Intron-mediated enhancement of gene expression in maize (Zea mays L.) and bluegrass (Poa pratensis L.)

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Abstract. We report a strength comparison of a large variety of monocot and dicot intron-containing fragments inserted in the S untranslated leader, between the CaMV 35S promoter and the wid3 gene (coding for the β-glucuronidase; GUS). Relative strengths of the intron-containing fragments were evaluated by comparing transient GUS expression after particle bombardment in embryogenic maize and bluegrass suspension cultures. Our results confirm a dramatic dependence on the presence of an intron for chimeric gene expression in both species. On average, the maize first intron of wid3 provided the highest enhancement of gene expression in maize and bluegrass (71- and 26-fold enhancement, respectively). Half of the introns tested affected gene expression differently in bluegrass and maize. This suggests that the intron-mediated enhancement of gene expression generally observed with maize may not be fully applicable to all monocots. We also report enhancement of gene expression (92-fold) in a monocot species by a dicot intron (chka4 intron).

Introduction

Introns can influence gene expression in both animals (Olamer and Leder, 1979; Gassen et al., 1982) and plants (Keith and Chua, 1986; Callis et al., 1987; Vasil et al., 1989). Enhancement of gene expression in plants was first demonstrated by Callis et al. (1987). Such enhancement requires that the intron be present in the transcriptional unit of the gene (Callis et al., 1987; Maccarena et al., 1990; Maas et al., 1991; Clancy et al., 1994) and is most likely based on post-transcriptional mechanisms (Luchscher and Walbot, 1991, 1994; Maas et al., 1991). The splicing process may stabilize pre-mRNA in the nucleus (Luchscher and Walbot, 1991, 1994) leading to a higher accumulation of mature mRNA in the cytoplasm (Callis et al., 1987; Tansaka et al., 1990; Luchscher and Walbot, 1991; Maas et al., 1991), which subsequently results in an increase in the amount of enzyme. Intron-mediated enhancement of gene expression has been reported in various monocotyledonous species using introns from maize: adh1-S (Callis et al., 1987), oc13 (Luchscher and Walbot, 1991), bx1 (Callis et al., 1987), sh1 (Vasil et al., 1989); rice: oc11 (McElroy et al., 1991); oat: phy3 (Bruce and Quail, 1990) and castor bean: cat1 (Tanaka et al., 1990; Takami et al., 1994). However, the situation in dicot species is less clear. Monocot introns do not stimulate (Last et al., 1991; Rahus et al., 1993) and often reduce (Leon et al., 1991; Maas et al., 1991; McElroy et al., 1991) gene expression in dicot species. Results obtained from introduction of dicot introns (rch3, cat, sh1, wb1, phaseolin, st-ls-) into dicot cells indicate that the effect ranges from no stimulation of gene expression (Kuhlman et al., 1988; Vancanneyt et al., 1990; Paszkowski et al., 1992) to a slight stimulatory effect (Tanaka et al., 1990; Leon et al., 1991; Morris et al., 1993).

Research on intron enhancement of gene expression is valuable for basic studies on RNA processing and stability. Introns are also crucial in the development of reliable, high level expression vectors for transformation of monocot species. All agronomically important cereals have been successfully transformed using intron-containing vectors (see Chua, 1994; Vasil, 1994, for reviews). A comparison of the relative strengths of the various intron-containing fragments commonly used in transformation studies would be beneficial for laboratories conducting experiments designed to express transgenes.

In this study, we evaluated the effects of seven different introns on transient gene expression in embryogenic cells of both maize and bluegrass. Introns were isolated from the translated or untranslated regions of monocot and dicot genes and were inserted in the S untranslated leader between the CaMV35S promoter and the wid3 gene (coding for β-glucuronidase; GUS). Various intron-containing constructions were introduced...
into rapidly-growing embryonic cells via particle bombardment. The relative strengths of the intron-containing fragments were then evaluated by measuring transient GUS expression.

