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Increased Gene Expression by the First Intron of Maize *Shrunken-1* Locus in Grass Species[1]

Vimla Vasil*, Maureen Clancy, Robert J. Ferl, Indra K. Vasil, and L. Curtis Hannah

Vegetable Crops Department, University of Florida, Gainesville, Florida, 32611

ABSTRACT

The first intron of the *shrunken-1* (*Sh1*) locus of maize was incorporated into constructs containing the chloramphenicol acetyltransferase gene (CAT) coupled with the nopaline synthase 3′ polyadenylation signal. Transcription was driven with the 35S promoter of the cauliflower mosaic virus (CaMV) or the *Sh1* promoter of maize. Transient gene expression was monitored following electroporation into protoplasts of *Panicum maximum* (guineagrass), *Pennisetum purpureum* (napiergrass), or *Zea mays* (maize). The 1028 base pair intron increased gene expression in cells of each species when transcription was driven with the 35S promoter. Eleven to 91-fold increases were observed. Expression levels observed in maize were two and eight times those observed in napiergrass and guineagrass, respectively. The 35S promoter gave CAT activity 10 to 100 times that observed with the *Sh1* promoter. Whereas expression driven by the 35S promoter was reproducible, that observed with the *Sh1* promoter proved quite variable. In similar constructs the first intron of the *alcohol dehydrogenase-1* (*Adh1*) gene of maize led to increased gene expression of only 7 to 10% of that observed with the *Sh1* first intron. The increased level of gene expression caused by the *Sh1* first intron is approximately 10 times higher than that caused by any other plant introns that have been used. Thus, the *Sh1* first intron may prove quite useful in increasing expression of foreign genes in monocots and possibly other plants.

Historically, the *Sh1*[2] locus of maize has been of interest in genetic, biochemical, and molecular investigations. The gene encodes the major endosperm sucrose synthase, an enzyme important in synthesis of starch (3). Loss of this enzymic activity gives rise to inadequate starch levels and, in turn, to shrunken or collapsed kernels at maturity. Because of the gene's importance in starch metabolism, its very abundant transcript (1), its pivotal role in the study of transposable elements (reviewed in refs. 5, 6, and 20), and the extent of natural variation within the gene (28), the *Sh1* locus has been cloned and its structure elucidated (21, 26).

The *Sh1* locus is large and complex. The gene is composed of 16 exons and is some 6 kb in length. A noteworthy feature of the *Sh1* gene, as proposed by Werr *et al.* (26), is the lack of protein coding information in the first exon. This is interesting in light of the hypothesis that introns are used in evolution as molecular handles to move about exons and thereby create new proteins (9) or, more frequently, to increase the rates of recombination within genes and thereby create new forms of existing proteins.

Another role for introns has also been demonstrated. It has been shown in mammalian systems (8, 13), in SV40 (12), and recently in plants (2, 22) that steady-state transcript levels and gene expression are increased by the presence of certain introns.

Previous studies have shown that the CaMV 35S promoter is 10- to 40-fold more effective than the NOS promoter in driving the expression of foreign genes introduced into plant cells (7, 16). These studies also demonstrated that the CaMV 35S promoter was 10- to 100-fold less effective in grass cells than in dicotyledonous species such as petunia and carrot. In our attempts to obtain efficient expression of the introduced genes in grass cells, we have previously used promoters of the *Adh1*, *Adh2*, and *Sh1* genes of maize (15). The *Adh2* and *Sh1* promoters gave no detectable expression in grass protoplasts, while the levels of expression obtained with the *Adh1* promoter were approximately 30% of the CaMV 35S promoter. In continuation of these studies, we now report that the incorporation of the first intron of *Sh1* increases expression of a reporter gene up to 91-fold. The increase is 10-fold greater than that caused by the first intron of the *Adh1* gene of maize and is observed in protoplasts of a number of grasses.

