

Assessment of the Efficiency of Various Gene Promoters *via* Biolistics in Leaf and Regenerating Seed Callus of Millets, *Eleusine coracana* and *Echinochloa crusgalli*

Promila GUPTA, Saurabh RAGHUVANSHI and Akhilesh K TYAGI*

*Department of Plant Molecular Biology, University of Delhi South Campus,
Benito Juarez Road, New Delhi-110021, India*

*Corresponding author E-mail address: aktpmb@hotmail.com

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Abstract

A simple regeneration protocol has been developed for two millets, *Eleusine coracana* and *Echinochloa crusgalli*. The plantlet regeneration in both the millets is *via* somatic embryogenesis as evidenced by histological studies. In the case of *E. coracana*, up to 340 plants could be regenerated per 100 seed calli while up to 2266 plants could be regenerated per 100 seed calli of *E. crusgalli*. Subsequently, the regenerating seed callus as well as leaf segments from these two millets have been used as explants for assessing the suitability of five gene promoter constructs for transformation *via* biolistic means. Transient GUS histochemical as well as spectrofluorometric assays reveal the high efficiency of *Ubiquitin I* gene promoter from maize in terms of bringing about maximum GUS activity in both the millets. The activity of *Ubiquitin I* promoter from maize was highest in leaf lamina followed by leaf sheath and seed callus. Other four promoters were found to be much less efficient for both millets.

Keywords: Biolistics, *Echinochloa crusgalli*, *Eleusine coracana*, gene promoters, somatic embryogenesis, transient expression.

Millets are important crops of the African and Indian subcontinent for food and animal feed. The millets like sorghum (*Sorghum bicolor*), finger millet (*Eleusine coracana*), barnyard millet (*Echinochloa crusgalli*) and pearl millet (*Pennisetum glaucum*) form an essential component of the staple diet of the natives of South Africa and India (Sastri, 1952). The solubilization of dietary fibres and β -glucans present in the mature cell walls of endosperm of these native grain crops has been implicated in bringing about hypocholesterolemic effect (Newman *et al.*, 1989; Anderson *et al.*, 1990) and also in improving glycemic response (Wolever and Jenkins, 1986; Wolever, 1990). According to one study, consumption of food products based on millets and legumes like fenugreek seeds brings about desirable hypoglycemic effect (Pathak *et al.*, 2000). Several of such studies have led to an upsurge in the development of new techniques for genetic engineering of crop plants so that an ideal plant type combining various desirable qualities can be produced. Among cereals, rice is considered more amenable to manipulations *in vitro* and al-

ready regeneration and transgenic systems exist for several varieties (Mohanty *et al.*, 1999; Tyagi *et al.*, 1999). Although there are a number of reports describing plant regeneration in millets (Rangan, 1976; Mohanty *et al.*, 1983; Wang and Yan, 1984; Cobb *et al.*, 1985; Tyagi *et al.*, 1985; Wazizuka and Yamaguchi, 1987; Eapen and George, 1989, 1990; Talwar and Rashid, 1989; Sivadas *et al.*, 1990; Poddar *et al.*, 1997), reports regarding genetic transformation of millets are relatively few in comparison to other cereals. The genetic engineering of millets is desirable because in spite of their small size, they are capable of surviving in some of the most inhospitable ecosystems of the world providing food and fodder to the millions where other quality cereals can not be grown. Hence, with an ultimate aim of supplementing conventional breeding efforts, the genetic transformation protocols for millets need to be developed so that important quality traits may be incorporated across the barriers of incompatibility. Some of the recent reports of production of transgenic millets include pearl millet (Lambe *et al.*, 2000) and sorghum (Casas *et al.*,

1993, 1997; Zhu *et al.*, 1998; Rathus and Godwin, 2000; Zhao *et al.*, 2000). In this paper, we present the analysis of efficiency of various gene promoters in bringing about transient *uidA* gene expression in leaf lamina, leaf sheath and regenerating seed callus explants of the two millets, *Eleusine* and *Echinochloa*.

