

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

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Received 22 May 1995/Revised version received 17 August 1995 – Communicated by I. K. Vasil

Abstract. We report a strength comparison of a large variety of monocot and dicot intron-containing fragments inserted in the 5' untranslated leader, between the CaMV 35S promoter and the *uidA* 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 *ubi1* 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 obtained 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 (*chsA* intron).

Introduction

Introns can influence gene expression in both animals (Hamer and Leder, 1979; Gasser *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; Mascarenhas *et al.*, 1990; Maas *et al.*, 1991; Clancy *et al.*, 1994) and it is most likely based on post-transcriptional mechanisms (Luehrsen and Walbot, 1991, 1994; Maas *et al.*, 1991). The splicing process may stabilize pre-mRNA in the nucleus (Luehrsen and Walbot, 1991, 1994) leading to a higher accumulation of mature mRNA in the cytoplasm (Callis *et al.*, 1987; Tanaka *et al.*, 1990; Luehrsen 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), *act3* (Luehrsen and Walbot, 1991), *bz1* (Callis *et al.*, 1987), *sh1* (Vasil *et al.*, 1989); rice: *act1* (McElroy *et al.*, 1991); oats: *phyA3* (Bruce and Quail, 1990) and castor bean: *cat1* (Tanaka *et al.*, 1990; Takumi *et al.*, 1994). However, the situation in dicot species is less clear. Monocot introns do not stimulate (Last *et al.*, 1991; Rathus *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 (*rbcS-3A*, *cat*, *ubi3*, *ubi10*, *phaseolin*, *ST-LS1*) into dicot cells indicate that the effect ranges from no stimulation of gene expression (Kuhlemeier *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 Christou, 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 5' untranslated leader between the CaMV35S promoter and the *uidA* gene (coding for β -glucuronidase:GUS). Various intron-containing constructions were introduced

into rapidly-growing embryogenic 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 Maize Tissue. Embryogenic suspension cultures of maize (*Zea mays* A188 x B73) were initiated from type II embryogenic callus as described previously (Vain *et al.*, 1993a). Embryogenic suspension cultures were maintained by weekly subculture at low density in a medium containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1968), 2% sucrose, and 1.5 mg/l 2,4-dichlorophenoxyacetic acid (pH 5.7). Prior to bombardment, embryogenic maize cells were filtered through a 500 µm filter and 100 µl packed cell volume (~100 mg FW of cells) was evenly dispersed on a 7 cm filter paper disc (Whatman #4) to form a very thin layer of cells. Discs were stored on the same MS-based medium solidified with 0.6% agarose (Sigma, Type I) but containing 0.2 M mannitol and 0.2 M sorbitol. This osmotic treatment was performed 4 h before and 16 h after bombardment in order to enhance bombardment efficiency (Vain *et al.*, 1993a).

Preparation of Bluegrass Tissue. Seeds of bluegrass (*Poa pratensis* L. cv. 'Adelphi') 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 maize embryogenic suspension cultures but with 0.8% agarose (pH 5.7). Seeds were cultured at 27°C in the dark. After 2-3 months, embryogenic calli were removed from the germinated seeds and subcultured to fresh medium. Establishment, maintenance, and bombardment conditions for embryogenic suspension cultures of bluegrass were the same as those previously described for maize but the bluegrass cells were plated on glass filters (Fisher G8) instead of the filter paper discs.

Plasmid constructions. The plasmid p35S-GUS (originally described as "pUCGUS" by Finer & McMullen, 1990 and renamed "p35S-GUS" to be consistent with the other plasmids described in this work), and pJJ3411 (Jones *et al.*, 1992) both containing the CaMV35S promoter:GUS coding region: nopaline synthase terminator were used as cassettes for the introduction of various intron containing fragments in the 5' untranslated leader, between the CaMV35S promoter and the GUS coding sequence.

p35SactGUS: the pBY510 plasmid containing the *act1* intron1 from rice was generously provided by R. Wu (Cornell University, Ithaca, NY). The 448 nt *act1* intron1 with short upstream (33 nt) and downstream (7 nt) exon flanking sequences was excised from pBY510 with *EcoR1* and *XbaI*. The fragment ends were filled with Klenow and inserted into the unique *SmaI* restriction site of p35S-GUS between the CaMV35S promoter and the GUS coding sequence.

p35SubiGUS: the pAHC30 plasmid containing the *ubil* intron1 from maize was generously provided by A. Christensen (George Mason University, Fairfax, VA; Christensen *et al.*, 1992). The 1010 nt *ubil* intron1 with short upstream exon flanking sequence (41 nt) was excised from pAHC30 with *BglII* and *HindIII*. The fragment ends were filled with Klenow and inserted into the unique *SmaI* restriction site of p35S-GUS between the CaMV35S promoter and the GUS coding sequence.