MATERIALS AND METHODS

Cell Lines, Protoplast Isolation, and Electroporation

Protoplasts were isolated from cell suspension cultures of *Panicum maximum* Jacq. (guineagrass), *Pennisetum purpureum* Schum. (napiergrass), and *Zea mays* L. (maize). Guineagrass protoplasts were isolated from cell line Pm85 as previously described (23). Napiergrass protoplasts were isolated from cell line Pp86 (D Lu, IK Vasil, unpublished data) 5 d following subculture. The maize cell line Mpp was established from protoplast-derived callus of *Zea mays* Dekalb XL82 (25), maintained on a 4 to 5 d subculture schedule and used for protoplast isolation 3 d after subculture.

The basic cell wall digesting enzyme solution contained 1% Cellulase Onozuka RS (Yakult Honsha) and 1% Pectinase (Serva). In addition, 0.5% Macerozyme R10 (Yakult Honsha) was added for napiergrass and 0.1% Pectolyase Y23 (Seishin)

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[2] Abbreviations: *Sh1*, *shrunken-1*; CAT, chloramphenicol acetyltransferase; CaMV, cauliflower mosaic virus; *Adh1*, *alcohol dehydrogenase-1*; bp, base pair; kb, kilobase pair; NOS, nopaline synthase.

for maize. The enzymes were dissolved in Mes buffer (23). Settled suspension culture cells (4–6 mL, 1.2–1.6 g fresh weight) were dispersed in 50 mL of enzyme and incubated on a gyrotory shaker (50–60 rpm) for 4–6 h at room temperature. The protoplasts were sieved, washed three times, and counted as previously described (24). Protoplast density was adjusted to 4 to 5 × 10^6/mL. The same number of protoplasts was used in experiments comparing the two promoters and the effect of the inserted introns. Twenty μL of plasmid DNA (concentration of each plasmid construct was determined spectrophotometrically and adjusted to 1 μg/μL with sterile TE) was added to 1 mL of protoplasts at room temperature. Electroporation was done over ice using a BRL Cell Porator with a single pulse of 200V and capacitance of 1180 μF. Protoplasts electroporated without plasmid DNA served as negative controls. Electroporated protoplasts were cultured according to methods described by Vasil *et al.* (23).

All experiments were repeated at least once and each treatment had at least one replicate sample within the same experiment. To accurately compare the level of gene expression between different plasmid constructs in one experiment, protoplasts from a single batch were used.

DNA Construction

All gene constructions were cloned in the *Escherichia coli* plasmid vector pUC19. Correct joining and orientation of the gene elements was confirmed by DNA sequencing and/or restriction analysis.

The reporter sequence used was the generous gift of H. Klee and C. Gasser and consisted of the CAT coding region and NOS 3′ polyadenylation signal (19; H Klee, unpublished data). The *Sh1* promoter fragment has been previously described (15). The promoter, 5′ flanking region, transcription start site, and 5′ untranslated leader sequences to +42 in exon 1 (26) were contained in the approximately 2 kb *Hin*cII fragment of the *Sh1* clone. A modified CaMV 35S promoter containing a duplicated enhancer region was the gift of C. Gasser and H. Klee. Construction of this modified 35S promoter was similar to one described elsewhere (17).

The *Sh1* first intron was excised from the clone p17.6 (28) with *Hin*cII and partial *Taq*I digestion. The 1028 bp intron with short flanking exon sequences was inserted in the 5′ untranslated region between the promoter and the CAT/NOS cassette. Constructions were made with the 35S and *Sh1* promoters and the *Sh1* intron fragment was cloned in both the normal and inverted orientations.

The first intron of *Adh1* has been described (4), and examined for its effect on gene expression (2). The plasmid clone pI_1 [911] was generously provided by M. Fromm. The 534 bp *Adh1* intron with short flanking exon sequences was excised with *Bcl*I and *Bam*H1 and inserted as described above for the *Sh1* intron.

