was excised as an *Eco*RI – *Xba*I restriction fragment and subcloned into the vector pSP72 (Promega) to produce plasmid p*Act1*-G72. Plasmid p*Act1*-G72 was then digested with *Cla*I and *Bst*EII to remove the *Act1* 5′-flanking region and first noncoding exon. The remaining plasmid was treated with Klenow enzyme prior to ligation to create the promoterless translational fusion constructs p*Act1*-F6, which contains the *Act1* 5′ intron (and associated splice site junction sequences) fused to the *Gus-nos* reporter gene.

Act1 intron 1 was isolated from plasmid p*Act1*-F2 as a 449 bp *Bst*EII – *Acc*I restriction fragment, treated with Klenow enzyme, and cloned into *Cla*I- and *Kpn*I-digested and Klenow enzyme-treated pSP72 vector DNA to reconstitute the p*Act1* exon 1–intron 1–exon 2 junction region, thus creating plasmid *Act1*-I72. The *Act1* intron was reisolated from p*Act1*-I72 as a 459 bp *Eco*RV – *Sma*I restriction fragment and then inserted in the reverse 3′ to 5′ orientation into *Bst*EII- and *Acc*I-digested and mung bean nuclease-treated p*Act1*-F2, to produce plasmid p*Act1*-F7. The same operation was carried out to insert the intron in the correct 5′ to 3′ orientation into the *Sst*I site (Klenow enzyme-treated) of *Act1* exon 1 in plasmid p*Act1*-R (McElroy et al. 1990c), creating the *Act1-Gus* translational fusion plasmid p*Act*-RI.

Construction of CaMV35S-Gus *fusion constructs*. The 3.0 kb *Hin*dIII – *Eco*RI restriction fragment, which contains the CaMV 35S promoter and a portion of the 5′ transcribed leader sequence fused to the *Gus* coding region and the *nos* polyadenylation sequence, was removed

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from plasmid pBI221.1 (Jefferson 1987) and inserted between the *Hin*dIII and *Eco*RI sites of pBluescriptII-KS to create plasmid pBCG. Plasmid pBCG-A was created by inserting *Act1* intron 1 from plasmid p*Act1*-F6 as a 459 bp *Eco*RV – *Sma*I restriction fragment into the *Sma*I site in the 5′ transcribed leader region of the *Gus* gene of plasmid pBCG. Plasmids pBCG-A4 and pBCG-A5 were constructed by replacement of the *Bst*EII – *Sna*BI restriction fragment in plasmid pBCG-A with the corresponding fragments (containing modified *Act1-Gus* junction regions) from plasmids p*Act1*-F4 and p*Act1*-F5, respectively.

Act1 intron 1 from p*Actl*-F6 was isolated as a 492 bp *Eco*RV – *Sma*I restriction fragment and four copies of the intron were cloned into the *Sma*I site of pBCG to create the translational fusion construct pBCG-4I. In this plasmid, the *Gus* translation initiation codon is fused in-frame (assuming the correct splicing of the four *Act1* introns) with the first ATG codon of the four *Act1* translation initiation codons. The plasmid pAI_1GusN (Klein et al. 1988) contains the promoter, modified first exon lacking the ATG initiation codon, and first intron of the maize *Adh1* gene fused to a *Gus-nos* reporter gene. Plasmid pCI_1GusN was created by removal of the *Adh1* promoter from plasmid pAI_1GusN after digestion with *Eco*RI and *Hin*dIII and replacement with a *Hin*dIII fragment from $pCaMVI_1CN$ containing the CaMV 35S promoter (Callis et al. 1987).

Construction of Act1*-based expression vectors.* A 2.2 kb *Eco*RV restriction fragment from p*Act*-A (McElroy et al. 1990c) containing the *Act1* 5′ region was subjected to in vitro oligonucleotide-directed mutagenesis (Kunkel 1985) to create an overlapping *Nco*I restriction site at the *Act1* translation intiation codon (**AG**ATG**G** to **CC**ATG**G**). The in vitro modified *Act1* sequence was cloned in both possible orientations into the *Eco*RV site of vector pBluescriptII-KS (Stratagene, La Jolla, Calif.) to produce plasmids pCOR101 and pCOR102. A 1.4 kb *Xho*I – *Eco*RV restriction fragment from pCOR101 was isolated and subcloned into *Xho*I – *Sma*I digested pBluescriptII-KS to create plasmid pCOR103 for use in constructing *Act1*-based translational fusions.

A 2.2 kb *Apa*I – *Nco*I restriction fragment containing the *Act1* 5′ region was excised from pCOR101 and subcloned between the *Apa*I and *Nco*I sites of pGEM – 5Zf(+) (Promega) to produce the plasmid pCOR106. Plasmid pCOR106 was digested with *Xho*I to remove the 5′-most 0.8 kb of *Act1* DNA and intervening polylinker sequence, and the remaining plasmid was ligated to create pCOR107 for constructing *Act1* translational fusions. Plasmid pCOR101 was digested with *Xho*I to remove the 5′-most 0.8 kb of *Act1* DNA and intervening polylinker sequence, and the remaining plasmid ligated to create the *Act1* translational fusion vector pCOR104. Plasmid pCOR104 was linearized by digestion with *Nco*I, and the *Act1* translation initiation codon was removed with exonuclease III and mung bean nuclease digestions followed by ligation to produce plasmid pCOR105 for constructing *Act1* transcriptional fusions.

Plasmid pCOR102 was digested with *Nhe*I and *Spe*I to remove the 5′-most 0.8 kb of *Act1* DNA and intervening polylinker sequence, and the remaining plasmid was ligated to create the *Act1* translational fusion plasmid pCOR108. A 2.2 kb *Eag*I – *Nco*I restriction fragment containing the *Act1* 5′ region from pCOR102 was excised and subcloned between the *Eag*I and *Nco*I sites of pGEM – 5Zf(+) to produce the plasmid pCOR109. A 1.4 kb *Xho*I – *Sal*I restriction fragment containing the *Act1* 5′ region from pCOR109 was excised and subcloned into the *Xho*I site of pBluescriptII-KS in both possible orientations to create plasmids pCOR110 and pCOR111. Plasmid pCOR111 was digested with *Eco*RV to remove the intervening polylinker sequence, creating plasmid pCOR112 for use in constructing *Act1* translational fusions. Plasmid pCOR111 was also digested with *Nco*I and *Cla*I, subjected to exonuclease III and mung bean nuclease digestions, and ligated to create plasmid pCOR113 for constructing *Act1* transcriptional fusions. Finally, plasmid pCOR110 was digested with *Hin*dIII and *Eag*I, subjected to exonuclease III and mung bean nuclease digestions, and ligated to create the *Act1* translational fusion plasmid pCOR114.