Mature seeds of *Eleusine coracana* Gaertn. cv PR-202 and *Echinochloa crusgalli* (L.) cv VL-29 were obtained from the University of Agricultural Sciences, Hebbal, Bangalore. The seeds were dehusked, surface-sterilized with 0.1% HgCl_2 solution for 20 min and thoroughly washed with sterile distilled water in aseptic conditions. For callus induction, seeds were cultured on MS (Murashige and Skoog, 1962) semi-solid medium containing 30 g l^{-1} sucrose and 2,4-D (1–5 mg l^{-1}). The medium was gelled with 8 g l^{-1} agar and the pH was adjusted to 5.8 prior to autoclaving at 120 °C for 15 min. The cultures were kept at 26 ± 2 °C in 16 h photoperiod. The light intensity of 100–125 $\mu\text{mol m}^{-2}\text{s}^{-1}$ was provided by fluorescent tube lights (Philips India Ltd, New Delhi).

For regeneration of plantlets, the MS basal medium was used. The regenerated plants were transferred to earthen pots containing a mixture of soilrite (Kel perlite, Bangalore, India) and soil (1:1) and kept under cover to maintain humidity for 10–15 days. Later, they were transplanted to the field and grown until maturity.

For histochemical studies, the proliferating calli at various stages of development were fixed in FAA (formalin : acetic acid : ethanol, 1:1:9), dehydrated in ascending series of ethanol and embedded in paraffin wax (Feder and O'Brien, 1968). Serial sections were cut at 8–10 μm and stained with Toluidine Blue O (a metachromatic stain for paraffin sections). The selected sections were photographed using Nikon Optiphot -2 microscope (Nikon Corporation, Tokyo, Japan).

The plasmids used in this study contained *uidA* gene encoding β -glucuronidase enzyme driven by promoters from rice *Actin I* (McElroy *et al.*, 1995), maize *Ubiquitin I* (McElroy *et al.*, 1995), rice *RbcS* encoding small subunit of ribulose 1, 5-biphosphate carboxylase (McElroy *et al.*, 1995), cauliflower mosaic virus 35S (Jefferson, 1997) and *Flaveria trinervia* (a C_4 dicot plant) *ppcA-L-Ft* encoding *ppcA* subgroup of phosphoenolpyruvate carboxylase (*PepC*) gene family (Stockhaus *et al.*, 1994) (Fig. 1). The purified plasmid DNA was obtained by using plasmid isolation kit (Qiagen, Germany).

Fifty milligrams of tungsten particles (1.1 μm) in 1 ml 100% ethanol (HPLC grade) were incubated in boiling water bath for about 2 h in a tightly capped

microcentrifuge tube. Particles were then centrifuged at a high speed for 1 s. Ethanol was replaced with 1 ml fresh aliquot and the sample was vortexed and again centrifuged for 1 s. Ethanol was discarded and 1 ml sterile distilled water was added. The particles were vortexed vigorously and then centrifuged for 1 s. The supernatant was discarded and a fresh aliquot of water was added. The particles were washed with water thrice and finally they were suspended in 1 ml sterile distilled water and stored at -20 °C. The particle suspension was thawed when required and vortexed vigorously to resuspend the particles. Fifty microliters of particle suspension was taken and the following components were added in the given order: 10 μl plasmid DNA (1 $\mu\text{g } \mu\text{l}^{-1}$), 50 μl 2.5 M CaCl_2 and 20 μl 0.1 M spermidine (free base). The mixture was vortexed gently for about 30 min in the cold room (maintained at 4 °C). Later, 200 μl ethanol was added to the mixture and it was centrifuged for 1 s at 10,000 rpm. The supernatant was discarded and 200 μl fresh ethanol was added. In this way, the pellet was washed with ethanol at least thrice and finally resuspended in 30 μl ethanol. The coated particles were kept at 4 °C and used within 1 h. Six microliters of the coated particle suspension was spread onto the macrocarrier membrane and allowed to dry for about 10 min. For negative control, particles were prepared in exactly the same manner without addition of plasmid DNA.

Four-week-old proliferating calli and leaf lamina as well as leaf sheath from 10-day-old *in vitro* raised seedlings of both millets were used as explants for shooting. The explants were transferred to the Petri plates containing MS medium gelled with 0.8% agar. They were arranged in the center of the plate in a circle having a diameter of about 2–3 cm. The bombardment was carried out using Biolistic™ PDS-1000/He particle delivery system (Bio-Rad laboratories, California, USA). Different combinations of pressure and target plate distance from macrocarrier launch assembly were tried to optimize the best combination (data not given). All bombardments were essentially done under a vacuum of 27.5 inches of mercury. A combination of 1100 psi pressure and distance of 6 cm was chosen for further analysis. After bombardment, the Petri dishes containing the explants were incubated in light for 2 days.

