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Evaluation of Exogenous Promoters for Use in *Brachiaria brizantha* Transformation

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Abstract

***Brachiaria* (*Poaceae*) is the most important forage genus for cattle production in Brazil. The genetic breeding of this genus is limited by the incompatibility among species, differences in ploidy level and the natural cloning of plants by apomixis (Valle and Miles 1992). However, plant regeneration via tissue culture methods and genetic engineering provide an opportunity to introduce new characteristics in plants of this genus. We have developed methods for the genetic modification of *Brachiaria brizantha* cv. Marandu via biolistic transformation. A higher number of shoots was obtained whith 4 mg/L 2.4-diclorophenoxyacetic acid and 0.2 mg/L benzylaminopurine in calli induction medium and 0.1 mg/L naphtaleneacetic acid and 4.0 mg/L kinetin in shoot regeneration medium. A selection curve for mannose was determined to use phospho mannose isomerase (PMI) gene of *Escherichia coli* as a selection marker. Calli formation was inhibited from 5 g/L mannose, even in the presence of sucrose while calli that were formed in the presence of mannose failed to develop embryos showing that PMI gene can be used for selection of transformants of this grass. Different promoters were tested to evaluate the efficiency based on the detection of the GUS gene expression (Jefferson et al. 1987). The monocot promoters, act1-D and ubi-1, resulted in higher expression levels than dicot promoters, ubi-3 and act-2, or the CaMV35S and CVMV promoters.**

Key words: Apomixis, biolistics, forage grass, somatic embryogenesis

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Introduction

Brachiaria (*Poaceae*) is cultivated in at least 30 million hectares in Brazil due to its qualities of forage grass and good adaptability to Cerrado (dry-tropical savanna, Brazil; Miles et al. 1996). *Brachiaria brizantha cv. Marandu* combines palatability and nutritional quality with spittlebug resistance, important requirements to grow this pasture in American tropical regions (Keller-Grein et al. 1996). In *B. brizantha* most accessions reproduce by apomixis (Valle and Savidan 1996).

Apomixis is an asexual reproduction through seeds (Nogler 1984) that is found in many other genera of the *Poaceae* family and in more than 300 angiosperms species (Hanna and Bashaw 1987). This mode of reproduction is characterized by the formation of the embryo directly from cells of the ovule without fertilization of the egg cell. In *Brachiaria* species, apomixis is found to be of the aposporic type (Gobbe et al. 1981; Lutts et al. 1984; Dusi and Willemse, 1999; Naumova et al. 1999; Araujo et al. 2000), where an unreduced embryo sac develops from a somatic nucelar cell. In *Brachiaria*, all apomicts are polyploids, while the sexual ones are diploids, making hybridization techniques difficult (Valle and Miles 1992). Plant transformation can contribute to the breeding of *Brachiaria* since new characteristics can be introduced with genetic engineering techniques, independently of sexual compatibility. The possibility of controlling the mode of reproduction on natural apomicts would allow recombination with sexual accessions, increasing the gene pool of these forage grasses.

The success of transformation techniques depends on factors such as an efficient regeneration and transformation proto-

col and selection of the transformed cells.

Regeneration of *Brachiaria* cells can be achieved by somatic embryogenesis from mature embryos (Tohme et al. 1996) or by organogenesis from meristematic basal explants from in vitro plants (Pinheiro et al. 2000). For somatic embryogenesis, the auxin 2,4-diclorophenoxiacetic acid (2,4-D) was the most effective in calli induction (Tohme et al. 1996; Lenis-Manzano 1998). The combination of naphthalene acetic acid (NAA) and kinetin (KIN) was the most efficient in regenerating shoots from somatic embryos and meristematic explants (Tohme et al. 1996; Pinheiro et al. 2000). In this work, different concentrations of these growth regulators were tested in the regeneration of mature embryos aiming to obtain a higher number of shoots and improve the transformation by biolistics.