A 1.4 kb *Nhe*I – *Spe*I restriction fragment containing the *Act1* 5′ region was excised from plasmid pCOR109 and subcloned into the vector-containing fragment of *Spe*I-digested pCOR109 in both possible orientations, to create the *Act1* translational fusion plasmids pCOR115 and pCOR116. Plasmid pCOR116 was then linearized by *Nco*I digestion and the *Act1* translation initiation codon was removed by exonuclease III deletion and mung bean nuclease digestions before being ligated to produce plasmid pCOR117 for constructing *Act1* transcriptional fusions.

Transformation and β-glucuronidase assays. Rice (*Oryza sativa* "japonica" cv. B-42) protoplasts were isolated from finely dispersed, 2-year-old cell suspension cultures, transformed with supercoiled plasmid DNA (20 μg), and cultured as previously described (Zhang and Wu 1988; Jenes and Pauk 1989). Rapidly dividing maize (*Zea mays* L.) Black Mexican Sweet (BMS) suspension culture cells (200 mg) were collected on Whatman no. 4 filter paper by vacuum filtration and the filters were laid on solid Murashige-Skoog (MS) medium. Plasmid DNA (2.5 μg) was precipitated onto M-10 tungsten particles (Sylvania-GTE, Towanda, Pa.) with an average diameter of 1.1 μm as described previously (Klein et al. 1988) and each plate was bombarded twice using the Biolistics™ PDS-1000 Particle Delivery System (DuPont). After 3 days of incubation in darkness at 25° C, the bombarded cells were scraped off the filter paper and resuspended in 0.5 ml of lysis buffer for assay (Jefferson 1987). Exponentially growing *Nicotiana tabacum* NT1 suspension culture cells were collected as described above (400 mg per filter) and the filters placed on solid NT1 medium. Cells were bombarded and incubated as described above.

Spectrofluorometric analysis of GUS activity was carried out essentially as described by Jefferson (1987). Protein extracts were prepared by sonication to disrupt

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the cells followed by centrifugation at 4° C to remove the cellular debris. A 50 µl aliquot of the extract was added to 500 µl of lysis buffer containing 1 mM 4-methyl umbelliferyl β-D-glucuronide (MUG) and GUS activity assayed by fluorometric detection of 4-methylumbelliferone (MU) in a SLM-Aminco spectrofluorometer. Protein concentrations were determined by the method of Bradford (1976).

Results

Investigation into the roles of intron 1, the translation initiation region, and the N-terminal amino acid addition in Act1*-mediated* Gus *expression*

Figure 1 shows the restriction map of the *Act1* 5′ region in plasmid p*Act1*-B (Fig. 1A), which was employed in the construction of the *Act1*-based expression vectors described in this report. Also shown are the restriction map (Fig. 1B) and 5′ transcribed sequence (Fig. 1C) of the *Act1-Gus* fusion gene in plasmid p*Act1*-D, which has previously been used in rice transformation experiments (McElroy et al. 1990c; Zhang et al. 1991). The *Gus* transcript of p*Act1*-D contains two in-frame translation initiation codons, one each from the *Act1* and *Gus* genes, thereby adding 15 N-terminal amino acids to the GUS protein (Fig. 1C). An ACT1-GUS fusion peptide of the predicted molecular mass (72.5 kDa) has been observed in immunoblot analysis of proteins extracted from transgenic rice plants (Y.R. Mawal and R. Wu, unpublished result). To examine further the role of the *Act1* 5′ transcribed region in *Act1*-mediated gene expression, additional *Gus* fusion plasmids were constructed (Fig. 2) and their respective GUS activities were measured in transformed rice protoplasts and cultured maize suspension cells (Table 1). The sequences surrounding the translation initiation region for several of these constructs are shown in Fig. 3.

Our reference plasmid, p*Act1*-F (McElroy et al. 1990c), was derived from p*Act1*-D (Fig. 1B) and contains 0.84 kb of *Actl* 5′ flanking sequence along with the 79 bp 5′ noncoding exon, the 448 bp intron 1, and 25 bp of the ATG-containing second exon of the *Act1* primary transcript, fused to a promoterless *Gus-nos* reporter gene. The transcript of p*Act1*-F (like p*Act1*-D) contains two in-frame translation initiation codons, one each from the *Act1* and *Gus* genes (Fig. 3). Plasmid p*Act1*-F produces 5- and 8-fold increased GUS activity in rice protoplasts and maize cells, respectively, than is observed with plasmid pAI_1GusN, which contains a maize *Adh1-Gus* fusion. In addition, 20-fold more GUS activity is observed in both monocots after introduction of plasmid p*Act1*-F compared to plasmid pBCG, which contains a CaMV 35S-*Gus* fusion (Table 1).

Plasmid p*Act1*-F2 (Fig. 2) was created to investigate the influence of the sequence context surrounding the *Act1* translation start codon on *Gus* expression. Plasmid p*Act1*-F2 was derived from p*Act1*-F by in vitro mutagenesis, in which the sequence around the *Act1* start codon was altered from GTAGAAGATGG to GTAGACCATGG to match that of the eukaryotic translation initiation codon consensus sequence (Kozak 1985). This sequence modification also created *Nco*I (GTAGA***CCATGG***) and *Acc*I (***GTAGAC***CATGG) restriction sites around the sequence of the start codon