Histochemical GUS assay was carried out after 48 h of particle bombardment. The explants were incubated overnight in GUS histochemical stain (Jefferson *et al.*, 1987) at 37 °C. After removing chlorophyll by keeping in destaining solution (acetone : ethanol, 1:3), the number of blue foci per

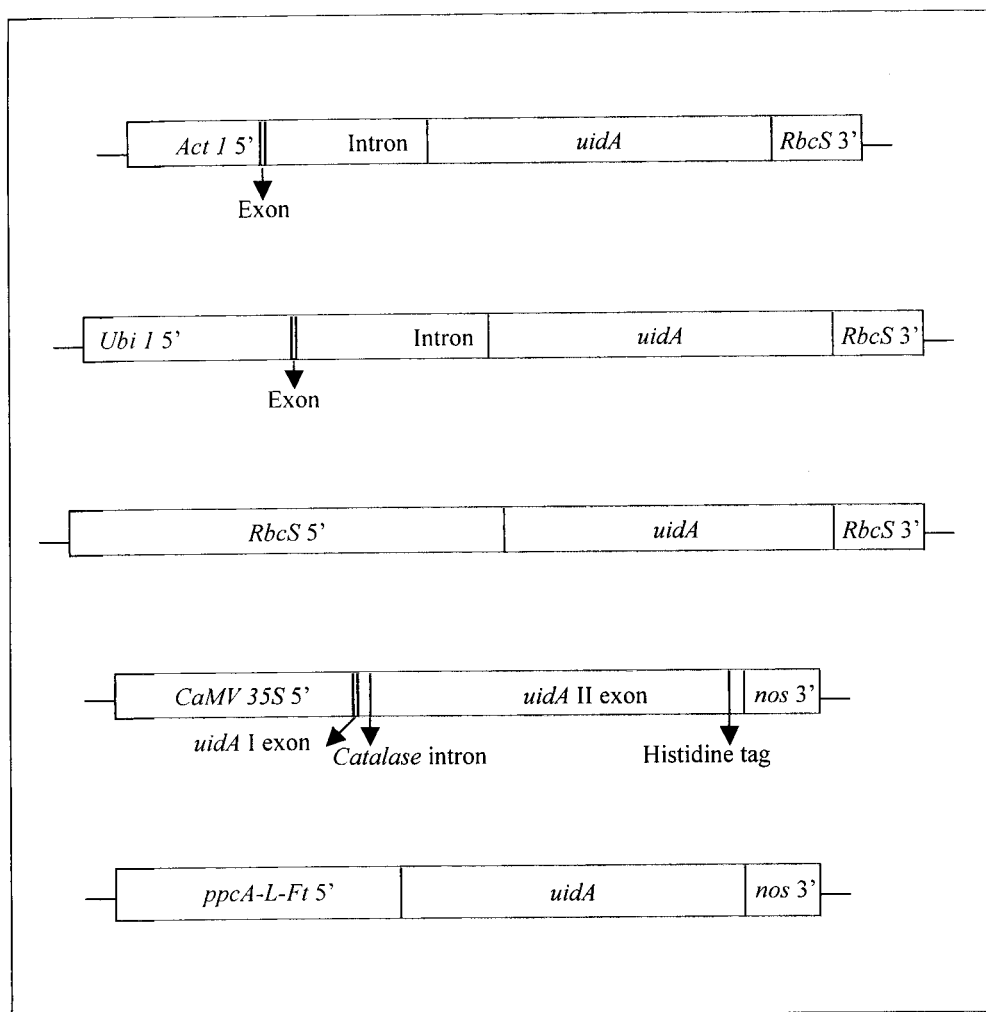


Fig. 1 Schematic maps of five promoters - *uidA* gene constructs used. *Act I 5'*, rice *Actin I* gene promoter; *uidA*, locus of *E. coli* encoding β -glucuronidase enzyme; *RbcS 3'*, transcription termination region of rice ribulose 1, 5-biphosphate carboxylase gene; *Ubi I 5'*, maize *Ubiquitin I* gene promoter; *RbcS 5'*, rice *RbcS* gene promoter; *CaMV 35S 5'*, cauliflower mosaic virus 35S promoter; *nos 3'*, transcription termination region of nopaline synthase gene from *Agrobacterium tumefaciens*; *ppcA-L-Ft 5'*, promoter of C_4 isoform of phosphoenolpyruvate carboxylase gene in *Flaveria trinervia* encoded by *ppcA* subgroup of *PepC* gene family.

explant were counted under microscope.