Biolistics, bombardment of microparticles carrying DNA, has been widely used for genetic transformation of different monocotyledonous species (Klein et al. 1988; Christou et al. 1991; Bower and Birsh 1992). This technique was developed for *B. brizantha*, *B. decumbens* and *B. ruziziensis* (Lenis 1998) and involves physical factors that can be optimised, such as: pressure of helium gas, vacuum pressure, distance between the retention screen and the material to be bombarded, and also the distance between the gas chamber of high pressure and the carrier membrane with DNA (Sanford et al. 1991; 1993). Appropriate vectors carrying promoters that induce high expression of the genes of interest and a selection system are also important factors for the efficiency of transformation. Selection of transformed cells with the phospho mannose isomerase gene of *Escherichia coli*, PMI, (Joersbo et al. 1998) can be an alternative to antibiotic resistance ones such as hygromycin gene (Lenis-Manzano 1998). A selection curve for mannose was determined in an attempt to use PMI as a selection marker.

The promoters Act1-D from *Oriza sativa* (Mc Elroy et al. 1990), Act2 from *Arabidopsis thaliana* (Yong-Qiang et al. 1996), Ubi1 from *Zea Mays* (Christensen et al. 1992), Ubi3 from *A. thaliana* (Burke et al. 1988.) CaMV35S (Guilley et al. 1982), and CVMVp (Verdaguer et al. 1996) were tested to evaluate their efficiency based on the detection of the GUS gene expression.

Materials and Methods

Plant materials

Seeds of *Brachiaria brizantha* cv. Marandu tetraploid apomictic (cultivar BRA000591 from Embrapa) (2n=4X=36) were kindly provided by Dr. do Valle-Embrapa Beef Cattle-MS-Brazil. After excision of the seed coat, the seeds were disinfested in 70% ethanol for three minutes, 20 minutes in 1% sodium hypo chloride and washed three times with sterile distilled water. Embryos were excised under stereoscopic microscope.

Somatic embryogenesis

The excised embryos were sown either in M1.1 (Lentini et al. 1999) or M1.2 calli induction media, both containing MS salts and vitamins (Murashige and Skoog 1962), 100 mg/L casein hydrolizate, 3% sucrose, 0.7% agar, supplemented with 0.2 mg/L benzylaminopurine (BAP) and 2.0 mg/L 2.4-diclorophenoxyacetic acid (2,4-D) in medium M1.1 or 4.0 mg/L 2.4-diclorophenoxyacetic acid and 0.2 mg/L BAP in M1.2, pH 5.8. The embryos were cultivated in the dark at $27 \pm 2^{\circ}C$ for 30 days. Embryogenic calli were transferred to regeneration medium MS 1 (Lentini et al. 1999) or MS 2 containing MS salts and vitamins (Murashige and Skoog 1962) 100 mg/L myo-inositol, 3% sucrose, 0,7% agarose, 200 mg/L activated charcoal. MS 1 medium is supplemented with 0,1 mg/L naphtaleneacetic acid (ANA) and 0.4 mg/L kinetin (Kin) and MS 2 supplemented with 4 mg/L Kin. The embryogenic calli were cultivated under diffused fluorescent light (10-15 $\mu mE^{-2}s^{-1}$) with 16 h photoperiod, at 27 $\pm 2^{\circ}C$, until regeneration. Regenerated plantlets were rooted in glass culture tubes containing B medium (half strength MS macro salts, 1 mg/L $ZnSO_4$, 0.03 mg/L $CuSO_4.5H_2O$, 0.03 mg/L $AlCl_3$, 0.03 mg/L $NiCl_2.6H_2O$, 1 mg/L H_3BO_3, 0.01 mg/L KI, 0.1 mg/L $MnSO_4.4H_2O$, 100 mg/L myo-inositol, 1 mg/L calcium pantothenate, 1 mg/L thiamine, 0.01 mg/L biotin; Bourgin et al. 1979) supplemented with 30 g/L sucrose. The plantlets were maintained under diffused fluorescent light (10-15 $\mu mE^{-2}s^{-1}$) with 16 h photoperiod, at $27 \pm 2^{\circ}C$.