p35SadhGUS and p35Sadh-invGUS: the p11 plasmid containing the *adh1-S* intron1 from maize was generously provided by M. Fromm (Monsanto, St Louis MO). The 434 nt *adh1-S* intron1 with short upstream (15 nt) and downstream (6 nt) exon flanking sequences was excised from p11 with *BclI* and *BamHI*. The fragment was inserted into the unique *BamHI* restriction site of p35S-GUS between the CaMV35S promoter and the GUS coding sequence. Constructions were made with the *adh1-S* intron1 in both sense (p35SadhGUS) and inverted (p35Sadh-invGUS) orientations.

p35SshGUS (originally described as pGB5 in Finer *et al.*, 1992): a plasmid containing the *sh1* intron1 (Vasil *et al.*, 1989) from maize was generously provided by L. C. Hannah (University of Florida, Gainesville, FL).

The 1028 nt *sh1* intron1 with short upstream (10 nt) and downstream (17 nt) exon flanking sequences and short regions of flanking polylinker sequences was excised from this plasmid with *EcoR1* and *Sph1*. The fragment was inserted into the unique *BamHI* restriction site of p35S-GUS between the CaMV35S promoter and the GUS coding sequence after both the intron fragment and *BamHI*-cleaved p35S-GUS ends were blunt ended with T4 polymerase.

p35SshHPT: the 1028 nt *sh1* intron1 with short upstream (10 nt) and downstream (17 nt) exon flanking sequences was excised from the plasmid containing the *sh1* intron1 (L. C. Hannah, University of Florida) with *EcoR1* and *Sph1*. The fragment was inserted into the *XbaI* restriction site of pHygr (Finer *et al.*, 1992) between the CaMV35S promoter and the HPTII coding sequence, after both the intron fragment and *XbaI*-cleaved pHygr ends were made blunt with T4 polymerase.

p35SwxGUS: the *wx* intron1 (Kloesgen *et al.*, 1986) was amplified from maize genomic DNA by polymerase chain reaction using the primers GGTACCATGGCTCGAGCTCATCTCGTCGACGACCA and GGTACCATGGCCCGCCATGCCGATTAATCCAC with 40 cycles of amplification using a Precision GTC2 thermocycler set to run with the following parameters: denature 1 sec at 99°C, anneal 30 sec at 50°C, extend 60 sec at 72°C. The amplified fragment contained the 139 nt *wx* intron1 with 21 nt upstream and 21 nt downstream exon fragments. The 5' primer extensions incorporated *XhoI* and *NcoI* restriction sites such that the intron could be inserted in sense orientation between the unique *XhoI* and *NcoI* restriction sites present in the plasmid pJJ3411 vector, between the CaMV35S promoter and the GUS coding sequence. This replaces the Cab leader of pJJ3411 with the intron-containing fragment.

p35SbzGUS: the pAGS528 plasmid (Ralston *et al.*, 1988) containing the unique *bz* intron from maize was generously provided by E. Ralston (DNAP, Oakland, CA). The 105 nt *bz* intron with 19 nt upstream and 20 nt downstream exon flanking sequences was amplified from the pAGS528 plasmid by polymerase chain reaction using the primers GGTACCATGGCTCGAGGACGTCGGCGACCAGGGT and GGTACCATGGCCTCGTCCACCCTGTTTGC GGCT with 25 cycles of amplification using a Precision GTC2 thermocycler set to run with the following parameters: denature 1 sec at 99°C, anneal 30 sec at 50°C, extend 60 sec at 72°C. *XhoI* and *NcoI* restriction sites were incorporated into the PCR fragment which was cloned between the CaMV35S promoter and the GUS coding sequence as described above (p35SwxGUS).

p35SchsGUS: the unique *chsA* intron (Koes *et al.*, 1989) was amplified from petunia DNA by polymerase chain reaction using the primers GGTA CCATGGCTCGAGAAATTTAAGCGCATGTG and GCTACCATGCC CTCTGTTAAGTGCATGTACC with 25 cycles of amplification using a Precision GTC2 thermocycler set to run with the following parameters: denature 1 sec at 99°C, anneal 30 sec at 50°C, extend 60 sec at 72°C. The amplified fragment contained the 1347 nt *chsA* intron with 19 nt upstream and 45 nt downstream exon fragments. *XhoI* and *NcoI* restriction sites were incorporated into the PCR fragment which was cloned between the CaMV35S promoter and the GUS coding sequence as described above (p35SwxGUS).