Materials and Methods

Preparation of Mice Tissues. Embryonic suspension cultures of mouse (C3H/HeJ x 129/Sv) embryos were initiated from type II embryonic cells as described previously (Vain et al., 1994). Embryonic suspension cultures were maintained by weekly subculture at low density as a medium containing 15% antisera (Benzolag and Sambrook, 1982), 15% vitamins (Genschow et al., 1983), 2% sucrose, and 1.5 mg/l 2,6- dideoxythymidine as antibiotic (5:1). Prior to bombardment, embryonic mouse cells were filtered through a 50-μm filter and 100 μl packed cell volume (100 μg FW of cells) was evenly dispensed in a 7-mm filter paper disc (Whatman 44) to form a very thin layer of cells. Discs were stored on the same MS-based medium solidified with 0.4% agarose (Sigma, Type I) but containing 0.2 M mannitol and 0.2 M sucrose. This oenotic treatment was performed 4 h before and 16 h after bombardment in order to enhance bombardment efficiency (Vain et al., 1993a).

Preparation of Blue Transgene Tissues. Seeds of blue transgene (Pou prototype, L. cv. 'AdePhs') were surface sterilized in a 30% bleach solution for 20 minutes. After rinsing 5 times with sterile distilled water, seeds were placed on the same medium as for mouse embryonic suspension cultures but with 0.8% agarose (pH 7.7). Seeds were cultured at 27°C in the dark. After 2-3 months, mouse cells were removed from the germinated seeds and subcultured to fresh medium. Establishment, maintenance, and bombardment conditions for embryonic suspension cultures of blue transgene were the same as those previously described for mouse but the blue transgene discs were plated on glass filters (Fisher OR) instead of the filter paper discs.

Plasmid Constructions. The plasmid p35S−GUS (originally described as p50GUS) by Fiers & McMillan, 1990 and renamed "p35S−GUS" to be consistent with the other plasmids described in this work, and p3541I (Jones et al., 1992) both containing the CaMV 35S promoter-GUS coding region: neptunyl synthase terminator were used as cassette for the introduction of various intron containing fragments in the T3 unrearranged leader, between the CaMV 35S promoter and the GUS coding sequence. p35SαGUS: the pHV10 plasmid containing the α;TIR1 from rice was generously provided by Dr. Wu (Cornell University, Ithaca, NY). The 448 nt α;TIR1 with short upstream (33 nt) and downstream (7 nt) even flanking sequences was excised from pHV10 with EcoRI and XhoI. The fragment ends were filled with Klenow and inserted into the unique SalI site of p3541I which was cut with BglII and Klenow. The fragment ends were filled with Klenow and inserted into the unique SalI restriction site of p35S−GUS between the CaMV 35S promoter and the GUS coding sequence. p3SαH3O: the pHC30 plasmid containing the α;TIR1 from maize was generously provided by Dr. Chinnappan at the University of Illinois, Urbana, IL. The 1010 α;TIR1 with short upstream even flanking sequence (44 nt) was excised from pHC30 with BglII and XhoI. The fragment ends were filled with Klenow and inserted into the unique SalI restriction site of p35S−GUS between the CaMV 35S promoter and the GUS coding sequence. p3SαH3O and p3SαH3O−GUS: the α;TIR1 containing the α;TIR1 from maize was generously provided by M. Furem at Monash, Victoria, Australia. The 15 α;TIR1 and downstream (6 nt) even flanking sequences was excised from pHC30 with BglII and XhoI. The fragment was inserted into the unique SalI restriction site of p35S−GUS between the CaMV 35S promoter and the GUS coding sequence. Constructions were made with the α;TIR1 intron in both sense (p3SαH3O−GUS) and antisense (p3SαH3O−GUS) orientation.

p35SαGUS (originally described as p351H in Fiers et al., 1990): a plasmid containing the α;TIR1 (Vain et al., 1989) from tobacco was generously provided by Dr. L. C. Hanash (University of Florida, Gainesville, FL). The 1028 α;TIR1 with short upstream (10 nt) and downstream (17 nt) even flanking sequence and short regions of flanking polynucleotide sequences was excised from this plasmid with EcoRI and SphI. The fragment was inserted into the unique SalI restriction site of p35S−GUS between the CaMV 35S promoter and the GUS coding sequence after both the intron fragments and XhoI-cleaved p35S−GUS ends were blunt ended with T4 polymerase. p35SαSAPF: the 1028 α;TIR1 with short upstream (10 nt) and downstream (17 nt) even flanking sequence was excised from the plasmid containing the α;TIR1 (C. L. Hanash, University of Florida, with EcoRI and SphI. The fragment was inserted into the unique SalI restriction site of pHV10 (Fiers et al., 1992) between the CaMV 35S promoter and the HPRTI coding sequence, along with both upstream fragment and XhoI-cleaved p35S−GUS ends were blunt ended with T4 polymerase.