The differences between the treatments were estimated by comparing the number of embryos that developed calli and shoots.

Determination of mannose selection curve

Different concentrations of mannose were tested in M1.1 calli induction medium with 0 g/L or 15 g/L sucrose and 0, 5, 10, 15 and 20 g/L of mannose. The embryos were cultivated in the dark, at $27 \pm 2^{\circ}C$. Evaluation of embryogenic calli development was carried out after two weeks of culture.

Vector construction

To construct the plasmid pU3G, the vector pNOV1443 (NOVARTIS, Inc.) was digested with *Hin*dIII and *Bam*HI to isolate the ubiquitin gene promoter of *A. thaliana*, Ubq3, together with its intron. This fragment was then ligated to the vector pBluescript SK^+ and named pU3. The GUS gene with the nopaline synthetase terminator was extracted from pAct1-D (McElroy et al. 1990) by digestion with the enzymes *Bam*HI and *Xba*I. The

resulting fragment was ligated to pU3.

To obtain the vector p35M, the plasmid pBI426 was digested with *Hin*dIII and *Bam*HI to extract the doubled 35S promoter from CaMV (Datla et al. 1991) and this fragment was ligated to pBluescript SK^+. The *Bam*HI/*Xba*I fragment from pNOV1443, which contains the coding sequence of the gene PMI with the nos terminator, was fused to the 35S promoter. Similarly, the ubiquitin promoter of maize was extracted from pAHC27 (Christensen and Quail 1996) with the enzymes *Hin*dIII and *Bam*HI and inserted in the pBluescript SK^+ containing the PMI gene. All vectors, pU3G, p35M and pUP, were analysed through digestion profile with the following enzymes:*Bam*HI, *Hin*dIII and *Xba*I.

Bombardment of mature embryos of *B. brizantha*

For the analysis of promoter efficiency, approximately 50 mature embryos of *B. brizantha* were bombarded with each of the six plasmids outlined in Table 1. Experiments were repeated twice for the plasmids pAct1-D and pAHC-27, with approximately 150 embryos tested per plasmid. The embryos were placed in Petri dishes containing M1.1 (10 embryos of *B. brizantha* and two embryos of *Phaseolus vulgaris* as control) and were incubated in the dark, 24 hours before bombardment.

The bombardment protocol was modified from Lenis (1998). The pressure of helium gas used was of 900 psi and the distance of the explant in relation to the carrier membrane was 6 cm.

Histochemical detection of *β*-glucuronidase

After 24 hours of culture in the dark, the bombarded embryos were placed into a solution of X-Gluc ($NaH_2PO_4.H_2O$ 100 mM; $K_4Fe(CN)_6.3H_2O$ 0.5 mM; $Na_2EDTA.2H_2O$ 10 mM; Triton X-100 0.1%; X-Gluc 1 mM; McCabe et al. 1988) for 24 hours at 37°C.

Table 1. Outline of the plasmids used in bombardment of *Brachiaria brizantha* cv Marandu mature embryos.

Plasmid	Promoter	Species of the promoter	Coding region	Termina-tor	Size (Kb)	Reference
pAct1-D	Act1	*Oryza sativa*	GUS	nos	8.0	McElroy 1990
pAHC27	Ubi1	*Zea mays*	GUS	nos	7.0	Christiansen 1996
pAG1	Act2p	*Arabidopsis thaliana*	GUS	nos	6.5	Aragão 2000
pBI426	CaMV35S	Cauliflower mosaic virus	GUS: nptII	nos	6.2	Datla 1991
pILTAB 308	CsVMV	Cassava vein mosaic virus	GUS	nos	5.3	Verdarguer 1996
pU3G	Ubi3	*Arabidopsis thaliana*	GUS	nos	6.7	this paper

The GUS gene expression analysis to evaluate the activity of the different promoters was determined by the number of GUS positive embryos among the bombarded embryos. For this analysis only embryos with intense blue spots were considered as GUS positives (Figure 1E).