Determination of CAT Activity

Approximately 48 h after electroporation, protoplasts/cells were collected by centrifugation and frozen at −70°C. Total cellular protein was determined by the Bio-Rad Protein Assay and equal amounts of protein were generally used within a single experiment. To keep the CAT assay in the range of enzyme activity where no diacetylated product was formed, it was necessary to use lower amounts of protein in the assays of some maize cell extracts. A nonlinear correction factor determined from a CAT activity standard curve of protein amounts assayed was used to adjust CAT activities for the other cell extracts in the same experiment. CAT activity was determined from protoplast/cell extracts (heated at 60°C for 10 min to inactivate any plant derived background) according to Gorman *et al.* (11), using ^{14}C-chloramphenicol (0.3 μCi, New England Nuclear) and acetyl-CoA (20 μL from a solution of 10 mg/mL; Sigma) as substrates. Radioactive spots of chloramphenicol and its monoacetylated products were visualized from the TLC plates by autoradiography (x-ray film exposed 24 h) and quantitated by liquid scintillation counting to determine the percentage of acetylated chloramphenicol. Correction for background was made by counting a scraped area of the TLC plate showing no signal on the autoradiograph. CAT activity is expressed as the percentage of acetylated chloramphenicol.

RESULTS

All plasmid constructs used contained the CAT gene coupled to the NOS 3′ polyadenylation signal. Two promoters, *Sh1* of maize and CaMV 35S, were monitored for their ability to promote transcription of the CAT gene. In addition, the first intron of the *Sh1* locus and of the *Adh1* locus of maize were incorporated into the constructs between the promoter and the CAT coding region to determine if the expression of the CAT gene could be increased.

The plasmid constructs used are given in Figure 1. Protoplasts of *Panicum maximum* were electroporated with constructs 1 through 5 and 8 of Figure 1. CAT activity was detected with all constructs except pShIrSCN and p35SIrSCN. These constructs contain the *Sh1* first intron in the reverse orientation relative to the direction of transcription. The experiment was repeated with those plasmid constructs that showed positive CAT activity. Data from two experiments are presented in Table I. Each CAT activity value is the average of duplicate assays performed on electroporated cells from a single preparation of protoplasts. These duplicate assays were quite reproducible. For example the difference from the mean in eight sets of duplicated assays with *P. maximum* (Table I) was an average of ±19% of the mean.

The construct with the CaMV 35S promoter (p35SCN) gave 16- to 29-fold more CAT activity than did the construct with Sh1 promoter (pShCN). CAT activity was increased several-fold when the *Sh1* first intron was incorporated (pShIfSCN, p35SIfSCN). A 5- to 58-fold increase in CAT activity was seen with pShIfSCN relative to its control plasmid pShCN. With the CaMV 35S promoter and the *Sh1* first intron (p35SIfSCN), CAT activity was increased 23- to 28-fold relative to the construct without the *Sh1* intron (p35SCN). The increase in the expression of CAT was much more consistent when transcription was driven by the CaMV 35S promoter (p35SIfSCN) in comparison to the *Sh1* promoter (pShIfSCN, Table I). This pattern of variable expression by the *Sh1* promoter was also exhibited in the other grass species (see below).

We also investigated whether the elevated gene expression caused by the *Sh1* first intron occurred in other grass species.