All DNA constructions were isolated after transformation into *E. coli* and plasmid DNA was purified by CsCl₂ gradient centrifugation (Maniatis *et al.*, 1982).

Particle Bombardment. Plasmid DNA was precipitated on tungsten particles (M10, Sylvania) by mixing 6 µl of DNA (1 µg/µl), 14 µl of H₂O, 10 µl of particles (1 mg/10 µl), 25 µl of 2.5 M CaCl₂, and 10 µl of 100 mM spermidine (free base). After 5 min at 4°C, 45 µl of the supernatant was removed and discarded. Bombardments were performed using the Particle Inflow Gun (Finer *et al.*, 1992) with a helium pressure of 60 PSI and the solenoid set at 50 ms. Embryogenic cells were covered with a 500 µm baffle and placed 17 cm from the filter unit containing the particles (Vain *et al.*, 1993b). Histochemical analyses were made using a total of 6-10 replications for maize and 4-6 for bluegrass. Fluorometric analyses consisted of 3-4 replications for maize and 3-5 for bluegrass.

Histochemical and fluorometric analysis of GUS activity. Two days after bombardment, the bombarded cells were removed from each filter and assayed both histochemically and fluorometrically for β-glucuronidase

activity according to Jefferson (1987). Histochemical analysis was performed by incubating tissue for 12 hours at 37°C in a 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-gluc) solution and counting the number of blue foci using a dissecting microscope. The fluorometric GUS assay was performed using 4-methylumbelliferyl β -D-glucuronide (MUG) as the substrate (Jefferson, 1987). Fluorescence was measured using a Hoefer TKO mini-fluorometer. Protein content was determined using a PIERCE BCA protein assay kit. The fluorescence data were expressed as pmoles of 4-methylumbelliferone (MU)/min/mg of extracted protein.

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 (Last *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 pmoles/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* (Lyznik *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.

When the p35SshHPT plasmid containing no *uidA* gene was introduced into maize cells, no fluorometric GUS activity was detected (Table 1). However, two of the six bombardments performed with p35SshHPT 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 p35Sadh-invGUS plasmid, containing the *adh1-S* intron1 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 (Vasil *et al.*, 1989; Maas *et al.*, 1991; McElroy *et al.*, 1991) or drastically reduce (Callis *et al.*, 1987) gene expression. Low levels of gene expression from use of inverse 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 *uidA* gene (Weising *et al.*, 1988).

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

In most cases, expression of the *uidA* gene was higher in maize than in bluegrass tissues for each DNA construction tested (Table 1). When the intron-less p35S-GUS plasmid was used for bombardment, a GUS fluorometric activity of 2.9 and 15.4 pmoles/min/mg protein was measured in maize and bluegrass, respectively. These values are consistent with previous reports on biolistic transformation of suspension cultures of maize (McElroy *et al.*, 1991), wheat (Chibbar *et al.*, 1991), and barley

Table 1. Effect of different intron-containing fragments on *uidA* gene expression in maize and bluegrass.

Plasmid	Maize		Bluegrass	
	GUS histochemical assay*	GUS fluorometric assay [#]	GUS histochemical assay*	GUS fluorometric assay [#]
no bombardment	0.0 e	control	0.0 e	control
p35SshHPT	1.0 e	0.0 c	NA	NA
p35Sadh-invGUS	22.3 d	0.8 c	0.3 e	2.9 c
p35S-GUS	1479.4 bc	2.9 c	124.7 c	15.4 b
p35SwxGUS	932.1 c	12.3 bc	24.0 d	17.4 b
p35SbzGUS	1944.9 b	6.9 c	618.0 b	26.2 b
p35SactGUS	4040.7 a	39.5 ab	412.0 b	39.4 b
p35SadhGUS	5977.8 a	101.0 a	122.3 c	18.0 b
p35SshGUS	5631.5 a	142.5 a	507.3 b	30.9 b
p35SchsGUS	5393.1 a	272.9 a	347.3 b	26.3 b
p35SubiGUS	6230.7 a	211.3 a	1204.7 a	407.4 a

*number of blue foci per 100 mg FW of tissue.

[#]measured in pmoles/min/mg protein. "0.0" indicates that no GUS activity above the background was detected for this construction.

a, b, c, d, e : Entries within columns followed by different letters are significantly different at P=0.05 by one way analysis of variance. Data were transformed by log10 in order to meet the ANOVA assumptions.