p35SαSAPF: the α;TIR1 (Klösgen et al., 1984) was amplified from maize genomic DNA by PCR. The flanking sequence was excised from the plasmids containing the α;TIR1 (C. L. Hanash, University of Florida, with EcoRI and SphI. The fragment was inserted into the unique SalI restriction site of pHV10 (Fiers et al., 1992) between the CaMV 35S promoter and the HPRTI coding sequence so that the intron could be inserted in one orientation between the unique BglII and XhoI restriction sites present in the plasmids p3541I vector, between the CaMV 35S promoter and the GUS coding sequence. This replaces the CaMV 35S promoter of p3541I with the intron-containing fragment.

p3SGE: the pGEM8B plasmid (Kloos et al., 1993) containing the unique 5′ intron from maize was generously provided by E. Kloos (Frisch, CA). The 101 nt 5′ intron with 19 nt upstream and 20 nt downstream even flanking sequences was amplified from the plasmid pGEM8C−GUS by polymerase chain reaction using the primers (50′GATTCACCACCCGGTTTCTTGCAT3′ and 5′CATGTGGTTGTTAAGCAGCAGACGATCATAG3′) with 45 cycles of amplification using a Precision GT2C thermocycler set to run with the following parameters: denaturation 1 sec at 96°C, anneal 30 sec at 50°C, extend 60 sec at 72°C. The BglII and XhoI restriction sites were incorporated into the PCR fragment which was then cloned into the CaMV 35S promoter and the GUS coding sequence as described above (p3SGE).

p35SαE: the unique 3′ end of α;TIR1 (Klösgen et al., 1984) was amplified from maize genomic DNA by PCR. The flanking sequence was excised from the plasmids containing the α;TIR1 (C. L. Hanash, University of Florida, with EcoRI and SphI. The fragment was inserted into the unique SalI restriction site of p35S−GUS between the CaMV 35S promoter and the GUS coding sequence. Constructions were made with the 3′α;TIR1 intron in both sense (p35SαE−GUS) and antisense (p35SαE−GUS) orientation.

p35SαE−GUS: the unique 3′ end of α;TIR1 (Klösgen et al., 1984) was amplified from maize genomic DNA by PCR. The flanking sequence was excised from the plasmids containing the α;TIR1 (C. L. Hanash, University of Florida, with EcoRI and SphI. The fragment was inserted into the unique SalI restriction site of p35S−GUS between the CaMV 35S promoter and the GUS coding sequence. Constructions were made with the unique 3′α;TIR1 intron in both sense (p35SαE−GUS) and antisense (p35SαE−GUS) orientation.

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p35SαE−GUS: the unique 3′ end of α;TIR1 (Klösgen et al., 1984) was amplified from maize genomic DNA by PCR. The flanking sequence was excised from the plasmids containing the α;TIR1 (C. L. Hanash, University of Florida, with EcoRI and SphI. The fragment was inserted into the unique SalI restriction site of p35S−GUS between the CaMV 35S promoter and the GUS coding sequence. Constructions were made with the unique 3′α;TIR1 intron in both sense (p35SαE−GUS) and antisense (p35SαE−GUS) orientation.

All DNA constructions were initiated after transformation into E. coli and plasmid DNA was purified by CaCl2 gradient centrifugation (Maniatis et al., 1982).

Plasmid Characterization. Plasmid DNA was precipitated on tannic particu- lates (M5, Sylbea) by using 5 μg of tannic particulates, 1 μl of 10× pol (10 μl of particles (1 mg/ml) in 0.25 μl of 2.5 M CaCl2 and 10 μl of 50 μl with volume (1 μl). After 2 min at 4°C, 45 μl of supernatant was removed and discarded. Electromedias were then performed using the Pacllue Lown Gy (Fiers et al., 1992) with a helium pressure of 60 psi and the standard set at 100 μm. Embryonic cells were aquired with a 300 μm mouth plate and placed 17 cm from the filter unit containing the parti- cles (Vain et al., 1993a). After 48 hours, plasmid analyses were made using a total of 3-6 replicates for sense and 4-6 for the bluntron. Fluorometric analy- ses were performed at -3-4 replicates for sense and 3-4 for the bluntron.