Results

Somatic embryogenesis efficiency

The main steps of *B. brizantha* somatic embryogenesis are shown in Figure 1. Somatic embryogenesis was improved when 4 mg/L 2,4-D and 0.2 mg/L BAP were present in the calli induction medium (M1.2) and 4 mg/L Kin in regeneration medium (MS2) (Table 2).

Although there was no difference in the percentage of calli formation in both media (M1.1 and M1.2), calli formed in M1.2 were consistently larger than in M1.1 (0.4 mg/L 2,4-D and 0.2 mg/L BAP), and yet with visible more embryogenic calli regions than friable ones. When embryos cultivated in M1.1 were transferred to MS1, the percentage of shoot formation was 13% and when embryos from M1.2 were transferred to MS2 the percentage of shoot formation was 67% (Table 2), representing an increase in 54%. These data show that enhancing auxin concentration during calli induction alone was not sufficient to

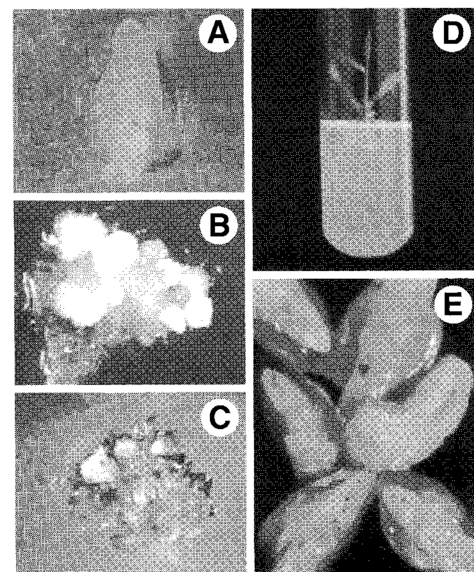

Figure 1. A-D) Process of calli induction and plant regeneration of *Brachiaria brizantha*: A) Embryo isolated from mature seed (23X). B) Embryogenic calli formed from embryo cultivated 20 days in M1.2 medium (15X). C) Regenerated shoots from embryogenic calli cultivated in MS2 medium (15X). D) Plant regenerated from embryogenic calli in B medium (0,6X). E) Embryos of *B. brizantha* 24 hours after bombardment with pAHC27 incubated with x-gluc solution (23X).

Table 2. Response of *B. brizantha* mature embryos cultured in medium containing different concentration of auxin/citokinin.

Medium	N° of embryos	N° of embryos that developed embryogenic calli (% embryogenic calli/embryos)	N° of total shoots (% total shoots/ embryos)
M1.1/MS1	80	59 (74%)	8 (13%)
M1.1/MS2	40	39 (97%)	6 (15%)
M1.2/MS1	112	69 (62%)	18 (26%)
M1.2/MS2	112	82 (73%)	55 (67%)

improve regeneration. Furthermore, a higher level of cytokinin in shoot regeneration medium improved somatic embryogenesis of *B. brizantha*, in two different and well-defined steps, calli induction and regeneration of plants. Thus, the combination M1.2 and MS.2 is recommended for use in further experiments of *B. brizantha* somatic embryogenesis.

Mannose selection curve

In M1 medium, containing 30g/L sucrose, the efficiency of calli formation was 69% (Table 3). This number was drastically reduced when no sugar was added to the culture medium. In M1 medium, containing mannose as the only carbon source, *B. brizantha* mature embryos were still capable of forming calli (Table 3). Even though, the calli brownished and dried in a short period of time (data not shown). In culture medium containig 15 g/L sucrose, the addition of 5 g/L mannose was sufficient to reduce the size and viability of the calli and they did not develop into somatic embryos.

