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pEmu: an improved promoter for gene expression in cereal cells

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Summary. A recombinant promoter, pEmu, has been constructed to give a high level of gene expression in monocots. It is based on a truncated maize *Adh1* promoter, with multiple copies of the Anaerobic Responsive Element from the maize *Adh1* gene and ocs-elements from the octopine synthase gene of *Agrobacterium tumefaciens*. The pEmu promoter was one of 12 different promoter constructs that were linked to the β-glucuronidase (*GUS*) marker gene. Promoter activity was measured 48 h after introduction of the constructs into protoplasts of five different monocot species [wheat, maize, rice, einkorn (*Triticum monococcum*), and *Lolium multiflorum*] and one dicot (*Nicotiana plumbaginifolia*). In suspension cell protoplasts, the most highly expressing construct (pEmuGN) gave 10- to 50-fold higher expression than the CaMV 35S promoter in all the monocot species. The pEmu promoter should be valuable where a high level of gene expression is required in monocots. The pEmu promoter showed instability in several widely used *Escherichia coli* strains but was stable in a *recA*, *recD* strain AC001, which is described. Another construct, p4OCSΔ35SIGN, gave a tenfold increase in expression over the CaMV 35S promoter in dicot (*Nicotiana plumbaginifolia*) protoplasts.

Key words: Promoter – Enhancers – Monocot – Electroporation – Plant transformation

Introduction

One of the most important considerations in developing a plant transformation procedure is the availability of a promoter which provides reliable high level expression of introduced genes in the target cells. For the transformation of plant cells with DNA encoding an antibiotic resistance marker, high level expression of the introduced gene should facilitate efficient selection of transformants. In cases where the untransformed tissue shows a relatively high level of natural resistance to the antibiotic (e.g., wheat and maize embryo tissue selected on kanamycin) (Hauptmann et al. 1988; and E. L. Marsh, unpublished data), a strong selection system would be critical for the successful production of transformed plants.

The 35S promoter (Guilley et al. 1982; Odell et al. 1985) of Cauliflower Mosaic Virus (CaMV) is one of the most frequently used promoters in plant transformation procedures. This dicot virus promoter directs the expression of genes introduced into protoplasts of dicots and monocots (Fromm et al. 1985). However, it has relatively low activity in the agriculturally significant graminaceous plants such as wheat (Lee et al. 1987; Hauptmann et al. 1987). Conversely, the monocot promoter from the *Adh1* gene of maize gives very low expression in protoplasts of the dicot, *Nicotiana plumbaginifolia* (Ellis et al. 1987a). These observations suggest that there may be differences between monocots and dicots with respect to transcription factors and the recognition of promoter sequences.

The level of expression of a gene can often be increased by the addition of *cis*-acting sequences, also called enhancer elements, which increase the level of transcription from a promoter (Banerji et al. 1981). The addition of extra copies of an enhancer element already associated with a gene can increase expression of the gene shown, for example, by the addition of multiple copies of the Anaerobic Responsive Element (ARE) to the maize *Adh1* gene (Olive et al. 1990). Alternatively, enhancers

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can be obtained from other sources, such as viral or bacterial genomes, and combined with the gene of interest. In one such case, the species-specificity of a promoter was modified by the addition of the octopine synthase (*OCS*) enhancer from *Agrobacterium tumefaciens* to the maize *Adh1* promoter (Ellis et al. 1987a). After addition of the *OCS* enhancer, the maize *Adh1* promoter was able to give strong anaerobically inducible expression in transgenic tobacco plants. When the same construct was introduced into maize protoplasts, expression was high, irrespective of whether or not the protoplasts were anaerobically induced (Peacock et al. 1987).

The aim of the work described here was to use the knowledge detailed above to construct a promoter giving high level expression in monocot protoplasts. Such a promoter should make possible the development of an effective selection system for transformed monocot cells and could improve the expression of introduced genes in transgenic tissues. Some of the promoters consistently gave much higher expression in monocot protoplasts than did the 35S promoter and one construct was superior to the 35S promoter in the dicot test system.

Materials and methods

Plasmid construction

Standard molecular biological techniques were carried out according to Sambrook et al. 1989. The plasmid p35SGN (K. Singh, personal communication) was derived by ligating the 2.9-kbp HindIII/EcoRI fragment from pBI121 (Jefferson et al. 1987) into pUC118 (Vieira and Messing 1987). Plasmids pIGN, p6AREIGN (renamed p6ARE*Δ*ADHIGN in this paper), and p35SIGN have been described elsewhere (Olive et al. 1990).