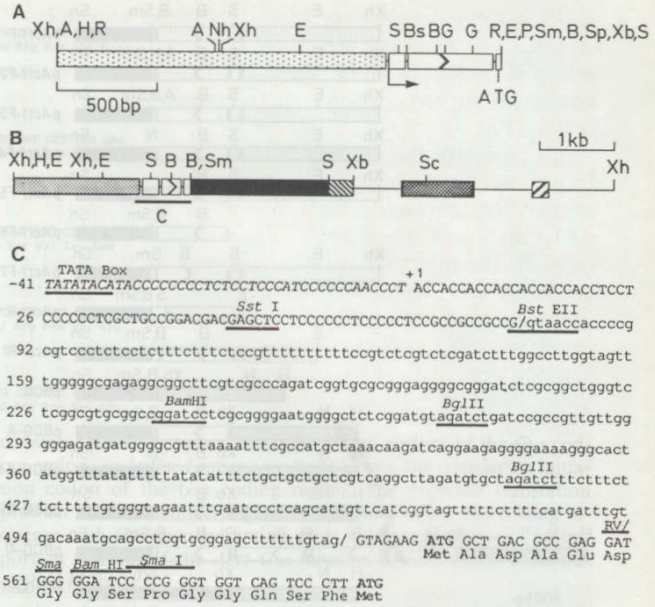


Fig. 1A–C. Diagrammatic representation of **A** rice actin 1 (*Act1*) genomic clone p*Actl*-B, **B** *Act1-Gus* fusion construct p*Act1*-D, and **C** the nucleotide sequence of the 5′ transcribed region of the rice *Act1-Gus* gene fusion in p*Act1*-D and p*Act1*-F. **A** Partial restriction map of the 2.2 kb p*Act1*-B insert (McElroy et al. 1990c), containing the 5′ transcribed and 5′ flanking region of the rice *Act1* gene, from which the *Act1-Gus* and *Act1*-based expression vectors were constructed. The transcription initiation site and direction of transcription are indicated by an *arrow* below the *Act1* 5′ noncoding exon of p*Act1*-B. **B** Restriction map of the 7.4 kb *Act1-Gus* fusion construct p*Act1*-D (McElroy et al. 1990 c), containing the 5′ transcribed and 5′ flanking region of the rice *Act1* gene fused to a *Gus-nos* reporter sequence. The different regions of p*Act1*-B and p*Act1*-D are indicated as follows: *light stippled box*, *Act1* 5′-flanking region; *open boxes*, *Act1* exons; *open box with dark arrowhead*, *Act1* intron 1; *filled box*, *Gus* coding region; *light striped box*, 3′ noncoding region of the nopaline synthase gene (*nos*); *heavy stippled box*, ampicillin resistance gene; *heavy striped box*, bacterial colE1 origin of replication. The region whose nucleotide sequence is presented in **C** is indicated by a *line below* the restriction map of p*Act1*-D. The indicated restriction sites in **A** and **B** are abbreviated as follows: A, *Acc*I; B, *Bam*HI; Bs, *Bst*EII; E, *Eco*RI; R, *Eco*RV; G, *Bgl*II; H, *Hin*dIII; Nh, *Nhe*I; S, *Sst*I; Sc, *Sca*I; Sm, *Sma*I; Sp, *Spe*I; Xb, *Xba*I; Xh, *Xho*I. **C** Nucleotide sequence of the *Act1-Gus* 5′ transcribed region in p*Act1*-D and p*Act1*-F. Nucleotides are numbered from nucleotide A of the *Act1* transcription initiation site as +1. Restriction sites used in the construction of the various *Act1-Gus* and *Act1*-based expression vectors are indicated and *underlined*. The different regions of the *Act1* 5′ transcribed sequences are: *upper case italic letters*, *Act1* 5′ region sequence; *upper case letters*, *Act1* exon sequence; *lower case letters*, *Act1* intron 1 sequence; *slashed lines*, intron splice sites between the noncoding exon/noncoding intron, and between the intron/first coding exon, respectively; *bold letters*, *Act1* and *Gus* translation initiation codons. The 15 amino acids of the *Act1-Gus* junction are shown below their respective codons

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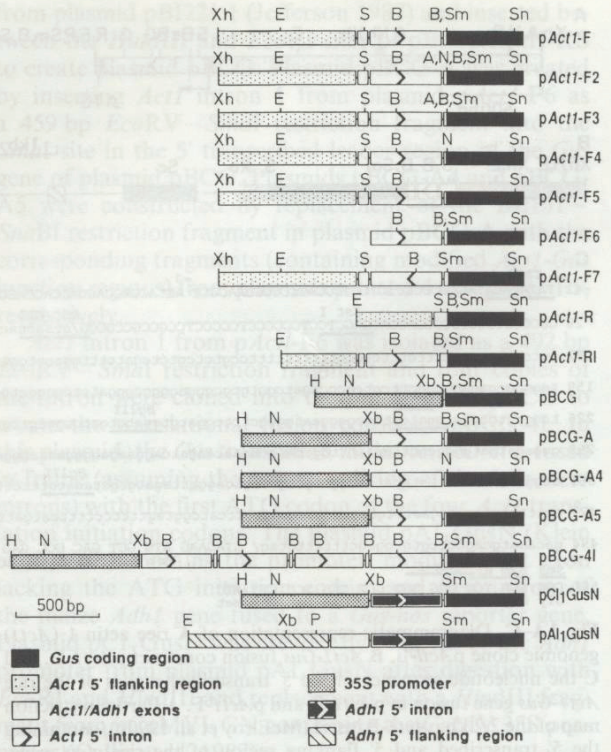


Fig. 2, Diagrammatic representation of the chimeric *Gus* constructs used in the transient gene expression assays. These plasmids contain different components from the 5′ regions of the rice actin 1 gene (*Act1*), maize alcohol dehydrogenase 1 gene (*Act1*) and/or CaMV 35S promoter, fused to the β-glucuronidase coding region (*Gus*) and nopaline synthase polyadenylation region (*nos*). The different sequence elements (see key) are as described in the legend to Fig. 1 except for the following: *dark stippled boxes*, CaMV 35S promoter; *striped box*, 5′-flanking region of the *Adh1* gene; *open boxes*, exons of rice *Act1* or maize *Adh1* genes; *filled boxes with light arrowheads*, 5′ intron of the maize *Adh1* gene; *filled boxes*, 5′ portion of the *Gus* coding region. The indicated restriction sites are abbreviated as given for Fig. 1 except for the following: N, *Nco*I; Sn, *Sna*BI. The details of each plasmid construction are described in the text

(Fig. 3). This modification of the translation initiation region actually resulted in a slight decrease in GUS activity in rice and maize cells when compared to p*Act1*-F (Table 1).

Plasmid p*Act1*-F3 was created to confirm that translation was initiated at the first ATG codon (*Act1*) of the *Act1-Gus* transcript in p*Act1*-F and p*Act1*-F2. Plasmid p*Act1*-F3 contains a deletion of the *Act1* start codon such that translation would now be expected to initiate from the next ATG codon located 29 bp upstream of, and out-of-frame with, the *Gus* initiation ATG codon (Fig. 3). Since this start codon is followed by a termination codon 39 bp downstream, no GUS activity was expected. If initiation starts with the internal *Gus* ATG codon, a low level of GUS activity was expected since eukaryotic ribosomes initiate translation at low efficiency from internal ATG codons (Liu et al. 1984; Kozak 1984). Table 1 shows that GUS activity was not detectable in either rice protoplasts or maize cells transformed with p*Act1*-F3.