Histological and Fluorometric analysis of GUS activity. Two days after bombardment, the bombard marker cells were removed from each filter and assayed both histologically and fluorometric in situ.
Results and discussion

GUS expression in embryogenic maize and bluegrass cultures

In monocot species, enzymatic transient expression assays have been reported to be variable due to differences in the target material and gene transfer efficiency (Isha et al., 1991; Schledzewski and Mendel, 1994). Both histochemical and enzymatic GUS assays were performed in this study (Table 1). The fluorometric assay reflects the overall GUS enzymatic activity. The histochemical GUS assay is limited to the detection of transformed cells displaying some threshold level of GUS expression but can provide a very reliable measure of expression (Vain et al., 1993b).

In the absence of particle bombardment, none of the maize or bluegrass cells exhibited blue coloration after GUS staining. However, a background level of fluorometric glucuronidase-like activity of 6.6 and 0.5 pmol/min/mg protein was measured in maize and bluegrass cells, respectively. These values are comparable to those previously reported for monocot tissues cultured in vitro (Czyzyn et al., 1991; Fennell and Hauptman, 1992). The level of specific GUS activity, resulting from the use of the different plasmids after correcting for the background, is shown in Table 1.

Table 1. Effect of different introcon-destroying fragments on GUS gene expression in maize and bluegrass.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Maize GUS histochemical assay*</th>
<th>Maize GUS fluorometric assay#</th>
<th>Bluegrass GUS histochemical assay*</th>
<th>Bluegrass GUS fluorometric assay#</th>
</tr>
</thead>
<tbody>
<tr>
<td>no bombardment</td>
<td>0.0 e</td>
<td>control</td>
<td>0.0 c</td>
<td>control</td>
</tr>
<tr>
<td>p3SShHPT</td>
<td>1.0 e</td>
<td>0.0 c</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>p3SSadv-invGUS</td>
<td>22.3 d</td>
<td>0.8 c</td>
<td>0.3 e</td>
<td>2.9 c</td>
</tr>
<tr>
<td>p35S-GUS</td>
<td>1479.4 bc</td>
<td>2.9 c</td>
<td>124.7 c</td>
<td>15.4 b</td>
</tr>
<tr>
<td>p35SwwGUS</td>
<td>932.1 c</td>
<td>12.3 bc</td>
<td>24.0 d</td>
<td>17.4 b</td>
</tr>
<tr>
<td>p35SshGUS</td>
<td>1044.9 b</td>
<td>6.9 c</td>
<td>618.0 b</td>
<td>26.2 b</td>
</tr>
<tr>
<td>p33anGUS</td>
<td>4040.7 a</td>
<td>39.5 ab</td>
<td>412.0 b</td>
<td>39.4 b</td>
</tr>
<tr>
<td>p35SadvGUS</td>
<td>5977.8 a</td>
<td>101.0 a</td>
<td>122.3 c</td>
<td>18.0 b</td>
</tr>
<tr>
<td>p35SshGUS</td>
<td>5631.5 a</td>
<td>142.5 a</td>
<td>507.3 b</td>
<td>30.9 b</td>
</tr>
<tr>
<td>p35SadvGUS</td>
<td>5393.1 a</td>
<td>272.9 a</td>
<td>347.8 b</td>
<td>26.3 b</td>
</tr>
<tr>
<td>p35SubGUS</td>
<td>6230.7 a</td>
<td>211.3 a</td>
<td>1204.7 a</td>
<td>407.4 a</td>
</tr>
</tbody>
</table>