The plasmid pGN was derived from pIGN by replacement of the BamHI/SacI fragment containing Intron1 of maize *Adh1* and *GUS* by the BamHI/SacI *'GUS'* fragment from p35SGN. This procedure was also used for p6ARE*Δ*ADHIGN to generate p6ARE*Δ*ADHGN. Plasmids p*Δ*35SGN and p*Δ*35SIGN were derived from pGN and pIGN by addition of the SalI/BamHI '*Δ*35S' fragment from p*Δ*35S(-90) (Olive et al. 1990). A plasmid, p4OCSAdhCAT, containing four tandem copies of the ocs-element from the *OCS* gene (Ellis et al. 1987b) was kindly provided by J. G. Ellis and E. J. Finnegan. The 4OCS element (deteailed in Fig. 1c) was prepared as a SalI/XhoI fragment from p4OCSX, a derivative of p4OCSAdhCAT made by blunt-end ligation of an XhoI linker into the SmaI site. The 4OCS element was added at the SalI site in p*Δ*35SIGN to give p4OCS*Δ*35SIGN and at the SalI site in p6ARE*Δ*ADHIGN to give p6ARE4OCS*Δ*ADHIGN, both with the same orientation of the 4OCS array (as in Fig. 1e). The plasmid p6ARE*Δ*35SIGN was derived from p6ARE*Δ*ADHGN by replacing the SalI/EcoRI fragment containing '*Δ*ADHGN' with the SalI/EcoRI fragment from *Δ*35SIGN. The plasmid p6ARE4OCS*Δ*35SIGN was derived from p6ARE4OCS*Δ*ADHIGN by replacing the SalI/EcoRI fragment containing '*Δ*ADHIGN' with the SalI/EcoRI fragment of p*Δ*35SIGN. The plasmid p6ARE*Δ*35SGN was derived from p6ARE*Δ*ADHGN by replacing the SalI/BamHI '*Δ*ADH' fragment with the SalI/BamHI '*Δ*35S' fragment from p*Δ*35S(-90).

Plasmid DNA of the above constructs was prepared from *E. coli* JM109 (Yanisch-Perron et al. 1985) and purified by two rounds of centrifugation in CsCl gradients. The final preparations were resuspended at 10 mg/ml in 10 m*M* TRIS-HCl (pH 8.0), 1 m*M* Na_2EDTA, and aliquots were checked by DNA sequencing using a Pharmacia T7 kit and by the sizing of restriction fragments in a triple digestion with HindIII/SalI/BamHI and a double digestion with PvuII/SmaI, to ensure that no sequence rearrangements had occurred. Only those preparations showing no spurious bands in gel electrophoresis were used in the electroporations. Subsequent DNA manipulations, were performed in *E. coli* AC001 (*recD1009*, *hsdr2*, *zjj*::*Tn10*, *recA*::*Cam*^{R}, *supF58*, *trp89*::*Tn5*).

Plant cell culture and protoplast isolation

Protoplasts were isolated from the following established cell lines: TM, an established line of einkorn (*Triticum monococcum*) (Kao et al. 1970); BMS from *Zea mays* cv Black Mexican Sweet (Chourey and Zurawski 1981); L1 from mature embryos of hexaploid *Triticum aestivum* cv Vilmorin 27 with two additional chromosomes of *Thinopyrum intermedium* (P. J. Larkin and J. M. Gibson, unpublished results); LM, a line derived from endosperm of *Lolium multiflorum* (Smith and Stone 1973); NpT_5, which originally derived from a leaf protoplast culture of *Nicotiana plumbaginifolia* (A. Tassie, unpublished results); and ER, an embryogenic culture of *Oryza sativa* cv Taipei 309 initiated from immature embryos and maintained in liquid suspension culture for 4 months. The media used for the maintenance of the cell suspensions, the enzymes used for isolation of protoplasts, and the media used for protoplast culture are given in Table 1. Culture medium CM1 is the CS5 medium described by Scowcroft and Adamson (1976) with the pH adjusted to 5.8. CM2 contains the mineral salts of Murashige and Skoog (1962), 170 mg/l L-asparagine, 0.77 mg/l glycine, 0.13 mg/l nicotinic acid, 0.025 mg/l calcium pantothenate, 0.025 mg/l thiamine.HCl, 0.025 mg/l pyridoxine.HCl, 4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 20 g/l sucrose (pH 5.8). CM3 is WtM1 (Young et al. 1989). CM4 contains the major inorganic salts of White (1963), the minor salts and vitamins of Murashige and Skoog (1962), 100 mg/l myo-inositol, 5 g/l yeast extract, 10 mg/l ferric citrate, 1 mg/l indole-3-acetic acid (IAA), 40 g/l sucrose (pH 5.5). CM5 contains the major and minor inorganic elements of R2 medium (Ohira et al. 1973) with 9 mg/l $FeCl_3$, 11.2 mg/l Na_2EDTA, 1 mg/l thiamine.HCl, 2 mg/l 2,4-D, 20 g/l sucrose (pH 5.9).