For spectrofluorometric analysis, approximately 100 mg tissue of each explant was used for protein extraction. Protein was quantified using Bradford's method (Bradford, 1976). Twenty micrograms of protein was incubated with 1 mM MUG (4-methyl umbelliferyl β -D-glucuronide) for 16 h. Relative fluorescence units were measured using a spectrofluorophotometer Model RF 540 (Shimadzu, Kyoto, Japan). GUS specific activity was calculated in terms of pmoles or nmoles of 4-MU (mg protein) $^{-1}$ h $^{-1}$.

After four weeks of inoculation on medium supplemented with 2,4-D (3-4 mg l $^{-1}$), the seeds of both the millets showed profuse callusing. In case of *Eleusine coracana*, maximum callusing response was observed at 4 mg l $^{-1}$ 2,4-D while in *Echi-*

nochloa crusgalli, 3 mg l $^{-1}$ 2,4-D was found most effective (Table 1). The response in terms of callusing was better in case of *E. crusgalli*. Two types of calli could be distinguished: embryogenic (nodular and compact) and non-embryogenic (watery and friable). Only the embryogenic calli were subcultured after every four weeks. In case of *Echinochloa*, the callusing was so profuse that after eight weeks of seed inoculation (i.e. at the time of second subculture), each single seed-derived callus was divided into four parts for subsequent subculture and again each growing callus was divided into three pieces during third subculture. In case of *Eleusine*, however, the callus became more compact and it was not divided during subcultures.

Organogenesis was considered as the most common pathway of plant regeneration in Gramineae

Table 1 Regeneration of plantlets via somatic embryogenesis in *Eleusine coracana* and *Echinochloa crusgalli* from seed-derived calli.

Plant	Concentration of 2, 4-D (mg l ⁻¹)	Number of seeds used for callusing	Number of seed calli (% callusing)	Number of plantlets per 100 seed calli
<i>Eleusine coracana</i>	3	392	125(31.8)	280
	4	648	295(45.5)	340
<i>Echinochloa crusgalli</i> ¹⁾	3	392	240(61)	2266
	4	648	225(34)	1680

¹⁾ Each seed-derived callus was divided into four pieces in second subculture and again into three pieces during third subculture on regeneration medium, resulting into 12 calli per seed callus.

until 1980. Since then, somatic embryogenesis has been reported in majority of the cereals and grasses (Vasil, 1982, 1985; Vasil and Vasil, 1986). When the proliferating calli were subcultured on MS medium for regeneration, they ceased to proliferate but showed differentiation of pale-green somatic embryo like structures in *E. coracana* as well as *E. crusgalli* after a lapse of four weeks. The microtome sections of regenerating calli reveal various stages of somatic embryo (Se) differentiation and maturation in *E. coracana* as well as *E. crusgalli*. The somatic embryos differentiate mostly from the peripheral cells of the callus which are generally small and densely cytoplasmic (Fig. 2 A-C). Either small pro-embryogenic masses (Pem) proliferate and bear numerous somatic embryos all around them (Fig. 2A, C) or the somatic embryos directly differentiate from the peripheral cells of the parent callus (Pc) (Fig. 2B). These cells undergo well-defined divisions to achieve near globular structure (Fig. 2A, C). At the time of initiation of scutellum, there is formation of a lateral depression or notch characteristic of monocot embryos (Fig. 2B). The shoot apex develops in this lateral notch followed by differentiation of root apex and embryonic vascular bundle (Fig. 2B, D). The fully differentiated somatic embryo resembles a typical zygotic monocot embryo (Fig. 2B, D). Secondary somatic embryo differentiation can be observed in certain instances if cultures are maintained on induction medium for a long time (Fig. 2D).