Table 1. β-Glucuronidase (GUS) specific activities in rice and maize cells transformed with *Gus* fusion constructs containing 5′ regions of the rice actin 1, CaMV 35S, and/or maize alcohol dehydrogenase 1 genes

Constructs	GUS specific activity (pmol MU/h per mg protein)			
	Rice protoplasts		Maize cells	
	Mean	SE	Mean	SE
p*Actl*-F	2676	151	1603	526
p*Actl*-F2	2122	149	637	126
p*Actl*-F3	ND	–	ND	–
p*Actl*-F4	2970	155	2434	718
p*Actl*-F5	1170	126	696	223
p*Actl*-F6	ND	–	ND	–
p*Actl*-F7	ND	–	ND	–
p*Actl*-R	ND	–	ND	–
p*Actl*-RI	2210	–	1432	421
pBCG	128	21	83	32
pBCG-A	5318	346	4701	1634
pBCG-A4	4913	273	5432	1608
pBCG-A5	1350	118	541	87
pBCG-4I	5814	321	4405	1032
pCI_1GusN	542	55	464	174
pAI_1GusN	515	62	184	78

The results of three experiments, assayed 3 days after transformation, were used to estimate the mean GUS specific activity (pmol 4-methylumbelliferone/h per mg protein) and associated standard error (SE), in rice and maize cells, after correcting for background ND, no GUS specific activity above background was detected for this construct

Plasmid p*Act1*-F5 was designed to investigate the influence on *Gus* expression of the 15 N-terminal amino acids added to the GUS protein from the *Act1-Gus* junction region in both p*Act1*-F and p*Act1*-F2 (Fig. 3). Plasmid p*Act1*-F5 contains an *Act1-Gus* transcriptional fusion in which all upstream ATG codons are deleted such that translation would now be expected to initiate at the *Gus* start codon. This change resulted in a specific activity for GUS from p*Act1*-F5-transformed cells, which was approximately 40% of that obtained for p*Act1*-F (Table 1). This result initially implied that the addition of 15 amino acids had a slight enhancing effect on GUS activity. However, upon closer inspection, it can be observed that the sequence context surrounding the *Gus* initiation codon in p*Act1*-F5 has a suboptimal eukaryotic translation initiation motif. To restore this motif, plasmid p*Act1*-F4 was created as an *Act1-Gus* transcriptional fusion that deletes the extra-N-terminal amino acids but retains the eukaryotic translation initiation site consensus sequence (**ACC**ATG**G**) of p*Act1*-F2 rather than the bacterial sequence (**CTT**ATG**T**) of p*Act1*-F5 (Fig. 3). These modifications resulted in restoration of GUS activity in p*Act1*-F4 transformed cells to levels that were somewhat higher than those observed for p*Act1*-F and p*Act1*-F2 (Table 1). Taken together

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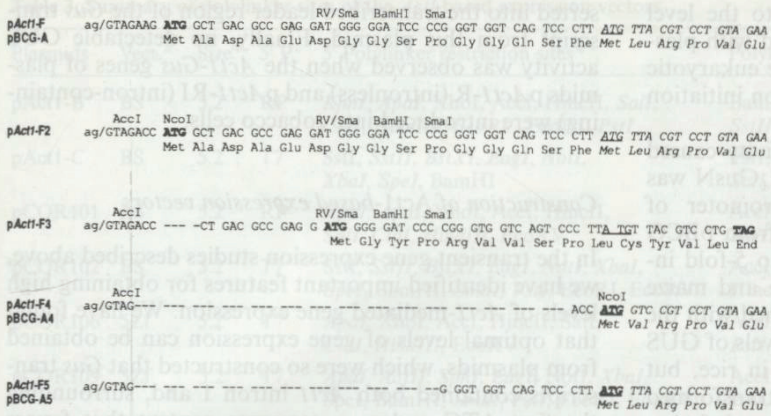


Fig. 3. Nucleotide sequences surrounding the translation initiation codon in the chimeric *Gus* constructs; the sequence context of the translation initiation codon at the *Act1-Gus* junction region in the chimeric *Gus* constructs is presented. Restriction sites used in the construction of the translational fusions are indicated. The sequences are presented as in Fig. 1 except for: *upper case letters*, first coding exon of the *Act1-Gus* fusion; *dashed lines*, nucleotides deleted with respect to the junction sequence of the *Act1-Gus* fusion construct p*Act1*-F; *upper case italic letters*, codons of the *Gus* coding region; *underlined upper case italic letters*, the translation initiation codon of the *Gus* coding region. The expected translation product for each chimeric gene is indicated below the coding sequence with *Gus*-encoded amino acids indicated in *italics*. The important features of the *Act1-Gus* junction in each plasmid are described in the text

these results suggest that the extra 15 N-terminal amino acids on the GUS protein had relatively little effect on its enzymatic activity but that translation of the transcript was influenced by the RNA sequence surrounding the ATG initiation codon.

To investigate further the influence of *Act1* intron 1 on gene expression, three plasmids with intron 1 modifications were tested. Plasmid p*Act1*-F6 (Fig. 2) was created by deleting 0.88 kb of *Act1* sequence upstream of intron 1 to test for any possible promoter activity in the intron itself. Plasmid p*Act1*-F7 (Fig. 2) contains *Act1* intron 1 in an inverted orientation (3′→5′) and was designed to investigate the orientation requirement for intron-dependent gene expression. Plasmid p*Act1*-R (Fig. 2) is an intronless *Act1-Gus* gene, which should produce an mRNA that is identical to the spliced mRNA from p*Act1*-F (McElroy et al. 1990c). p*Act1*-R was created to measure the basal level of activity (no intron-mediated effects) from the *Act1* promoter. Transient assays showed no detectable GUS activity in rice protoplasts or maize cells transformed with either p*Act1*-F6, p*Act1*-F7 or p*Act1*-R plasmids (Table 1). To demonstrate that p*Act1*-R was potentially functional, *Act1* intron 1 was re-inserted into the transcribed leader region of p*Act1*-R to create the plasmid p*Act1*-RI (Fig. 2). In rice protoplasts and maize cells transformed with p*Act1*-RI, GUS activity was restored respectively to levels 82% and 89% of that observed with p*Act1*-F transformed cells.