Protoplast washing solution PW1 consists of 0.3 *M* mannitol, 156 m*M* NaCl, 3.5 m*M* KCl, 9.4 m*M* $MgSO_4$, 8.4 m*M* $MgCl_2$, 3.4 m*M* $CaCl_2$, 0.9 m*M* $NaHCO_3$ (pH 6.0). PW2 contains the major and minor mineral salts of B5 medium (Gamborg et al. 1968), 27 m*M* mannitol, 109 m*M* KCl, 105 m*M* $MgCl_2$, 33 m*M* $CaCl_2$, 3 m*M* 2-(N-morpholino)ethanesulphonic acid (MES) (pH 5.7). PW3 consists of 0.568 *M* mannitol, 80 m*M* $CaCl_2$, 0.2% MES (pH 5.8). PW4 is PW2 with the concentration of mannitol raised to 49 m*M*.

Enzyme digestion mixture ED1 consists of 1% (w/v) Cellulysin (Calbiochem), 1% (w/v) Driselase, 1% (w/v) Macerozyme (Onozuka R-10) in washing solution PW1 with the pH adjusted to 5.8. ED2 contains 1% w/v Cellulysin (Calbiochem), 0.5% (w/v) Hemicellulase (Sigma), 0.02% Pectolyase Y-23 (Seishin Pharmaceutical), 50 m*M* $CaCl_2$, 10 m*M* sodium acetate, 0.3 *M* mannitol (pH 5.8). ED3 contains 1% (w/v) Cellulase RS (Yakult Honsha), 0.1% (w/v) Driselase, 0.06% (w/v) Pectolyase Y-23, 0.2% (w/v) Hemicellulase, 0.2% (w/v) Macerozyme R-10, 0.495 *M* mannitol, 0.189 *M* glucose, 2 m*M* ascorbic acid, 14 m*M* $CaCl_2$, 3 m*M* MES (pH 5.8). ED4 is 0.5% (w/v) Cellulase RS, 0.68% (w/v) Driselase, 0.05% Pectolyase Y-23, 6.5 m*M* MES, 0.325 *M* mannitol, 40 m*M* $CaCl_2$, to which 0.5% (w/v) activated charcoal was added. After gentle agitation

Table 1. Media used in the preparation of protoplasts from the cell lines. The composition of the media is detailed in the text

Plant	*Nicotiana*	Maize	Wheat	Einkorn	*Lolium*	Rice
Name of cell line	NpT_5	BMS	L1	TM	LM	ER
Cell suspension culture medium	CM1	CM2	CM3	CM3	CM4	CM5
Enzyme digestion mixture	ED1	ED2	ED3	ED3	ED4	ED5
Protoplast washing solution	PW1	PW2	PW3	PW3	PW1	PW4
Protoplast culture medium	PC1	PC2	PC3	PC3	PC4	PC5

for 30 min, the charcoal was removed by centrifugation at 10,000 *g*. The solution was then adjusted to pH 5.9 and sterilized by filtration. ED5 is 1% (w/v) Cellulase RS, 0.1% (w/v) Driselase, 0.1% (w/v) Pectolyase Y-23, 0.35 *M* mannitol, 80 m*M* $CaCl_2$, 3 m*M* MES (pH 5.9).

The protoplast culture media were all sterilized by filtration before use. Protoplast culture medium PC1 consists of the medium of Kao and Michayluk (1975) without the free amino acids, adenine, guanine, thymine, uracil, hypoxanthine, xanthine, riboflavin and vitamin B12, as suggested by Vasil and Vasil (1980), but containing 0.4 *M* glucose, 0.1 *M* sucrose, 1 mg/l 2,4-D, 0.2 mg/l zeatin, pH adjusted to 5.6. The medium was ultrafiltered through an Amicon YM10 membrane (Davies et al. 1989) prior to filter sterilization. PC2 consists of the inorganic elements of Murashige and Skoog (1962), 7.7 mg/l glycine, 1.3 mg/l nicotinic acid, 0.25 mg/l thiamine.HCl, 0.25 mg/l pyridoxine.HCl, 0.25 mg/l calcium pantothenate, 167 mg/l L-asparagine, 1 g/l L-glutamine, 66 g/l mannitol, 20 g/l sucrose, 1.67 g/l glucose, 2% (v/v) coconut water (Gibco), 4 mg/l 2,4-D, and 0.1 mg/l 6-benzylaminopurine (pH 5.8). PC3 consists of the major and minor minerals of Kao and Michayluk (1975), 1 mg/l nicotinamide, 1 mg/l pyridoxine.HCl, 1 mg/l thiamine.HCl, 1 mg/l calcium pantothenate, 0.4 mg/l folic acid, 0.02 mg/l p-aminobenzoic acid, 0.01 mg/l biotin, 400 mg/l m-inositol, 2% (v/v) coconut water, 750 mg/l casein hydrolysate, 200 mg/l L-glutamine, 150 mg/l L-aspartic acid, 10 g/l sucrose, 108 g/l glucose, 1 mg/l 2,4-D, 0.2 mg/l 1-naphthaleneacetic acid, 0.2 mg/l zeatin (pH 5.6). PC4 is CM4 with the addition of 73 g/l sorbitol. PC5 has the inorganic ingredients of CM5 plus the vitamins of B5 medium (Gamborg et al. 1968), the sugars and organic acids of Kao (1977), 137 g/l sucrose, 2 mg/l 2,4-D, and 0.1 mg/l kinetin (pH 5.7).