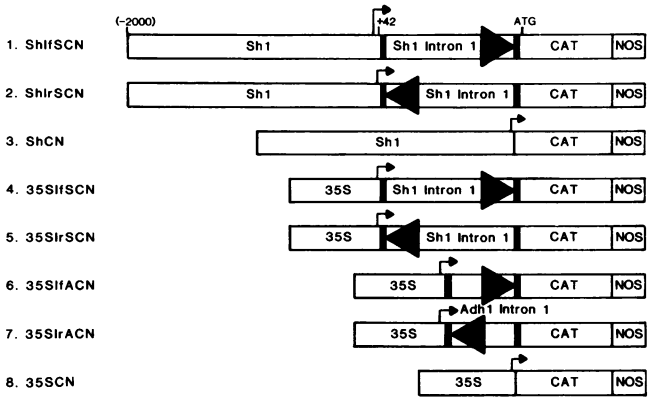

Figure 1. Gene constructions used for analysis of intron stimulation of gene expression and comparison of promoter strengths. Maps are drawn to approximate scale and the 2.7 kb plasmid vector pUC19 is not shown. Nucleotide numbering for the 5′ end of the *Sh1* promoter is approximate. For each construction the transcription start site is designated by the bent arrow. The translation initiation site in the CAT coding region is indicated by the ATG and the 3′ polyadenylation signal is from the nopaline synthase gene as described in "Materials and Methods." Intron orientation is indicated by the large arrow head and flanking exon sequences are shown as solid boxes. The *Sh1* intron 1 cassette contains the complete 1028 bp intron with 10 bp of exon 1 and 17 bp exon 2 sequence. The *Adh1* intron 1 fragment consists of 14 bp exon 1, the 543 bp intron, and 5 bp exon 2. No ATG trinucleotide is present in the polylinker or short exon regions flanking the *Adh1* intron inserted in either orientation. The inverted *Sh1* intron construction pShIrSCN (line 2) contains an ATG in the reversed exon 1 sequence approximately 50 bp 5′ to the CAT coding region ATG.

Protoplasts of *Pennisetum purpureum* were electroporated with the plasmid constructions. Data from one of three experiments are shown in Table I. Elevated levels of CAT activity were obtained with plasmid constructs containing the *Sh1* intron 1 (pShIfSCN, p35SIfSCN). However, elevation with the *Sh1* promoter was only 1.9-fold while a 12-fold increase was observed with the 35S promoter. Moreover, the level of stimulation caused by *Sh1* first intron was lower in *P. purpureum* relative to that observed in *Panicum*.

The ability of the *Sh1* first intron to elevate gene expression was also examined in maize protoplasts. Two experiments are presented in Table I. The *Sh1* first intron led to a 31- to 91-fold increase when transcription was driven by the 35S promoter, but stimulation of gene expression was quite variable (1- to 172-fold that observed without the intron) when transcription was driven by the *Sh1* promoter. As was the case with the other two grass species, the level of expression with the 35S promoter was much higher (16- to 100-fold) than that observed with the *Sh1* promoter. Among the three grass species, levels of expression with the 35S promoter were several-fold (2–8) higher in *Zea mays* in comparison to *P. maximum* or *P. purpureum*. Stimulation of CAT activity by the *Sh1* first intron was also highest in maize.

In all three grass species, duplicate CAT activity assays from a single preparation of protoplasts were consistent. As a measurement of variation between duplicate assays, the individual data differ, on average, from the mean by ±29% for constructs containing the *Sh1* promoter and a comparable value of 17% for constructs containing the 35S promoter. However, comparison of CAT activities measured with separate protoplast preparations shows substantial variability for constructs utilizing the *Sh1* promoter, whereas the level of stimulation generated by *Sh1* first intron in constructs containing the 35S promoter is quite reproducible. For example increases of 23-fold and 28-fold were found in *P. maximum* with the 35S promoter. Increases of 31- and 91-fold (Table I) and 36- and 50-fold (Table II) were observed in maize. In contrast, the stimulation with the *Sh1* promoter was quite variable; 5.4 to 58-fold in *P. maximum* and 1- to 172-fold in maize.

Because previous work showed that the first intron of *Adh1* increased gene activity (2), we synthesized 35S-CAT constructs containing this intron in both orientations (entry 6

Table I. *CAT Activity in Panicum maximum, Pennisetum purpureum, and Zea mays with CaMV 35S versus Sh1 Promoters, and the First Intron of Sh1 with Either Promoter*

Protoplasts were electroporated with plasmid constructs and assayed for transient expression 48 h after DNA delivery. CAT activity was determined after a 2 h reaction as described in "Materials and Methods." The data for the first and second experiments of *Panicum* are from duplicate assays utilizing 400 μg and 300 μg protein/sample, respectively. *Pennisetum* data are from duplicate assays with 140 μg protein/sample. Reported CAT activities in the maize cells have been adjusted to 5 μg protein per assay. Actual amounts used were 5 μg for all constructs containing the *Sh1* first intron and 75 μg (first experiment) and 200 μg (second experiment) for the constructs lacking the intron.