When the p3SSadvHPT plasmid containing no wild gene was introduced into maize cells, no fluorometric GUS activity was detected (Table 1). However, two of the six bombardments performed with p3SSadvHPT resulted in the production of 3 blue foci. This was probably due to cross-contamination with other plasmid molecules during the plasmid preparation or the bombardment process. The p3SSadv-invGUS plasmid, containing the adh1-S intron in the inverted orientation (relative to the direction of transcription) was also used as a negative control in maize and bluegrass. Minimal GUS enzymatic activity was detected using this DNA construction (Table 1). The presence of an intron in the inverted orientation in the 5' untranslated region has been reported to either eliminate (Vain et al., 1989; Maas et al., 1991; McElroy et al., 1991) or drastically reduce (Cai et al., 1987) gene expression. Low levels of gene expression from use of reverse orientation introns in monocots may result from either cryptic splicing or plasmid DNA rearrangements which can occur during the transformation process, leading to an expressible wild gene (Weisbe et al., 1998).

Identification of monocot introcon-containing fragments giving high levels of gene expression

In most cases, expression of the wild gene was higher in maize than in bluegrass tissue for each DNA construction tested (Table 1). When the introcon-less p35S-GUS plasmid was used for bombardment, a GUS fluorometric activity of 2.9 and 15.4 pmol/min/mg protein was measured in maize and bluegrass, respectively. These values are consistent with previous reports on bioassay transformation of suspension cultures of maize (McElroy et al., 1991), wheat (Chibbar et al., 1991), and barley
with intron-less constructions. On average, 1479 maize and 125 bluegrass cells exhibited a low intensity blue staining after particle bombardment with p35S-GUS. The wx (P3S:S:w22) and bc-w22 (p35S:hGUS) introns did not significantly enhance GUS expression compared to the intron-less control (p35S-GUS) in both species (Table 1). The slight increase in enzymatic activity observed with p35S:hGUS (1.7- to 2.3-fold) in both systems is somewhat less than previously reported by Callis (1987) where use of the h21 intron with the 35S promoter increased CAT activity 6.2-fold in maize protoplasts. The minor differences in the sequences of the two bronze introns (h21 vs. bc-w22, (Palston et al., 1988)) seems unlikely to be responsible for these differences. The short length of both bc-w22 and wx introns (105 and 139 nt respectively) is also unlikely to be responsible for their limited effect on gene expression. Introns as small as 70-150 nt have been reported to be efficiently spliced (Goeddel and Filipowicz, 1990) and to enhance gene expression (Callis et al., 1987) in monocots.

The act1 intron1 (p35S:S:act1), adhl-S intron1 (p35S:S:adhl1-GUS), shi intron1 (p35S:S:shi-GUS) and sbl1 intron1 (p35S:S:sbl1-GUS) significantly increased wild4 gene expression in bombarded maize cells compared to the intron-less construction p35S-GUS. These enhancements were manifested by increases in both GUS enzymatic activity and number/intensity of blue foci (Table 1). Enhancement of gene expression from the use of the act1 intron1, adhl-S intron1 and shi intron1 has previously been reported in maize by McElroy et al. (1990), Callis et al. (1987) and Vasil et al. (1989), respectively. Although the beneficial effect of the entire 5'S ubl1 region (promoter+introns1+exons1) on chimeric gene expression in many monocots has been well documented (Christensen et al., 1992; Fennell and Hauptman, 1992; Gallo-Meagher and Irvine, 1993; Taylor et al., 1993), the effects of a ubl1 intron-containing fragment alone have not yet been reported. In bombarded maize cells, no statistical differences were observed between the following four introns despite a sizable variation in their effect on gene expression when compared to the intron-less construction p35S-GUS: rice act1 intron1 (x133.3), maize adhl-S intron1 (x34), maize shi intron1 (x48.1) and maize ubl1 intron1 (x71.4). Most of our data are in agreement with previous studies. In maize, use of the maize adhl-S intron1 resulted in higher gene expression over the maize bc-w1 intron [1.3- to 3.4-fold (Callis et al., 1987) vs. 14.6-fold in this study] and the maize shi intron1 provided more enhancement than the maize adhl-S intron1 [2.5- to 24.2-fold (Vasil et al., 1989; Fennell and Hauptman, 1992; Clancy et al., 1994) vs. 1.4-fold in this study]. Although quantitative differences in gene expression were observed between this and previous studies, the only qualitative difference was obtained when the adhl-S intron1 and act1 intron1 were compared. McElroy et al. (1991) showed a 10-fold enhancement of gene expression with the rice act1 intron1 over maize adhl-S intron1 (vs. 0.4-fold in this study).