Electroporation of protoplasts

After complete digestion protoplasts were sieved through 328-, 110-, and 50-μm mesh sieves (twice through the 50-μm sieve in the case of the L1 and TM lines). Following sedimentation by slow speed centrifugation (80 *g* for 5 min), the protoplasts were resuspended in the washing solution found best suited to the particular cell type (Table 1).

The protoplasts were again sedimented, washed, sedimented, and resuspended in TBS9 buffer [3.63 g/l TRIS, 876 mg/l $CaCl_2 \cdot 2H_2O$, 8.78 g/l NaCl, 50 g/l mannitol (pH 9.0), Taylor and Larkin 1988] at a concentration of 2×10^6 protoplasts per millilitre. Immediately before electroporation, 200 μl of the protoplast suspension was added to a tube containing 5 μg of plasmid DNA dissolved in 5 μl of 10 m*M* TRIS-HCl (pH 8.0), 1 m*M* Na_2EDTA. The mixture was transferred to an electroporation chamber (2 mm between electrodes) and three pulses of 275 V (1,375 V/cm), with a pulse width of 5 ms and a delay of 100 ms, were applied between the electrodes from a 24 μF capacitor. After allowing the protoplasts to recover for 5 s, the protoplast suspension was pipetted back into a microfuge tube to which 600 μl washing solution was added. The tubes were spun gently (< 100 *g*) for 5 min, the supernatant was removed, and the protoplasts were resuspended in 1 ml of culture medium. The protoplast suspensions were transferred to 35-mm petri dishes, which were sealed with Parafilm and incubated at 25 °C in the dark to allow expression of the *GUS* gene.

Assay of GUS gene expression in electroporated protoplasts

After 44–48 h incubation, 400 μl washing solution was added to each dish and each protoplast sample was gently pipetted into a microfuge tube. The tubes were centrifuged at 100 *g* for 8 min and the supernatant was discarded. Protoplast pellets were either stored at −80 °C until required or used immediately. Each pellet was resuspended, with the aid of a vortex mixer, in 250 μl extraction buffer (Jefferson et al. 1987). The samples were sonicated on ice for 5 s using a Labsonic 1510 sonicator set at 55 W, equipped with a microtip probe. Debris was pelleted by centrifugation in a microfuge for 1 min and the clear supernatant was assayed for total protein using a Bio-Rad kit, according to the manufacturer's recommendations. For each set of constructs the fluorimetric *GUS* assay (Jefferson et al. 1987) was performed on an aliquot of the supernatant containing a fixed amount of total protein in the range of 5 to 50 μg dissolved in 100 μl extraction buffer. A further 100 μl extraction buffer containing 2 m*M* 4-methyl-umbelliferyl β-D-glucuronide (MUG) was added, and the mixture was vortexed briefly and incubated at 37 °C for a fixed time in the range of 20 to 160 min. The reaction was stopped by the addition of 1,000 μl 0.2 *M* Na_2CO_3, and fluorescence at 455 nm was measured using a Perkin-Elmer Spectrofluorimeter set at an excitation wavelength of 365 nm.

Results and discussion

Construction of plasmids containing hybrid promoters

A series of promoters was constructed based on either a truncated maize *Adh1* promoter (ΔADH), which contains the region −100 to +106 (Ellis et al. 1987b), or a truncated 35S promoter (Δ35S), which contains the region −90 to +3 (Olive et al. 1990). All the constructs compared in this study were based on the pUC118 (Vieira and Messing 1987)-derived plasmid shown in Fig. 1a, which consists of the promoter region 'X,' upstream of a β-glucuronidase (*GUS*) reporter gene (Jefferson et al. 1987) (abbreviated to G in plasmid names), with the transcription terminator/polyadenylation signal from the nopaline synthase gene (*nos3'*; abbreviated to N in plasmid names) (Bevan et al. 1983). The promoter region X varies in the different constructs according to