Construct	*Panicum maximum*				*Pennisetum purpureum*		*Zea mays*			
	First Experiment		Second Experiment				First Experiment		Second Experiment	
	CAT Activity	Activity Relative to ( )	CAT Activity	Activity Relative to ( )	CAT Activity	Activity Relative to ( )	CAT Activity	Activity Relative to ( )	CAT Activity	Activity Relative to ( )
	%		%		%		%		%	
pShCN	0.937	1.0 (pShCN)	0.1680	1.0 (pShCN)	0.4269	1.0 (pShCN)	0.0534	1.0 (pShCN)	0.0049	1.0 (pShCN)
pShIfSCN	5.436	57.96 (pShCN)	0.9196	5.474 (pShCN)	0.8281	1.94 (pShCN)	0.0564	1.055 (pShCN)	0.7739	172.2 (pShCN)
p35SCN	2.75	1.0 (p35SCN)	2.711	1.0 (p35SCN)	4.428	1.0 (p35SCN)	0.8814	1.0 (p35SCN)	0.4735	1.0 (p35SCN)
p35SIfSCN	62.49	22.72 (p35SCN)	74.7	27.55 (p35SCN)	50.092	11.50 (p35SCN)	27.30	30.79 (p35SCN)	42.90	90.60 (p35SCN)

Table II. *Levels of CAT Activity in Zea mays Protoplasts Electroporated with Plasmid Constructs Containing the First Intron of Either Sh1 (p35SIfSCN) or Adh1 (p35SIfACN)*

Activity is also expressed relative to the construct without the intron (p35SCN). CAT activity was assayed 48 h after electroporation as described in "Materials and Methods" and is expressed as the percentage of the ^{14}C chloramphenicol acetylated in a 2 h reaction. Values are based on 5 μg of protein assayed per sample. Data are single determinations from two experiments.

Construct	CAT Activity	
	%	Relative to p35SCN
p35SCN	1.46	1.0
	0.58	1.0
p35SISCN	51.9	35.5
	29.3	50.2
p35SIACN	5.7	3.9
	2.1	3.6

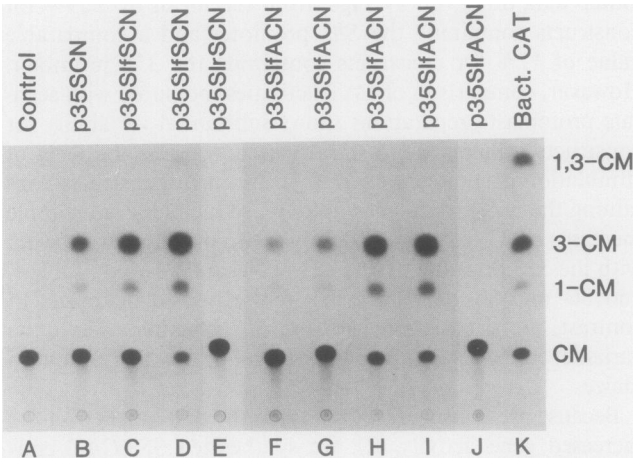

Figure 2. Comparison of CAT activity expression in protoplasts of *Zea mays* electroporated with plasmid constructs without introns (lane B), with *Sh1* intron 1 (lanes C and D) or with *Adh1* intron 1 (lanes F–I). The positions of unreacted chloramphenicol (CM) as well as I-acetyl chloramphenicol (1-CM), 3-acetyl chloramphenicol (3-CM) and 1,3-diacetyl chloramphenicol (1,3-CM) are indicated on the autoradiograph. Lane A is protoplasts electroporated without DNA. Lanes E and J are plasmids with inverted orientation of intron. Lane K, bacterial standard. Lanes A, B, E, I, and J were assayed at 200 μg protein/sample; lanes C and F, 5 μg protein/sample; lanes D and G, 10 μg protein/sample; lane H, 100 μg protein/sample.

and 7, Fig. 1). Results comparing expression with the two introns in maize cells are summarized in Table II and Figure 2. Neither intron stimulated CAT expression when cloned in the reversed orientation. The construct with the *Adh1* intron 1 in the correct orientation (p35SIfACN) showed an average 3.7-fold increase in comparison to an average 42.8-fold increase by the *Sh1* intron (p35SIfSCN) relative to the construct lacking the intron (p35SCN). It can be concluded, therefore, that in the maize protoplasts used in these experiments the *Sh1* intron 1 is approximately ten times more effective than the *Adh1* intron 1 in stimulating CAT expression.