Vector construction

The three vectors constructed at this work are shown in Figure 2. pU3G was created to test the activity of Ubq3 promoter from *A. thaliana*. In an attempt to have plasmids containing the PMI selection gene under control of different promoters the plasmids p35M and pUP were constructed.

Table 3. Calli formation in *B. brizantha* mature embryos cultured on M1 medium containing sucrose and mannose.

Sugar concentration (g/L) (sucrose/mannose)	N° of embryos	% of embryos that formed calli
0/0	37	8
0/5	37	16
0/10	38	10
0/15	39	29
0/20	39	25
15/0	39	61
15/5	39	34
15/10	38	47
15/15	39	33
15/20	38	36
30/0	16	69

GUS gene expression

The activity of *GUS* gene was analysed by β-glucuronidase histochemical assay in all the bombarded embryos (Figure 1E). No activity was detected in the embryos bombarded with the vector pU3G in *B. brizantha*. A great number of blue spots could be observed in the control, the bean explant, indicating high GUS activity. Embryos bombarded with pBI 426 and pILT AB 308 showed low or no detectable β-glucuronidase activity in the histochemical assay used. Again, intense blue spots were observed in the control. The embryos bombarded with vectors pAHC 27 and pAct 1D showed approximately the same number of blue spots (Table 4) indicating similar *GUS* expression levels.

Discussion

In this study, we report the improvement of several aspects of *Brachiaria brizantha* transformation protocol. Firstly, a more efficient regeneration protocol has been established by an increase in auxin concentration in calli induction medium from 2 mg/L to 4 mg/L 2.4-D and a 10-fold increase in cytokinin in shoot regeneration medium. Together these modifications yielded a 54% increase in the number of regenerated shoots. As it has been reported for other grasses (Vasil 1988), high concentrations of 2,4-D are sufficient to increase calli induction in *B. brizantha*. But, the balance between auxin and cytokinin was necessary for somatic embryo germination in the second step

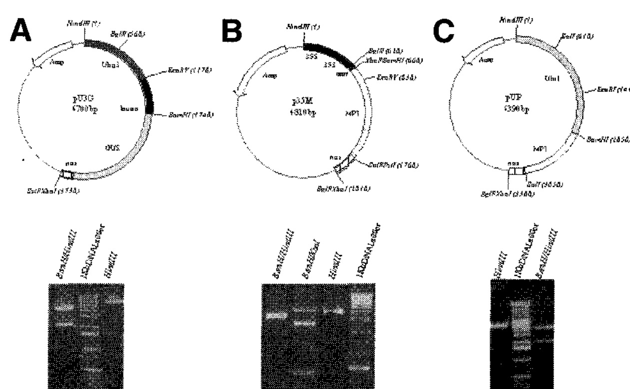

Figure 2. Outline of the constructed plasmids in pBluescript and restriction profile in 0.8% agarose gel stained with ethidium bromide. A) pU3G (ubiquitin promoter of *Z. mays* and GUS gene). B) p35M (duplicated 35ScaMV promoter and the PMI gene). C) pUP (ubiquitin promoter of *Z. mays* and the PMI gene).

Table 4. β-glucuronidase (GUS) response in mature embryos of *B. brizantha* 24 hours after biolistic transformation with pAct1-D and pAHC27. GUS expression was analysed by the distribution of blue spots per embryo as: + = < 5; ++ = from 5 to 10; +++ = > than 10.