In bombarded bluegrass cells, the only major enhancement of wild4 gene expression (26-fold) was observed when the shi intron1 (p35S:S:shi-GUS) was used for bombardment. The act1 intron1, adhl-S intron1, and shi intron1 did not significantly enhance wild4 gene expression when compared to the intron-less construction p35S-GUS. The results obtained with bluegrass did not always parallel the enhancement of gene expression observed in maize. This difference between maize and bluegrass suggests that the results of intron-mediated enhancement of gene expression may vary between different grass species. Differences in intron-mediated enhancement of gene expression were also seen between different species (maize, wheat, rice) by Taylor et al. (1993) and in two wheat species by Takami et al. (1994).

A dicot introcontaining fragment can also enhance gene expression in monocots

The petunia chel1 intron significantly enhanced expression of the wild4 gene in maize (52-fold) when compared to the intron-less control p35S-GUS. Its effect on gene expression was comparable, or greater than that of the most active monocot intron-containing fragment tested in this study (shi intron1, Table 1). Previously, the dicot intron, carl1 intron1 from castor bean (inserted in the coding sequence of the wild4 gene), increased gene expression 10- to 40-fold in rice (Tanaka et al., 1990) and 3- to 5-fold in wheat (Takami et al., 1994).

In bombarded bluegrass cells, the chel1 intron (p35S:S:chel1-GUS) did not significantly enhance wild4 gene expression compared to the intron-less construction p35S-GUS. However, a significantly higher number of blue foci was obtained. This difference in intron-mediated gene enhancement between maize and bluegrass was similar to that observed earlier with act1 intron1, adhl-S intron1, and shi intron1.

In monocots, species enhancement of gene expression can be achieved using both monocot and dicot introns. The level of enhancement is not related to the origin (homologous/heterologous species), length, or position (translated/untranslated region) of the intron in the native gene. But rather, it results most likely from a specific interaction between the intron, its surrounding sequences, and the species in which it is introduced (Callis et al., 1987; Fennell and Hauptman, 1992; Clancy et al., 1994).

In dicot species, the situation is less clear. The effects of introns on gene expression can vary from negative (with some monocot introns (Maas et al., 1991; Leon et al., 1993; McElroy et al., 1991)) to positive (with some dicot introns (Tanaka et al., 1990; Leon et
Intron-based gene expression enhancement in plants

Intron-mediated enhancement of gene expression is of considerable importance in the development of reliable, high level expression vectors for transformation of monocot species. In this study, intron-containing fragments with short native exon flanking sequences were utilized. The flanking sequences strongly influence intron processing (Luchsen and Walbot, 1991) and therefore have a large effect on the intron-mediated enhancement of gene expression (Mascarenhas et al., 1990; Fennell and Hauptman, 1992; Clancy et al., 1994). Moreover, the presence of residual exonic sequences after intron splicing results in minor differences in the mRNA leader sequence compared to the intronless gene (g355-GUS in this work). This can affect mRNA stability, transport or translation (Mascarenhas et al., 1990). Several studies have described the contribution of the adjacent exonic sequences to intron-enhancement of gene expression. Short exon sequences, without their respective introns, led to a limited enhancement of gene expression (1 to 22% of the total intron-mediated increase in gene expression (Callis et al., 1987; Maas et al., 1991; Fennell and Hauptman, 1992; Clancy et al., 1994)). When introns and native exons are used together, their interactions are likely to be responsible for most of the intron-mediated enhancement effect (Maas et al., 1991; Fennell and Hauptman, 1992). In this case, extensive deletion of exon flanking sequences (18 nt deletion out of 54 nt, (Mascarenhas et al., 1990)) or large modifications of the 5' untranslated leader (77 nt vs 146 nt (Luchsen and Walbot, 1991)) drastically affects intron enhancement of gene expression. In addition, exon-intron interactions increase with the size of the exon (Mascarenhas et al., 1990). Therefore, it is necessary to consider simultaneously the intron together with its native flanking sequences in gene expression studies.

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