When embryogenic calli were placed on MS basal medium in test tube, embryos could regenerate into independent plantlets in both millets (Fig. 2E, F). Each plantlet had its well-organized root and stem system. Ultimately, in case of *E. coracana*, up to 340 plantlets could be regenerated per 100 seed calli while up to 2266 plantlets could be regenerated per 100 seed calli in case of *E. crusgalli* (Table 1).

Somatic embryos have been shown to be bipolar structures having single cell origin which eliminates

the regeneration of chimeric plants besides producing identical plants from genetic transformation events (Jones and Rost, 1991). So far, there are no reports of stable genetic transformation in the finger millet and barnyard millet. Hence, with the availability of a simple regeneration protocol for these two millets, attempts were made to optimize the parameters of DNA delivery in the three explants by biolistic method. As we are interested in high expression of desirable genes in millets, further analysis was done to identify a strong promoter. The transient GUS expression assays help in rapid evaluation of gene delivery and expression as the activity can be measured within a few hours of DNA introduction (Tyagi *et al.*, 1999). Besides, it allows evaluation of various homologous/heterologous promoters to be utilized in different gene constructs in future stable transformation efforts (Tyagi *et al.*, 1999).

Five constructs were used in this study which had different gene promoters driving the *uidA* gene (Fig. 1). All these constructs have been shown to be functional in other explants and taxa (Stockhaus *et al.*, 1994; McElroy *et al.*, 1995; Jefferson, 1997). In case of *E. coracana*, *Actin I* and *Ubiquitin I* gene promoters resulted in intense blue spots in GUS histochemical assay (Fig. 2), whereas *RbcS* and *CaMV 35S* gene promoters mostly gave faint but detectable level of blue colour in leaf lamina and leaf sheath explants. Only *ppcA-L-Ft* promoter was found ineffective in bringing about detectable expression of GUS in histochemical assay in *Eleusine*. On the other hand, in case of *E. crusgalli*, only *Ubiquitin I* promoter causes intense blue colour in all three explants while other promoters turned out to be mostly ineffective (Fig. 2). Although *35S* promoter from cauliflower mosaic virus is the most frequently used promoter in dicot transformation (Benfey and Chua, 1990), this shows very low activity in monocots (McElroy and Brettell, 1994). To overcome such limitations, strategies