Experiment	pAct1-D					pAHC27				
	N° of embryos bombarded	N° of positive embryos (pe)	Distribution of GUS expression in pe			N° of embryos bombarded	N° of positive embryos (pe)	Distribution of GUS expression in pe		
	n	n	+	++	+++	n	n	+	++	+++
I	58	15	12	1	2	58	13	7	4	2
II	56	18	12	6	0	54	11	8	2	1
III	47	15	12	3	0	39	10	7	1	2
Total	161	48	46	10	2	151	34	22	7	5

of the regeneration procedure. In transformation experiments, the improvement in the regeneration system can increase the chances of transformed calli recovery after selection.

A second important aspect in plant transformation is the efficacy of the selection system. Concerning the use of antibiotics, *B. brizantha* explants are resistant to 100 mg/L kanamycin, so its use is not suggested. However, hygromicin has been reported as an efficient selection agent for *B. brizantha* (Lenis 1998), and transformed plants have been produced using the hygromicin phosphotransferase (hpt) gene (Lentini et al. 1999). Nevertheless, the introduction of antibiotic resistance in invasive plants such as *Brachiaria* species can raise biosafety issues. In an attempt to circumvent this problem, we have tested a mannose selection curve, to allow the use of this sugar as a positive selection agent, with the PMI gene. Ferguson and Street (1958) suggested that the toxic effect of mannose for some plants could be due to the accumulation of mannose-6-phosphate. That, in turn, would inhibit glycolysis. This toxicity was reported for *Beta vulgaris* and *Zea mays*, at concentrations of 2.25 g/L and 10 g/L respectively (Joersbo et al. 1998; Negrotto et al, 2000).

From a concentration of 5 g/L mannose calli formation was reduced even in the presence of 15 g/L sucrose in *B. brizantha*. Furthermore, calli that were formed in the presence of mannose failed to develop embryos. These results suggest that mannose has a toxic effect in somatic embryogenesis of *B. brizantha* and the concentration of 5 g/L can be used in transformation experiments with PMI gene.

Finally, plant transformation depends on the expression of the selection marker. We have tested the efficiency of several promoters using *GUS* reporter gene. The promoters derived from the monocot maize, ubiquitin-1, and rice, actin-1, resulted in higher expression levels than promoters derived from the dicot or the CaMV35S and CVMV promoter. The vector pU3G, carrying Ubi 3 promoter was also tested in other plants, such as: *Vitis sp.*, *Musa sp.* and *Carica papaya*, with expression level similar to bean (de Souza, personal communication).

Both of these monocot promoters have been extensively used to obtain transgenic plants of maize, rice, barley and wheat (McElroy et al. 1990; Christensen et al. 1992; Cornejo et al. 1993; Taylor et al. 1993). The CaMV35S promoter drives lower expression levels in monocot when compared to dicot species (Rathus et al. 1993). This observation was also confirmed for *B. brizantha.*

In *B. brizantha*, the ubiquitin and actin promoters of maize and rice respectively showed similar expression levels, as reported in maize (Taylor et al. 1993). These results contrast with previous reports in rice (Cornejo et al. 1993), *Panicum maximum* and *Pennisetum purpureum* explants (Taylor et al. 1993), where the ubiquitin promoter was consistently more efficient than other monocot promoters such as the Adh1 (*Z. mays*) and the Act-1 (*O. sativa*) as well as the CaMV35S promoter.

In bean explant, used as positive control, all promoters tested, including the ones derived from monocot plants, induced high GUS activity, comparable to CaMV35S promoter. In *A. thalianna* and *Nicotiana tobacco*, monocot promoters showed low expression levels when compared to CaMV35S (Christensen et al. 1992; Wilmink et al. 1995). Both Act1 and Ubi1 constructs contain an intron in the 5´ untranslated region, which enhances transcription. Nevertheless, the inefficiency of these promoters in dicots and other monocots, such as garlic and sugarcane, can be attributed to different sequence requirements for efficient splicing (Christensen et al. 1992; Lacorte and Barros 2000). The splicing efficiency in cereal monocot introns probably varies between other monocot or dicot species. Nevertheless, a more extensive analysis would be necessary to elucidate the molecular nature of these results.

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