Utilization of Act1 *5′ transcribed sequences to stimulate* Gus *expression from the CaMV 35S promoter*

To determine whether the *Act1* first intron could stimulate gene expression from a heterologous promoter, *Gus* gene constructs were created with the CaMV 35S promoter and transcribed sequences from the *Act1* gene (Figs. 2, 3). The reference plasmid, pBCG, contains a 35S-*Gus-nos* reporter gene (Fig. 2). The plasmid, pBCG-A, was created by inserting the *Act1* 5′ transcribed region from p*Act1*-F6 (Fig. 2), including *Act1* intron 1 and an optimal *Act1* translation initiation region, into the 5′ leader region of the *Gus* gene in pBCG. Plasmid pBCG-A is analogous to p*Act1*-F (Fig. 3) in that it encodes an ACT1-GUS fusion protein with an extra 15 N-terminal amino acids. GUS activity was stimulated 41- and 65-fold in pBCG-A-transformed rice protoplasts and maize cells, respectively, compared to pBCG-transformed cells (Table 1). These levels of GUS activity are approximately 2- to 3-fold higher than that obtained in p*Act1*-F transformed rice and maize cells (Table 1). The importance of intron orientation was also demonstrated as no detectable GUS activity was observed in rice or maize cells transformed with pBCG-A7 which contains *Act1* intron 1 inserted in an inverted (3′→5′) orientation into the transcribed leader region of the *Gus* gene (data not shown).

To separate the stimulatory effects of *Act1* intron 1 from that of the optimal *Act1* translation initiation codon, and to confirm the results that were obtained with the *Act1-Gus* fusions, analogous constructs were created with the appropriate components of the *Act1* gene under transcriptional control of the CaMV 35S promoter. Plasmids pBCG-A4 and pBCG-A5 were created by exchanging restriction fragments containing modified *Act1-Gus* junction regions from plasmids p*Act1*-F4 and p*Act1*-F5, respectively, with the *Act1-Gus* region of plasmid pBCG-A (Fig. 3). After transformation with the transcriptional fusion pBCG-A5, like p*Act1*-F5, GUS activities were significantly reduced (Table 1), presumably because translation starts at the suboptimal *Gus* ATG codon.

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GUS activity levels were indeed restored to the level of pBCG-A when the cells were transformed with plasmid pBCG-A4 (Table 1), which contains the eukaryotic consensus sequence around its *Gus* translation initiation codon (Fig. 3).

To compare the stimulation in GUS activity caused by the *Act1* and *Adh1* introns, plasmid pCI_1GusN was created by replacing the maize *Act1* promoter of pAI_1GusN with the CaMV 35S promoter from pBCG (Fig. 2). Plasmid pCI_1GusN, showed a 4- to 5-fold increase in GUS activity in transformed rice and maize cells over that observed from cells transformed with the intronless pBCG plasmid (Table 1). These levels of GUS activity were only 40% of those observed in rice, but approached those observed in maize cells transformed with pBCG-A5, the analogous transcriptional fusion containing the rice *Act1* 5′ intron.

Since the addition of a single *Act1* intron 1 element dramatically stimulated *Gus* expression from the CaMV 35S promoter, we investigated whether the addition of multiple introns would further enhance expression levels. Plasmid pBCG-4I contains four tandem copies of *Act1* intron 1 and associated flanking sequences, with translation expected to initiate at the first of the four *Act1* ATG codons. As can be seen in Table 1, rice protoplasts and maize cells transformed with pBCG-4I produced similar amounts of GUS activity compared to pBCG-A transformed cells, indicating that a single *Act1* intron 1 is sufficient to produce maximal stimulation of gene expression.

Analysis of GUS activity in tobacco cells transformed with Act1- *and 35S-*Gus *fusion constructs*

To determine if the *Act1* 5′ region would be useful for gene expression in dicot cells, four *Gus*-containing plasmids were tested for activity in bombarded tobacco suspension cultured cells. As can be observed in Table 2, plasmid pBCG (containing the 35S promoter with no intron) directed the highest levels of GUS activity. This activity was reduced 4-fold when *Act1* intron 1 was inserted into the transcribed leader region of the *Gus* transcript from this plasmid. Finally, no detectable GUS activity was observed when the *Act1-Gus* genes of plasmids p*Act1*-R (intronless) and p*Act1*-RI (intron-containing) were introduced into tobacco cells.

Table 2. GUS specific activites in tobacco suspension culture cells transformed with *Gus* fusion constructs containing 5′ sequences of the rice actin 1 gene and/or CaMV 35S promoter

Constructs	GUS specific activity (pmol MU/h per mg protein)	
	Mean	SE
p*Actl*-R	ND	–
p*Actl*-RI	ND	–
pBCG	438	29
pBCG-A	112	5

The results of two experiments, assayed 3 days after bombardment, were used to estimate the mean GUS specific activity in tobacco suspension cultured cells, after correcting for background

ND, no GUS specific activity above background was detected for this construct

Construction of Act1-*based expression vectors*

In the transient gene expression studies described above, we have identified important features for obtaining high levels of *Act1*-mediated gene expression. We have found that optimal levels of gene expression can be obtained from plasmids, which were so constructed that *Gus* transcripts contained both *Act1* intron 1 and, surrounding the first ATG codon, a sequence context that favors translation initiation. Based on these findings, a set of *Act1* 5′-based vectors for use in monocot transformation studies has been developed. Both translational and transcriptional fusion expression vectors are available, as shown in Table 3. These expression vectors contain a number of unique polylinker sites located both upstream (5′ polylinker sites) and downstream (3′ polylinker sites) of their respective *Act1* 5′ regions (Table 3) in order to simplify subsequent cloning procedures. The nucleotide sequence at the junction of the *Act1* transcribed region and polylinker region in each expression vector, and their relationship to the *Act1-Gus* fusion plasmids used in the transient gene expression assays are presented in Fig. 4.

Discussion

We have shown that the rice *Act1* 5′ region directs a high level of *Gus* expression in transformed rice and maize cells relative to the CaMV 35S RNA and maize *Adh1* promoters. In order to extend the utility of the rice *Act1* 5′ region for use in cereal transformation, we have developed and evaluated *Act1*-based expression vectors for their ability to promote high levels of *Gus* expression in transformed monocot cells. In this study, we have focused primarily on the influence of *Act1* intron 1 and the sequence context surrounding the translation initiation site on *Gus* expression.