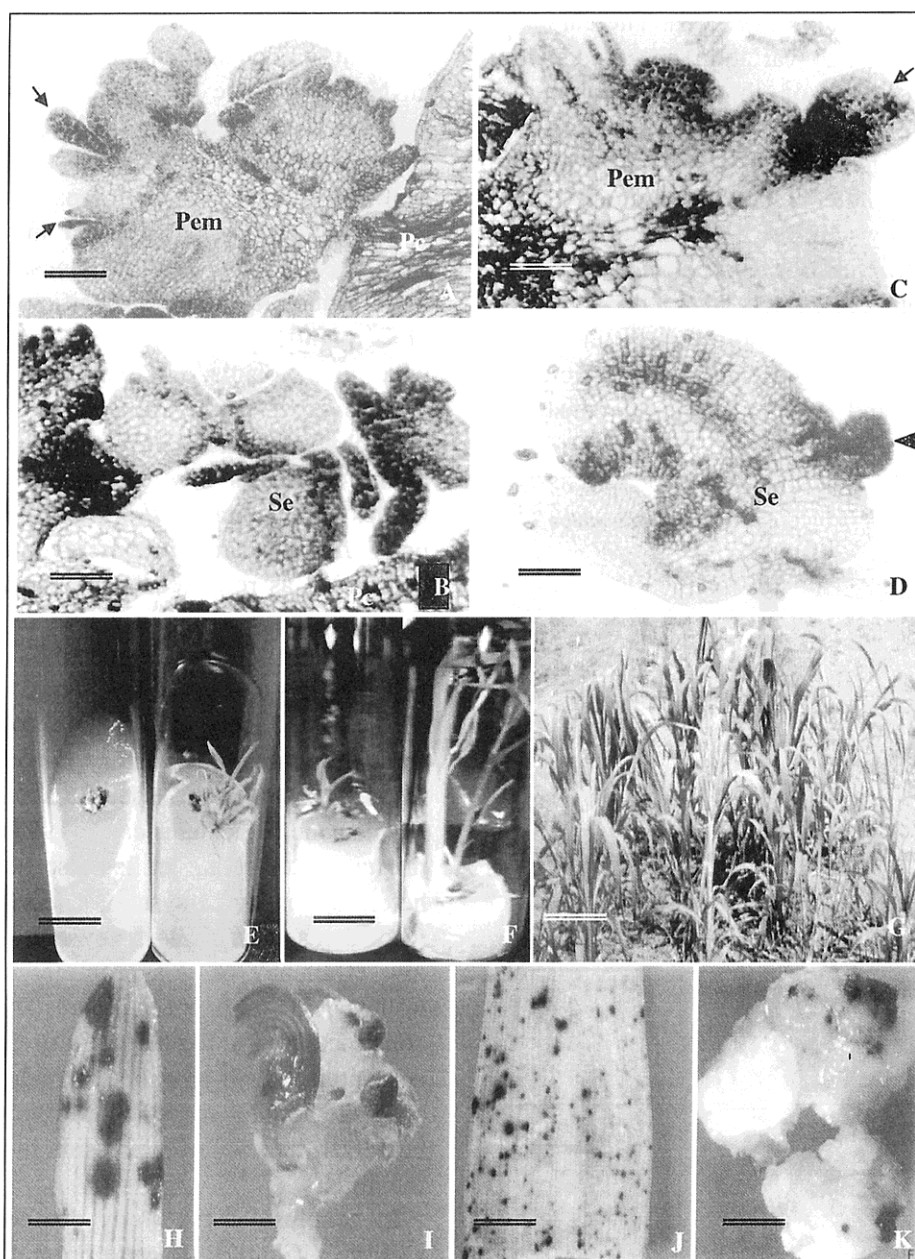


Fig. 2 Regeneration of plants from seed-derived calli and histochemical localization of GUS activity in leaf lamina and seed callus explants of *Eleusine* and *Echinochloa*. Transverse sections of regenerating calli showing differentiation of somatic embryos in *Eleusine* (A, B) and *Echinochloa* (C, D). Note the early globular (A) and late globular embryo (C) (arrows), which differentiate into typical zygotic embryo-like structure at maturity (B, D). Also, note the secondary somatic embryo (D) (arrowhead) arising from the epithelium of pre-existing somatic embryo. Culture tubes showing regenerating calli with multiple plantlet formation (E, F) which upon separation develop into independent plants. Tissue culture raised plants of *Echinochloa* at maturity after field transfer (G). Histochemical localization of GUS gene expression driven by maize *Ubiquitin 1* gene promoter in leaf lamina and callus explants of *Eleusine* (H, I) and *Echinochloa* (J, K). Pc, parent callus; Pem, pro-embryogenic mass; Se, somatic embryo. Bar: 128 μm (A–D); 1.4 cm (E); 1.2 cm (F); 16 cm (G); 830 μm (H); 2.5 mm (I); 660 μm (J); 2 mm (K).

have been devised to alter the promoters from monocots for enhanced constitutive expression in transformed cereal cells (McElroy *et al.*, 1994).

To account for the variations encountered in the size and intensity of the blue foci in the GUS

histochemical assay which could be correlated with constitutive/inducible nature or cell or tissue-specificity of the promoters, the delivery and expression of these gene constructs in various explants was also confirmed by more sensitive and quantitative GUS

spectrofluorometric analysis (Fig. 3). The bars represent the magnitude of variation observed in the two experiments. Out of the three constitutive promoters tested, *Actin I* gene promoter from rice and *Ubiquitin I* gene promoter from maize are the two monocot promoters showing naturally high constitutive activity (McElroy and Brettell, 1994). Among them, *Ubiquitin I* is the most efficient promoter for both the millets in all the three explants. *Actin I* and *35S* promoters also function in these millets albeit at significantly low efficiency. The *Ubiquitin I* and *Actin I* promoter constructs