(Chibbar *et al.*, 1993) 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* (p35S*wx*GUS) and *bz-w22* (p35S*bz*GUS) 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*bz*GUS (1.7- to 2.5-fold) in both systems is somewhat less than previously reported by Callis (1987) where use of the *bz1* 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 (*bz1* vs. *bz-w22*, (Ralston *et al.*, 1988)) seem unlikely to be responsible for these differences. The short length of both *bz-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 (Goodall and Filipowicz, 1990) and to enhance gene expression (Callis *et al.*, 1987) in monocots.

The *act1* intron1 (p35S*act*GUS), *adh1-S* intron1 (p35S*adh*GUS), *sh1* intron1 (p35S*sh*GUS) and *ubil* intron1 (p35S*ubi*GUS) significantly increased *uidA* gene expression in bombarded maize cells compared to the intron-less construction p35S-GUS. Such 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, *adh1-S* intron1 and *sh1* 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' *ubil* region (promoter+intron1+exon1) 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 *ubil* intron1-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 (x13.3), maize *adh1-S* intron1 (x34), maize *sh1* intron1 (x48.1), maize *ubil* intron1 (x71.4). Most of our data are in agreement with previous studies. In maize, use of the maize *adh1-S* intron1 resulted in higher gene expression over the maize *bz1* intron [1.3- to 3.4-fold (Callis *et al.*, 1987) vs. 14.6-fold in this study] and the maize *sh1* intron1 provided more enhancement than the maize *adh1-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 ob-

tained when the *adh1-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 *adh1-S* intron1 (vs. 0.4-fold in this study).

In bombarded bluegrass cells, the only major enhancement of *uidA* gene expression (26-fold) was observed when the *ubil* intron1 (p35S*ubi*GUS) was used for bombardment. The *act1* intron1, *adh1-S* intron1, and *sh1* intron1 did not significantly enhance *uidA* 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, sugarcane) by Taylor *et al.* (1993) and in two wheat species by Takumi *et al.* (1994).

A dicot intron-containing fragment can also enhance gene expression in monocots

The petunia *chsA* intron significantly enhanced expression of the *uidA* gene in maize (92-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 (*ubil* intron1, Table 1). Previously, the dicot intron, *cat1* intron1 from castor bean (inserted in the coding sequence of the *uidA* gene), increased gene expression 10- to 40-fold in rice (Tanaka *et al.*, 1990) and 3- to 5-fold in wheat (Takumi *et al.*, 1994).

In bombarded bluegrass cells, the *chsA* intron (p35S*chs*GUS) did not significantly enhance *uidA* 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, *adh1-S* intron1, and *sh1* intron1.

In monocot 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 into 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.*, 1991; McElroy *et al.*, 1991)] to positive [with some dicot introns (Tanaka *et al.*, 1990; Leon *et*

al., 1991; Morris *et al.*, 1993)]. An explanation for the differences in intron processing between monocot and dicot species was proposed by Goodall and Filipowicz (1989; 1991). They suggested that intron splicing is considerably more "permissive" in monocots than in dicots. Monocots (such as maize) were able to process natural and synthetic introns with various AU content, introns with a stem-loop structure, and even those of mammalian origin (Goodall and Filipowicz, 1991). In contrast, dicots were not able to similarly process these introns. Intron recognition and splicing in plants requires, in part, a 15 to 20% differential in AU content between intron and exon sequences. Due to the differences in average AU content of monocot introns and exons (59% and 44%, respectively) and dicot introns and exons (74% and 55%, respectively), monocot introns are usually inefficiently processed in dicot species (Keith and Chua, 1986; Goodall and Filipowicz, 1991; Leon *et al.*, 1991).

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 (Luehrsen and Walbot, 1991) and therefore have a large effect on 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 (p35S-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, used 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 [48 nt deletion out of 54 nt, (Mascarenhas *et al.*, 1990)] or large modifications of the 5' untranslated leader [77 nt vs. 146 nt (Luehrsen 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.

Acknowledgments

We thank A. Christensen, M. Fromm, L. C. Hannah, E. Ralston and R. Wu for providing intron-containing fragments. We gratefully acknowledge C. Nemes and L. Ringley for technical assistance. Salaries and research support were provided by State and Federal funds appropriated to OSU/OARDC, by a Kent State University/Regional Campus Summer Professional Development Award, and by the Ohio Turfgrass Foundation. Mention of trademark or proprietary products does not constitute a guarantee or warranty of the product by OSU/OARDC or KSU, and also does not imply approval to the exclusion of other products that may also be suitable. Journal Article No. 173-94

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