To elucidate further the role of the *Act1* intron 1 in gene expression, plasmids bearing *Act1-Gus* fusion genes with modified first introns were introduced into rice and maize cells. We had previously demonstrated that deletion of *Act1* intron 1 from an *Actl-Gus* gene (p*Act1*-R) resulted in a near complete loss of GUS activity in transformed rice cells (McElroy et al. 1990c). We have now observed an identical effect in transformed maize cells and have further demonstrated that GUS activity can be restored in transformed rice and maize cells if the *Act1* first intron is reinserted into the transcribed leader region of p*Act1*-R in the creation of p*Act1*-RI. Intron-mediated restoration of gene expression is orientation-dependent, as inversion of *Act1* intron 1 resulted in no detectable GUS activity in p*Act1*-F7 transformed rice and maize cells. Moreover, the observa-

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Table 3. Summary of polylinker sites of the *Act1*-based expression vectors

Plasmids	Vect.[a]	Size[b]	5′ P[c]	5′ Polylinker restriction sites[d]	Polylinker restriction sites[d]	3′ P[c]
pAct1-B	BS	5.2	RP	*KpnI, ApaI*, XhoI, AccI, HincII, *SalI, ClaI, HindIII, EcoRV*, EcoRI, *PstI*	BamHI, *SpeI, XbaI, NotI, EagI, BstXI, SstII*, SstI	T7
pAct1-C	BS	5.2	T7	SstI, *SstII, BstXI, EagI, NotI, XbaI, SpeI*, BamHI	*PstI*, EcoRI, *EcoRV, HindIII, ClaI, SalI*, HincII, AccI, XhoI, *ApaI, KpnI*	RP
pCOR101	BS	5.2	RP	*KpnI, ApaI*, XhoI, AccI, HincII, *SalI, ClaI, HindIII*, EcoRV	AccI, *NcoI*, EcoRV, EcoRI, *PstI, SmaI*, BamHI, *SpeI, XbaI, NotI, EagI, BstXI, SstII*, SstI	T7
pCOR102	BS	5.2	T7	SstI, *SstII, BstXI, EagI, NotI, XbaI, SpeI*, BamHI, *SmaI, PstI*, EcoRI, EcoRV	AccI, *NcoI*, EcoRV, *HindIII, ClaI, SalI*, HincII, AccI, XhoI, *ApaI, KpnI*	RP
pCOR106	5Zf	5.2	T7	*ApaI*, XhoI, AccI, HincII, SalI, *ClaI, HindIII*, EcoRV	AccI, *NcoI, SstII*, EcoRV, *SpeI, NotI, PstI*, AccI, HincII, SalI, SstI, *BstXI*	SP6
pCOR109	5Zf	5.2	T7	*ApaI, AatII, SphI, EagI*, NotI, *XbaI*, SpeI, BamHI, *SmaI*, PstI, EcoRI, EcoRV	AccI, *NcoI, SstII*, EcoRV, SpeI, NotI, PstI, AccI, HincII, SalI, SstI, *BstXI*	SP6
pCOR103	BS	4.4	RP	*KpnI, ApaI, XhoI*	AccI, *NcoI*, BamHI, *SpeI, XbaI, NotI, EagI, BstXI, SstII*, SstI	T7
pCOR104	BS	4.4	RP	*KpnI, ApaI, XhoI*	AccI, *NcoI, EcoRV*, EcoRI, *PstI, SmaI*, BamHI, *SpeI, XbaI, NotI, EagI, BstXI, SstII*, SstI	T7
pCOR107	5Zf	4.4	T7	*ApaI, XhoI*	AccI, *NcoI, SstII, EcoRV, SpeI, NotI, PstI*, AccI, HincII, *SalI*, SstI, *BstXI*	SP6
pCOR108	BS	4.4	T7	SstI, *SstII, BstXI, EagI, NotI, XbaI, XhoI*	AccI, *NcoI, EcoRV, HindIII, ClaI, SalI, HincII*, AccI, XhoI, *ApaI, KpnI*	RP
pCOR114	BS	4.4	T7	SstI, *SstII, BstXI, ClaI*, AccI, *HincII, SalI*, XhoI	AccI, *NcoI, SstII, EcoRV, SpeI, NotI, PstI, ApaI, KpnI*	RP
pCOR112	BS	4.4	RP	*KpnI, ApaI, XhoI*	*AccI*, NcoI, SstII, *EcoRV*, EcoRI, *PstI, SmaI*, BamHI, *SpeI, XbaI, NotI, EagI, SstII, BstXI*, SstI	T7
pCOR115	5Zf	4.4	T7	*ApaI, AatII, SphI, EagI, XbaI, XhoI*	AccI, *NcoI, SstII, EcoRV, SpeI, NotI, PstI*, AccI, HincII, *SalI*, SstI, *BstXI*	SP6
pCOR116	5Zf	4.4	SP6	*BstXI*, SstI, AccI, *SalI, PstI, NotI*	AccI, *NcoI, SstII, EcoRV, SpeI, XbaI, EagI, SphI, AatII, ApaI*	T7
pCOR105	BS	4.4	RP	*KpnI, ApaI, XhoI*	*AccI, EcoRV*, EcoRI, *PstI, SmaI*, BamHI, *SpeI, XbaI, NotI, EagI, BstXI, SstII*, SstI	T7
pCOR113	BS	4.4	RP	*KpnI, ApaI, XhoI*	AccI, *HindIII, EcoRV*, EcoRI, *PstI, SmaI*, BamHI, *SpeI, XbaI, NotI, EagI, BstXI, SstII*, SstI	T7
pCOR117	5Zf	4.4	SP6	*BstXI*, SstI, AccI, *SalI, PstI, NotI*	AccI, *SstII, EcoRV, SpeI, XbaI, EagI, SphI, AatII, ApaI*	T7

[a] Plasmid used in vector construction: BS, pBluescriptII-KS (2.96 kb); 5Zf, pGem-5Zf(+) (3.00 kb)
[b] Size of the vector/*Act1* 5′ region in kb
[c] Sequencing primer binding site at the 5′ and 3′ ends of the *Act1* region: RP, M13 reverse primer; T7, T7 17-mer primer; SP6, SP6 17-mer primer
[d] Polylinker restriction sites and the 5′ and 3′ ends of the *Act1* region; unique restriction sites in the expression vector are italic letters

tion that this intron did not restore gene expression when placed outside of the p*Act1*-R transcription unit suggested that the 5′ intron was not acting as an enhancer-like element (McElroy et al. 1990c). Finally, no *Gus* expression was detected in cells transformed with p*Act1*-F6, in which all *Act1* DNA upstream of the 5′ border of the intron had been deleted, suggesting that there is no promoter activity within the *Act1* intron1 itself. These findings further support our previous conclusion (McElroy et al. 1990c) that the rice *Act1* intron-mediated stimulation of gene expression is associated, in part, with an in vivo requirement for efficient intron splicing.