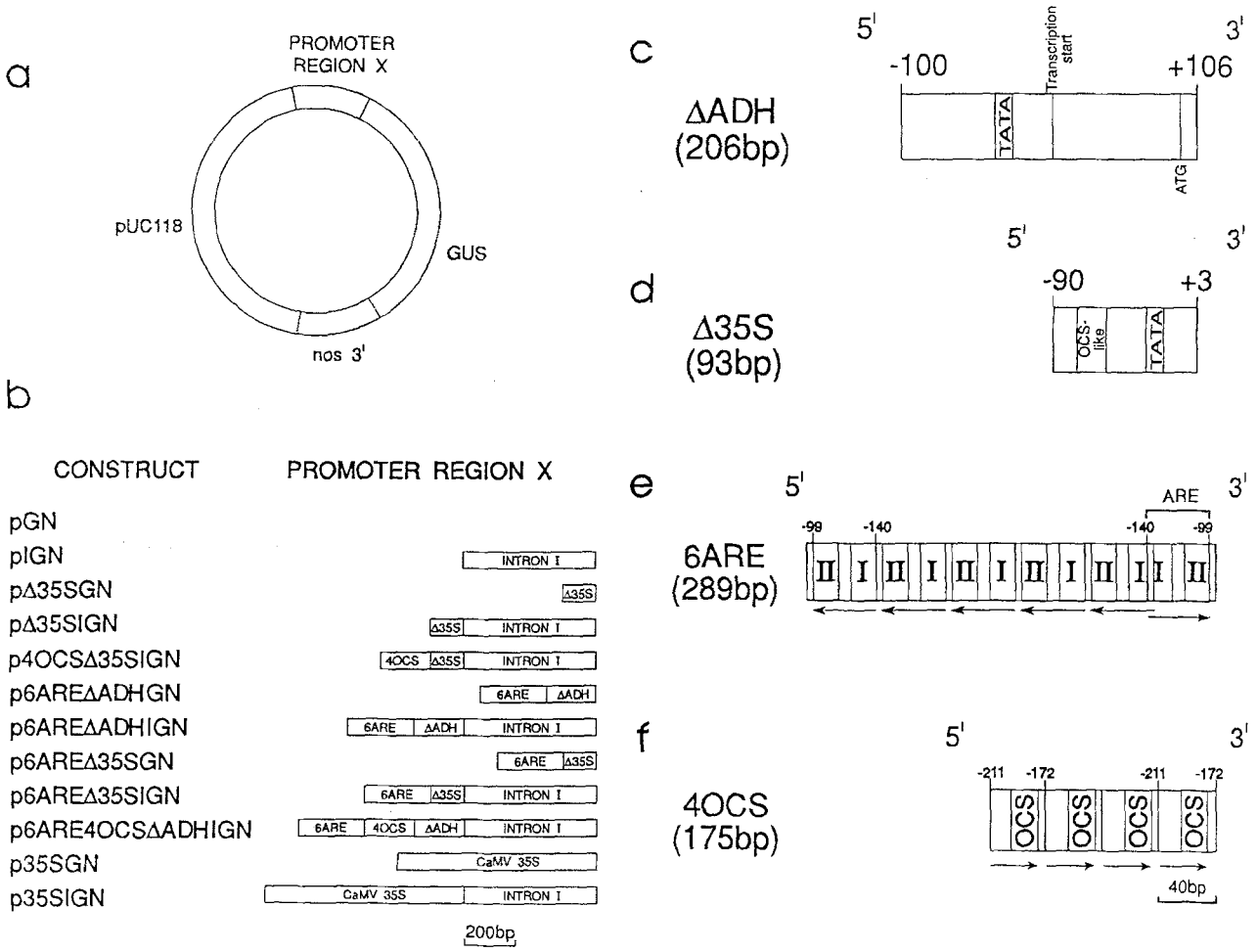

Fig. 1. **a** Generalized plasmid used for assaying promoter activities of promoter regions X. **b** Structures of promoter regions X in the different plasmids. The pEmuGN plasmid is represented as p6ARE4OCSΔADHIGN. **c** ΔADH promoter: TATA box and transcription start site are indicated. There are no ATG translation starts downstream of the transcription start site. **d** Δ35S promoter: TATA box and ocs-element are indicated. **e** 6ARE element: regions I and II of each ARE (−140 to −99 in the maize *Adh1* gene) are indicated. The 6ARE element consists of one ARE in the natural orientation preceded by five AREs in the reverse orientation. **f** 4OCS element: the element contains four 40-bp direct repeats of the −211 to −172 region from the *OCS* gene. All base numbering in **c** to **f** indicates the natural positions of bases in the sequences of the genes from which the constructs were derived