DISCUSSION

The studies reported here show that the first intron of the *Sh1* locus of maize greatly increases expression of a reporter gene when transcription is driven by the CaMV 35S promoter. Increased expression occurs in protoplasts of each of the three different grass species used although our data suggest that the maize intron is particularly active in maize protoplasts. Because the increased expression is dependent upon the orientation of the intron relative to the rest of the construct, it would appear that normal intron processing is required for increased gene expression. The *Sh1* first intron also led to increased CAT expression when the promoter of *Sh1* was employed. However, these enhanced levels of CAT were quite variable. In the experimental protocol used, the plasmids containing or lacking the intron were electroporated into aliquots of a homogenous preparation of protoplasts. Whereas different preparations of protoplasts expressed CAT derived from the 35S promoter-driven plasmid very reproducibly, these same preparations of cells gave quite variable enzyme levels when the *Sh1* promoter was employed.

Our results are of interest for at least three reasons. Most apparent is the possible application to studies involving expression of introduced foreign genes. Assuming that the *Sh1* first intron acts to increase gene expression at the transcriptional or post-transcriptional level, we envisage at least two applications. Increased gene expression should be useful in cases in which a potentially selectable gene is normally expressed at suboptimal levels (7, 14; see introduction). Furthermore, the increased gene expression caused by the *Sh1* first intron should be useful in those instances where the inserted gene codes for an agriculturally important trait. The data reported here also provide a ready explanation for a puzzling observation made earlier. As mentioned above, this intron is large and found in the 5′ leader of the pre-mRNA (26). While the original evidence for the existence of this first intron was based on primer extension and S1 protection data, subsequent isolation and sequencing of an almost full length cDNA clone (D McCarty, J Shaw, and LC Hannah, unpublished data) have provided definitive evidence for the existence of this intron, and indicate that the start of translation occurs in the second exon. Thus, the very large first intron does not separate protein-coding information. If one accepts the premise that introns were used in evolution to bring together functional polypeptide domains into different proteins (for example, refs. 9, 10), or that introns provide recombinational length to genes in order to create unique coding information from preexisting alleles, then one would not expect introns to fall in nonprotein-coding exon sequences. The finding that the first intron of *Sh1* greatly increases gene expression, therefore, provides an explanation for the existence of such an intron if this increased expression is of physiological relevance. Accordingly, it would be interesting to monitor the levels of *Sh1* expression in plants containing the *Sh1* gene which lacks the first intron.

The *Sh1* first intron is not unique in increasing gene expression in transient expression systems of plants. Callis *et al.* (2) reported that introns from the maize genes *Adh1* and bronze (*Bz*) increased gene expression in constructs similar to those

used in the experiments reported here. Furthermore Silva *et al.* (22) showed that the intron from a heat shock gene of maize increased expression of a reporter gene three- to sixfold when the 35S promoter was used to promote transcription.

While several maize introns function to increase gene expression, the extent of activation is quite variable and is also dependent on the sequences being used to promote transcription. For example, Callis *et al.* (2) showed that while the *Adh1* first intron increased gene expression 16- to 112-fold when the *Adh1* promoter was used, it increased expression only 5- to 22-fold in constructs containing the 35S promoter. The data reported here are in agreement with their finding in that we find only a 4-fold increase in our 35S promoter-containing constructs when the *Adh1* first intron is inserted between the promoter and the reporter gene. However, the *Sh1* first intron increases activity 42.8-fold in otherwise identical 35S constructs and host cells. To our knowledge, the first intron of *Sh1* is the most active in increasing gene activity in a plant transient expression system.

Finally, it has recently been shown that maize transposable elements can act as introns (reviewed by Wessler [27]). It is perhaps possible that introns evolved from transposable elements. The ability of transposable elements to insert into a gene and increase gene activity may have provided the variability in gene expression to give a selective advantage for plants to deal with sudden changes in their environment. Indeed, a regulatory role for transposable elements such as this would be consistent with McClintock's contention (18) that transposable elements act to control expression of their host gene.

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