This conclusion is further strengthened by the observation of intron-mediated stimulation of *Gus* gene expression from a heterologous plant promoter in transformed rice and maize cells. Addition of the rice *Act1* first intron to the transcriptional unit of the CaMV 35S-*Gus* gene in the construction of plasmid pBCG-A5 stimulated *Gus* expression more than 10- and 7-fold in transformed rice and maize cells, respectively, to levels similar to that achieved with the analogous *Act1-Gus* transcriptional fusion on plasmid p*Act1*-F5. Only a single intron was necessary to achieve maximal stimulation of *Gus* expression, as a 35S-*Act1-Gus* gene containing four introns in its transcribed leader region (pBCG-4I) directed levels of GUS activity similar to that obtained with the analogous single intron-containing pBCG-A construct. A similar intron-mediated enhancement of gene expression in transformed monocot cells has been reported for the maize *Adh1* (Callis et al. 1987; Oard et al. 1989; Kyozuka et al. 1990; Mascarenhas et al. 1990; Luehrsen and Walbot 1991) and *Sh1* introns (Vasil et al. 1989;

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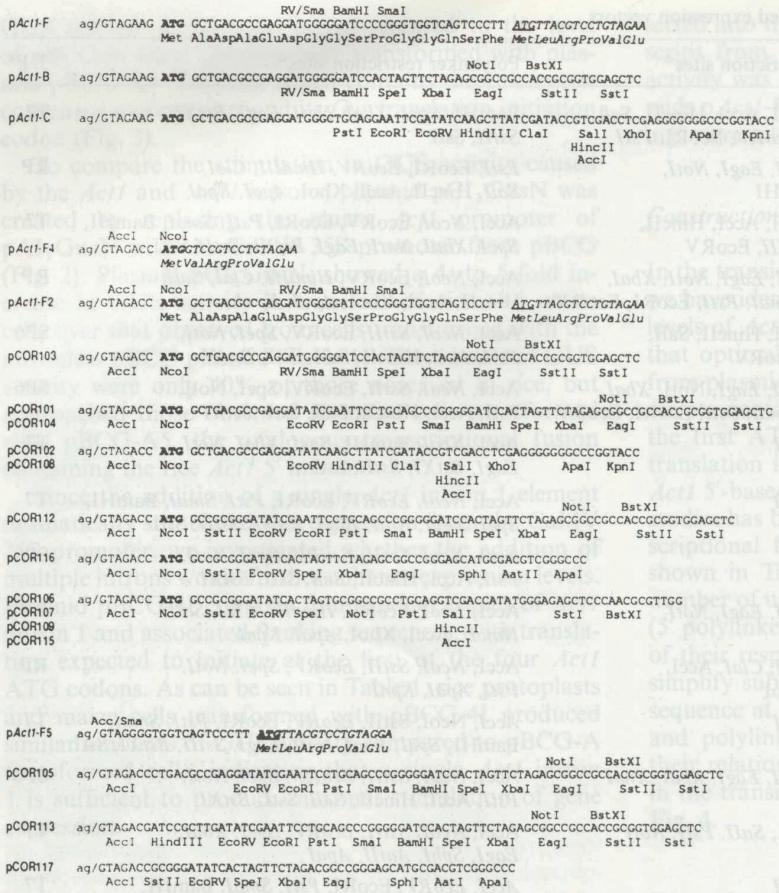


Fig. 4. Nucleotide sequences at the junction of the rice actin 1 gene (*Act1*) transcribed region and the polylinker region in *Act1* -based expression vectors. The *Act1*-based expression vectors have been grouped as translational or transcriptional expression vectors and the former are further subdivided according to the sequence context surrounding the translation initiation codon. Each plasmid group is derived from the *Act1-Gus* junction sequence shown on the first line of each group. The *Act1*-based translational expression vectors containing an unmodified *Act1* translation initiation codon region include p*Act1*-F, B, and C. The *Act1*-based translational expression vectors containing the modified (*Acc*I- and *Nco*I-containing) *Act1* translation initiation codon region include p*Act1*-F2, p*Act1*-F4 and pCOR101–104, 106–109, 112, and 115–116. The *Act1*-based transcriptional expression vectors are derived from p*Act1*-F5 and include pCOR105, 113, and 117. Polylinker restriction sites are indicated above and below each sequence. Features of the nucleotide sequence are indicated as presented in Fig. 3. The expected translation products, if any, are indicated below the coding sequence with *Gus*-encoded amino acids shown in *italics*. Details of construction for each expression vector are described in the Materials and methods

Maas et al. 1991) and even a dicot intron from the castor bean *cat-1* catalase gene (Tanaka et al. 1990). Addition of the maize *Adh1* intron 1 to the transcription unit of a CaMV 35S-*Gus* gene (pCI$_1$GusN) stimulated *Gus* expression more than 4-fold in transformed rice and maize cells to levels that approached or surpassed those achieved with the *Adh1-Gus* gene in plasmid pAI$_1$GusN. This intron-mediated enhancement of gene expression in monocot cells appears to be a general feature of most plant introns and has been attributed to increased mRNA levels and efficient intron splicing (Tanaka et al. 1990; Luehrsen and Walbot 1991).

In transformed tobacco cells, we observed that inclusion of the *Act1* intron in the CaMV 35S-*Gus* transcription unit caused a 4-fold decrease in *Gus* expression. Keith and Chua (1986) have reported that monocot and dicot introns are processed with different efficiencies in transgenic tobacco plants. Thus, the observed GUS activity in tobacco may be a measurement of the inefficiency of splicing of the rice *Act1* intron from the 35S-*Gus* fusion. Furthermore the rice *Act1* promoter, like the maize *Adh1* promoter (Ellis et al. 1987), is not active in transformed tobacco cells, regardless of whether the intron was present (p*Act1*-RI) or absent (p*Act1*-R). Taken together these findings indicate that the *Act1* gene would not be expected to be an efficient promoter in transgenic dicots. Therefore, the CaMV 35S promoter remains the promoter of choice for strong constitutive expression of genes in transgenic dicot plants.