the scheme shown in Fig. 1b. Details of the component fragments of region X are given in Fig. 1c–f. The plasmid p6AREΔADHIGN is p6AREIGN of Olive et al. (1990), renamed to be consistent with the other plasmid names. Various combinations of ocs-elements and AREs were added upstream of the truncated promoters in an attempt to make a highly expressing promoter. The array of four tandemly repeated ocs-elements (the 4OCS element) has been shown to have stonger activity than a single ocs-element in enhancing expression of a *CAT* gene driven by the *Adh1* promoter (J. G. Ellis, personal communication). Similarly, replacing the single ARE in the maize *Adh1* promoter with the 6ARE, gave an 11-fold increase in expression under anaerobic conditions (Olive et al. 1990). Replacement of part of the maize *Adh1* promoter (−35 to +106) with the CaMV 35S promoter truncated to −90 [which contains (Bouchez et al. 1989) a single ocs-element] produced a hybrid promoter that gives high expression without the need for anaerobic induction (Olive et al. 1990). It was therefore considered that p6AREΔ35SIGN might give close to the anaerobically induced level of expression of p6AREΔADHIGN without the need for anaerobic induction. Alternatively, since the 6ARE element is a stronger element than the single ARE, a stronger element such as 4OCS might be required to confer high level aerobic expression on a construct containing the 6ARE. The plasmid p6ARE4OCSΔADHIGN was constructed to test this assertion.

The inclusion of the maize *Adh1* Intron1 downstream of the *Adh1* promoter in the untranslated leader has been shown to increase expression tenfold from a chloram-

Table 2. Expression of the *GUS* gene in different plant cell lines, relative to the *GUS* expression obtained using p35SGN

Plant	*Nicotiana*	Maize	Wheat	Einkorn	*Lolium*	Rice
Name of cell line	NpT_5	BMS	L1	TM	LM	ER
Number of replicates	7	8	5	6	7	5
Construct						
pGN	0.004	0.026	0.000	1.6	0.17	0.000
pIGN	0.001	0.18	1.3	2.4	2.4	4.8
pΔ35SGN	0.69	1.1	0.10	1.2	0.16	6.2
pΔ35SIGN	1.7	2.2	0.59	1.2	0.39	4.2
p4OCSΔ35SIGN	8.4	6.1	0.088	2.3	3.9	6.5
p6AREΔADHGN	0.17	1.1	0.76	2.7	0.40	0.98
p6AREΔADHIGN	0.048	8.2	18	9.3	0.55	14
p6AREΔ35SGN	0.53	8.2	0.49	1.2	0.11	0.8
p6AREΔ35SIGN	2.0	5.2	1.3	1.0	0.65	1.4
p6ARE4OCSΔADHIGN (pEmuGN)	0.83	41	40	20	8.8	25
p35SGN	1	1	1	1	1	1
p35SIGN	1.2	1.6	0.59	1.2	0.5	1.3

Table 3. Specific activities of β-glucuronidase (*GUS*) in protoplast extracts following transient expression of p35SGN, p6ARE4OCSΔADHIGN, p4OCSΔ35SIGN and pGN

Construct	Mean specific activity (picomoles 4 MU/mg protein/min) with standard errors shown in brackets			
	p35SGN	p6ARE4OCSΔADHIGN (pEmuGN)	p4OCSΔ35SIGN	pGN
Cell line				
Nicotiana NpT5	157 (72)	131 (49)	1312 (600)	0.64 (0.44)
Maize BMS	17 (9.7)	698 (365)	103 (48)	0.45 (0.41)
Wheat L1	0.68 (0.38)	27 (5.4)	0.06 (0.06)	0.00 (0.00)
Einkorn TM	0.41 (0.17)	8 (1.4)	0.94 (0.30)	0.60 (0.22)
Lolium LM	1.7 (1.2)	15 (5.0)	6.6 (2.3)	0.29 (0.13)
Rice ER	0.69 (0.19)	17 (3.2)	4.5 (2.1)	0.00 (0.00)

phenicol acetyltransferase marker gene introduced into maize protoplasts (Callis et al. 1987). A fragment containing this intron was included between the promoter and the *GUS* gene in some of the constructs.

Expression assays in suspension cell protoplasts

The relative strengths of the promoters were assessed in protoplasts of five monocot and one dicot cell lines by assaying *GUS* enzyme activity 44–48 h after the DNA constructs were introduced into protoplasts by electroporation. The results presented in Table 2 have been normalized by taking the value for p35SGN to be 1.0. This normalization was made because a high degree of variation was observed in specific *GUS* activities between experiments carried out on different days with different protoplast preparations. Much less variation was seen between replica electroporations made with a given plasmid using the same batch of protoplasts. The ranking order of the plasmids was generally the same between experiments, within each plant species. Values shown are means of the least five and up to eight replica electroporations using protoplasts from at least three independent isolations. The range of specific activities of *GUS* produced using p35SGN, p4OCSΔ35SIGN, p6ARE-40CSΔADHIGN, and pGN are shown in Table 3. It should be emphasized that the standard conditions in this study employed a relatively low amount of DNA (1.2 μg/10^5 protoplasts). It was found that this concentration of DNA gave the clearest differential response in *GUS* enzyme activity between the different constructs. Higher *GUS* activities were observed for some of the less efficient plasmids when the DNA concentrations were increased (data not shown), but under such conditions the more efficient plasmids gave readings that were too high to measure on the same scale.