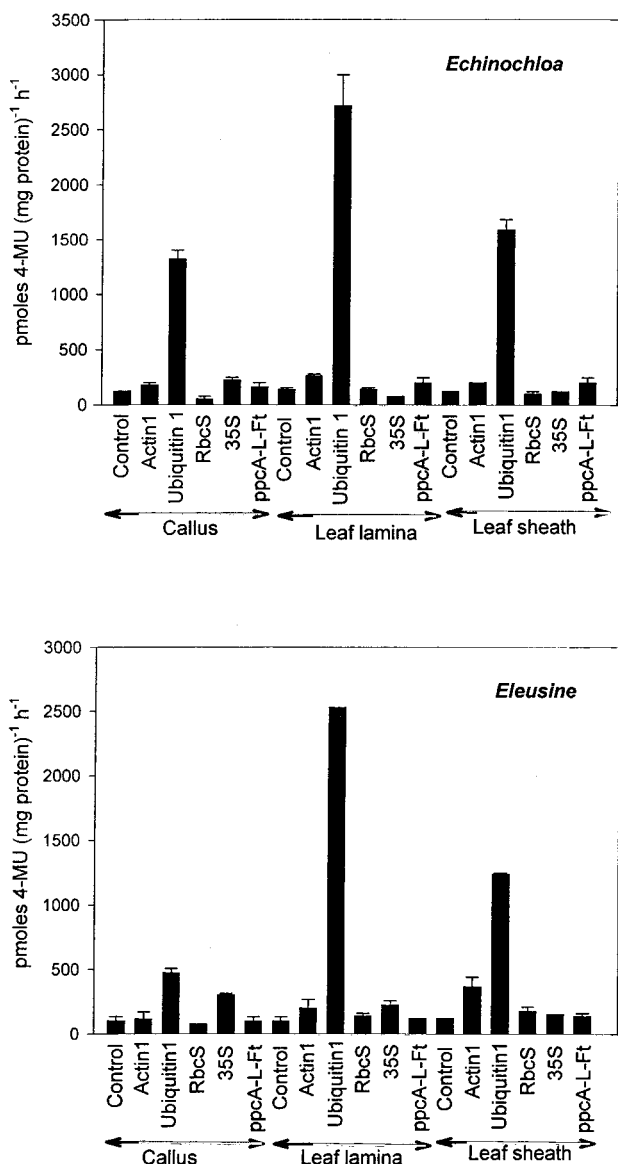


Fig. 3 GUS activity [pMoles 4-MU (mg protein)⁻¹ h⁻¹] in different explants after particle gun-mediated delivery of *uidA* gene constructs driven by promoters from rice *Actin I*, maize *Ubiquitin I*, rice *RbcS*, cauliflower mosaic virus *35S* and *Flaveria trinervia ppcA-L-Ft* genes respectively, in *Eleusine* and *Echinochloa*. Explants bombarded with particles without DNA coating served as control.

have one native intron incorporated in the transcription unit which has been implicated in elevating the mRNA abundance and enhancing the gene expression in transformed cereal cells (Callis *et al.*, 1987; Vasil *et al.*, 1989; Luehrsen and Walbot, 1991; McElroy *et al.*, 1991; Peterhans *et al.*, 1991). Other two promoter constructs used in this study were from *RbcS* and *ppcA-L-Ft* genes. Both of these are light-inducible and cell-specific promoters. In case of C₃ plants, *RbcS* is expressed in mesophyll cells while in C₄ plants, it is expressed in bundle sheath. On the other hand, *PepC* is highly expressed in mesophyll cells of C₄ plants while in case of C₃ plants, it shows low expression in a variety of tissues (Matsuoka *et al.*, 1994). In our study, the *ppcA-L-Ft* promoter confers detectable level of expression as reflected in the sensitive GUS spectrofluorometric assay in *Echinochloa*, a C₄ plant while in case of *Eleusine*, also a C₄ plant, its level of expression is extremely low. The 5' upstream region of the *ppcA1* gene of *Flaveria trinervia* has been shown to contain regulatory cis-elements that are responsible for the C₄ specific abundant expression of this gene (Stockhaus *et al.*, 1994). The activity of this promoter construct has also been checked in a heterologous dicot system, *Nicotiana*, a C₃ plant (Stockhaus *et al.*, 1994). However, in our study, the low efficiency of this promoter could be due to millets being monocot in nature. The *RbcS* promoter construct shows higher activity in leaf tissue as compared to calli in millets, which is in accordance with the reports made by McElroy *et al.* (1995). However, its level of expression is quite low in both the millets.

In conclusion, the study demonstrates the suitability of *Ubiquitin I* gene promoter in driving the high expression of transgenes in two millets, namely *Eleusine coracana* and *Echinochloa crusgalli*. This information along with efficient regeneration system would greatly help in stable transformation of millets by particle bombardment or by *Agrobacterium*-mediated gene delivery at desirable expression levels.

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