We have also investigated the influence of the sequence context surrounding the translation initiation site in *Gus*-containing transcripts. Kozak (1984) had previously noted a eukaryotic consensus sequence at the ATG initiation codon (**A**CC<u>ATG</u>**G**) with the most highly conserved positions being purines at −3 (usually an A) and at +4 (usually a G) (Kozak 1986, 1987). The *Act1* translation initiation site (**AAG**<u>ATG</u>**G**) closely matches this eukaryotic consensus sequence and the consensus sequence of **A**CA<u>ATG</u>**G** for plant transcripts (compiled by comparison of sequences from a rather limited subset of plant genes; Joshi 1987). Nonetheless, in an attempt to increase *Gus* expression we modified the *Act1* sequence from A**AG**<u>ATG</u>G in p*Act1*-F to A**CC**<u>ATG</u>G in p*Act1*-F2, to match precisely the animal consensus sequence for the translation initiation codon (Kozak 1984). This modification actually resulted in a slight reduction in GUS activity in transformed rice and maize cells.

The importance of the sequence context surrounding the translation initiation codon was most dramatically represented by the relative levels of GUS activity observed from cells transformed with plasmids p*Act1*-F4

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and p*Actl*-F5. Plasmid p*Act1*-F5 contains a transcriptional *Act1-Gus* gene fusion where translation is expected to initiate at the bacterial ATG codon embedded within the sequence **C**TTATG**T** (note the pyrimidine residues at −3 and +4). This plasmid directed levels of *Gus* expression that were reduced 4- and 9-fold relative to pBCG-A transformed rice and maize cells, respectively. However, when the eukaryotic consensus sequence (**A**CCATG**G**) was substituted for the bacterial sequence, GUS activity was restored to pBCG-A levels in both rice and maize cells. The intron and sequence dependence together are most dramatically represented by the observation that the CaMV 35 S-*Act1* intron 1-*Gus* gene (with the optimized ACCATGG) in plasmid pBCG-A4 produced 40- and 65-fold increased GUS activity in transformed rice and maize cells, respectively, than plasmid pBCG containing a generic 35S-*Gus* gene. Sleat et al. (1987) previously reported that the local sequence environment at the translation initiation codon had no influence on gene expression in a plant in vitro translation system; our results clearly demonstrate the importance of this feature for in vivo gene expression.

The sequence modifications to the translation initiation region occur immediately adjacent to the 3′ splice site junction of the *Act1* first intron, and thus could affect splicing efficiency as well as translation initiation. However, RNA gel blot analysis of mRNA from an *Act1*-expressed transgene (like p*Act1*-F4 with a modified *Act1* translation initiation region) in transformed maize calli showed no reduced efficiency in mRNA splicing (A.D. Blowers, unpublished result). We have also noted that the constructs tested in this report often differed in aspects other than the one parameter under investigation. These differences have included mRNA 5′-untranscribed leader length and composition, the distance between the 3′ splice site of the respective *Act1* intron 1 element and the translation start codon, and the sequence context surrounding the translation initiation codon. Although each of these features could potentially complicate the interpretation of our results, we would not be able to detect any potential effects without the use of precisely matched vectors that differ in just one parameter. It is worthwhile noting that Luehrsen and Walbot (1991) performed an exhaustive study on the effects of such features on maize gene intron splicing using matched vectors and observed only minor differences in transient gene expression.

With the knowledge that the *Act1* 5′ region can promote constitutive high levels of *Gus* reporter gene expression throughout the vegetative and gametophytic tissue of transgenic rice plants (Zhang et al. 1991), we have developed a set of *Act1*-based expression vectors for use in monocot transformation. From the results presented in this paper, we propose that constitutive high levels of foreign gene expression can be obtained from plasmids containing the *Act1* promoter and intron 1, and an optimized translation initiation codon sequence provided by the *Act1* gene or the foreign gene to be expressed.

The translational vectors described here would be suitable for the expression of proteins that can tolerate small N-terminal amino acid additions without affecting activity (such as GUS). Transcriptional vectors are available for situations where this is not the case, for example where the protein product contains an N-terminal signal peptide. If the gene of interest is of prokaryotic origin and contains a suboptimal region at the translation initiation site, an *Act1*-based expression vector which has a *Nco*I site overlapping the ATG codon can accommodate a suitably modified gene to produce a perfect in-frame fusion at the *Act1* initiation ATG codon. Some of the rice *Act1*-based expression vectors described here are already being used to direct foreign gene expression in stably transformed tissues of rice and maize plants. We have found that higher levels of gene expression can be obtained using the rice *Act1*-based vectors than were previously obtained with maize *Adh1*-based vectors. Moreover, high levels of *Act1* promoter activity have also been obtained in transient assays of *Act1-Gus* gene expression in cells from a number of other monocot species including wheat, oat, barley, and sorghum (A.D. Blowers, B. Jenes, J. Cao, D. McElroy, and R. Wu, unpublished results). In summary, we propose that these vectors hold great potential for the expression of foreign genes in monocot plants and should greatly facilitate the development of transgenic cereals with agronomically useful traits.

Acknowledgements. The authors wish to recognize Kin Ching Kong and James J. Mecca for technical assistance. The authors thank Drs. Elizabeth Earle and Rod Wing for critical reading of the manuscript. This research was supported (in part) by grants RF86058 Allocation No. 60 and RF 90031 Allocation No. 126 from the Rockafeller Foundation, by a grant from the Midwest Plant Biotechnology Consortium and by grant GM29179 from the National Institute of Health. D.M. was supported by a SERC/NATO studentship, a Fulbright Fellowship, scholarships from the British Universities North America Club and the St. Andrew's Society of Washington, DC, USA, a Cornell University East Asia Program Hu Shih Memorial Fellowship, and predoctoral fellowships from the Cornell University Biotechnology Program and the Cornell NSF Plant Science Center. A.D.B. was supported by the Cornell NSF Plant Science Center. The Cornell NSF Plant Science Center is a unit of the USDA-DOE-NSF Plant Science Centers Program and a unit of the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the US Army Research Office. B.J. was supported by a fellowship from the Agricultural Biotechnology Center, Hungary and the World Bank.

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Communicated by E. Meyerowitz

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