Identification of a promoter giving superior expression in monocots

The 35S promoter shows weak expression in all the monocot cell lines (Table 2), with *GUS* gene expression

comparable to that recorded for the promoterless constructs pGN and pIGN. In general, constructs based on the truncated *Adh1* promoter were expressed more highly in monocots than those based on the truncated 35S promoter. Consistently high levels of expression in the monocot cell lines were given by those plasmids in which the six AREs were linked to the maize *Adh1* promoter in the presence of the maize *Adh1* intron1. The plasmid p6ARE4OCSΔADHIGN, which includes four copies of the ocs-element, showed the highest expression in all the monocot cell lines. This plasmid gave a 10- to 50-fold increase in *GUS* expression over the CaMV 35S promoter in suspension cell protoplasts of the monocots maize, wheat, einkorn (*Triticum monococcum*), *Lolium multiflorum*, and rice.

The high expression obtained with this construct could result from several factors. The use of the monocot TATA box from the maize *Adh1* gene may make a positive contribution. The untranslated leader from the maize gene is long (106 bases) in this plasmid, a factor which may also be important as deletion of the leader sequence from the 3′ end beyond position +80 in the maize *Adh1* gene has been shown to abolish expression (J. C. Walker, personal communication). In general, the constructs based on ΔADH perform better than those based on Δ35S in the monocot cell lines. Conversely, the Δ35S-based promoters outperform the ΔADH-based promoters in the dicot (*Nicotiana plumbaginifolia*) cell line.

Inclusion of the maize *Adh1* intron1 gave an increase in expression with p6AREΔADHIGN and p6ARE-4OCSΔADHIGN, but no effect of the intron was observed in the CaMV 35S promoter-based constructs. In the case of the "promoterless" constructs, pGN gave no detectable *GUS* expression above the background observed for protoplasts electroporated in the absence of DNA. However, pIGN, which includes the maize *Adh1* Intron1, gave a low but measurable *GUS* activity, the reason for which is not known.

Another important factor contributing to the high level of expression obtained from p6ARE4OCSΔADH IGN may be the presence of multiple copies of ocs-elements and ARE. The ocs-element is a strong enhancer which functions in both dicots and monocots (Ellis et al. 1987b), and addition of five extra copies of the ARE has been shown to increase expression from the maize *Adh1* promoter when assayed in maize protoplasts (Olive et al. 1990). The cassette '6ARE4OCSΔADHI' may be useful where a high level of gene expression is required in cultured monocot cells. We have code-named it the 'pEmu cassette' and have accordingly abbreviated p6ARE-4OCSΔADHIGN to pEmuGN.

Table 4. Expression obtained in wheat L1 protoplasts using three different constructs under aerobic or anaerobic conditions

Construct	Mean specific activity (picomoles of 4 MU/mg protein/min) with standard errors shown in brackets	
	Aerobic	Anaerobically induced
p6AREΔADHIGN	1.4 (0.73)	4.3 (1.3)
p6AREΔ35SIGN	0	0.21 (0.04)
p6ARE4OCSΔADHIGN (pEmuGN)	29.3 (6.7)	28.7 (4.5)

Anaerobic induction is not required for maximal expression from pEmuGN

The plasmid p6AREΔADHIGN is anaerobically induced in maize (Olive et al. 1990), with anaerobically induced cells showing a more than tenfold increase in expression over aerobically grown cells.

The observation that pEmuGN gave a higher level of expression than p6AREΔADHIGN in the experiments described above suggested that addition of the ocs-elements might be allowing expression equal to the anaerobically induced level, even under aerobic conditions. To investigate this further, the anaerobic inducibilities of the above two constructs and also of p6AREΔ35SIGN were determined using wheat (L1) protoplasts and the same electroporation and *GUS* assay conditions as described above. Some samples were incubated in air at 25 °C (aerobic) with shaking whilst others (referred to as 'anaerobically induced') were placed in a vessel containing 5% O_2/95% N_2 and shaken at 25 °C for the duration of the incubation (44–48 h). The results, shown in Table 4, confirm that p6AREΔADHIGN was anaerobically induced about threefold in wheat (L1) protoplasts. On the other hand, pEmuGN gave a similar level of expression under both anaerobic and aerobic conditions, which was greater than the fully induced expression from p6AREΔADHIGN. Previous results have shown that adding the ocs-element to an *Adh1* promoter with one ARE conferred a high level of aerobic expression on the promoter when assayed in maize protoplasts (Peacock et al. 1987). The results presented here suggest that addition of the 4OCS element to p6AREΔADHIGN confers a high level of aerobic expression on the promoter, overriding the requirement for anaerobic induction. The analogue of p6AREΔADHIGN in which ΔADH was replaced by Δ35S (p6AREΔ35SIGN) did not give a high enough level of expression to allow any inferences concerning its anaerobic inducibility.

Expression of the constructs in a dicot test system

The results presented in Table 2 indicate that addition of the 4OCS element to the truncated promoter of pΔ35SIGN greatly increased expression in *Nicotiana plumbaginifolia* protoplasts. The promoter in p4OCSΔ35SIGN should prove valuable where a high level of gene expression is required in dicots. The presence of the maize *Adh1* intron1 in this and other highly expressed constructs indicates that successful splicing of the maize intron takes place in *Nicotiana*. A version of p4OCSΔ35SIGN without the intron (p4OCSΔ35SGN) gave a similarly high level of expression (data not shown), showing that the presence of the intron is not required for high level expression in *Nicotiana*. Constructs based on the truncated *Adh1* promoter give little or no expression in *Nicotiana*, except in the case of pEmuGN, which contains the 4OCS element and gives a similar level of expression to that given by the 35S promoter.

Variation in performance of the constructs between cell lines

Differences were noted between the relative performances of certain constructs in different cell lines. The construct pEmuGN gave a twofold increase in expression over p6AREΔADHIGN in wheat, *Triticum monococcum*, and rice, but in maize this ratio was fivefold and in *Lolium multiflorum* it was 16-fold, which is close to the value obtained for *Nicotiana plumbaginifolia* (17-fold). It is also interesting to note that the construct giving the highest expression in *Nicotiana* (p4OCSΔ35SIGN) is also relatively highly expressed in *Lolium* (44% of the expression obtained with pEmuGN), whereas the corresponding value for wheat is less than 1%. These differences probably reflect different complements of transcription factors in the different cell lines. The *Lolium* cell line may lie somewhere between the wheat and *Nicotiana* cell lines in this respect. As the cell lines were derived from widely differing tissues, it is not possible to draw any conclusions concerning the species-specificity of the promoters. In a study of promoter activity in protoplasts of a suspension cell line of sugar cane (*Saccharum officinarum*), pEmuGN gave a 50- to 100-fold increase in *GUS* expression over p35SGN (C. Rathus, BSES, Brisbane, personal communication).

Stability of the pEmu DNA in various E. coli strains

During the routine manipulation of the plasmids containing the pEmu promoter, as well as during the construction of plasmids containing the promoter linked to selectable genes, it was found that the promoter DNA underwent deletion in a number of *E. coli* strains. These included the widely used laboratory strins HB101, DH1, and DH5α (Sambrook et al. 1989). Since each of these strains has a impaired *recA* gene, the deletion was thought to take place either by *recA*-independent recombination or by a mechanism other than homologous recombination. Potential sources of instability are slipped mispairing during DNA replication of the 6ARE and 4OCS repeated DNA, or during intermolecular and/or intramolecular homologous recombination in the direct repeats of the 6ARE and 4OCS regions. Alternatively, the deletion could occur by the processing of the palindromic sequences formed at the inverted repeats in the ocs-elements or between the two AREs that are at inverse orientation to one another. The *recD* mutation of *E. coli* is known to stabilize a variety of unusual DNA structures cloned in bacteriophage λ (Wertman et al. 1986); however, the effect of this mutation in stabilizing similar sequences in plasmids has not been reported. To investigate whether the instability of the pEmu promoter is caused by the *recD* function, plasmids incorporating the pEmu promoter were introduced into *E. coli* AC001, which contains mutations in the *recA* and the *recD* genes. It was found that the pEmu promoter was stable in the strain AC001. The instability is likely to result from the nuclease activity of the RecBCD enzyme, absent in the *recD* mutant. This view is consistent with the observation that, during recent experiments, pEmu constructs have also been found to be stable in the strain JC8679 (Schultz et al. 1983), which has an impaired RecBCD enzyme because it contains mutation in both *recB* and *recC* genes.

Utility of pEmu for cereal transformation

The strength of the pEmu promoter in stably transformed plants has yet to be determined. However, if higher expression of antibiotic resistance genes can be achieved compared with that obtained with the commonly used 35S promoter, then improved selection of transformants should be possible. Plasmids containing the pEmu promoter linked to hygromycin, kanamycin, methotrexate, phosphinothricin, and chlorsulfuron resistance genes have recently been prepared and introduced into cereal cells by electroporation and microparticle bombardment. Selection of transformed cells is in progress.

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