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Genetic transformation of *Brachiaria brizantha* cv. Marandu by biolistics

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ABSTRACT

Brachiaria brizantha is a forage grass well adapted to tropical areas and cultivated in millions of hectares in Brazil. The apomictic mode of reproduction in this species, in addition to differences in ploidy between sexual and apomictic plants, impairs crossbreeding. The development of a methodology to transform apomictic cultivars will provide an option to introduce agronomic important traits to *B. brizantha* cv. Marandu. In addition, it will open the possibility to study *in vivo* the function of candidate genes involved in the apomictic reproduction. The objective of this work was to evaluate peeled seeds, isolated embryo from mature seeds, embryogenic calluses and embryogenic cell suspensions, as target explant for genetic transformation via biolistics. Plasmids bearing the marker genes *gus* and *hptII* under the control of the rice actin 1 promoter (pAct1-Os) or the maize ubiquitin 1 promoter (pUbi1Zm) were used. All the target-explants used were suitable for transient gene expression after bombardment, showing *gus* expression and resistance to hygromycin. Using embryogenic calluses and cell suspensions as target tissues, transgenic plants were regenerated and transgenes detected.

Key words: forage, *gus*, *hptII*, hygromycin.

INTRODUCTION

Brachiaria brizantha (*syn. Urochloa brizantha;* Shirasuna 2015) cv. Marandu is a forage grass well adapted to tropical areas. Only in Brazil *Brachiaria* is cultivated in millions of hectares. This cultivar is tetraploid and reproduces asexually by apomixis. Analysis of the reproductive mode, based on the embryo sac structure of 275 *B. brizantha* accessions from the Brazilian germplasm collection, displayed one single sexual diploid, the remaining being apomicts and polyploids (Valle and Savidan 1996). The difference in ploidy level between sexual and apomictic plants, in addition to the apomictic mode of reproduction, impairs breeding, reducing the chances of incorporating new traits to the cultivar. Interspecific crosses have been used to allow breeding, and more recently intraspecific crosses using an artificial tetraploid female progenitor (Monteiro et al. 2016). Considering all the bias resulting from these crosses, genetic transformation is an option to introduce agronomic important traits to the apomictic cultivated *B. brizantha* cv. Marandu. Another important aspect

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of *Brachiaria* transformation is the possibility to study the function of candidate genes involved in the apomictic reproduction (Silveira et al. 2012, Guimarães et al. 2013). Moreover, establishing methodology to express gene transiently may assist the investigation of promoters and genes using silencing and gain-of-function approaches in this species.

Transformation via biolistic resulted in the production of transgenic plants in many recalcitrant species of monocotyledonous that are not susceptible to *Agrobacterium* (Sharma et al. 2005, Sood et al. 2011). Embryogenic cultures or immature embryos are the preferred target explants to transform cereals and forage grasses (Harwood 2012). Actually, embryogenic calluses of the sexual diploid *B. ruziziensis* were transformed by biolistic with a vector containing a phosphinothricin resistance marker gene (*bar*) and the reporter gene *gus,* both under the control of the maize ubiquitin 1 promoter, pUbi1Zm (Ishigaki et al. 2012). Two transgenic plants were recovered, they had normal phenotype, however, they did not produce seeds. One fertile transgenic plant was obtained only from an artificially tetraploidized callus (Ishigaki et al. 2012).

In vitro plant regeneration protocols using as explants peeled seeds, isolated embryos from mature seeds, embryogenic calluses and embryogenic cell suspensions of *B. brizantha* cv. Marandu were developed (Cabral et al. 2011, 2015). Despite the progress in the development of tissue culture protocols, *B. brizantha* genetic transformation is still a challenge. The aim of this work was to evaluate the viability of these somatic embryogenesis systems for transient and stable expression of transgenes via biolistics using plasmids bearing the marker genes *gus* and *hptII*.

MATERIALS AND METHODS

Seeds of a natural tetraploid apomictic genotype of *Brachiaria brizantha* cv. Marandu (Embrapa's collection number BRA 000591) were used for all experiments. Four types of explants were evaluated for genetic transformation via biolistics: (i) embryo isolated from mature seed; (ii) mature dehusked seed; (iii) embryogenic callus; (iv) cell suspension.

The following plasmids were used for bombardment: a) pAct1-D, derived from pUC (McElroy et al. 1990) containing the *gus* gene under the rice actin 1 promoter pAct1-Os; b) pAHC27 (Christensen and Quail 1996) containing the *gus* gene under the maize ubiquitin 1 promoter (pUbi1Zm); pAHUG, derived from pUC19, containing the hygromycin phosphotransferase II (*hptII*) gene under pAct1Os promoter, and the *gus* gene under the pUbiZm promoter; two binary vectors pGPro1, and pGPro2 (Thilmony et al. 2006, 2009), containing the marker *hptII* gene under the control of pAct1Os promoter (Thilmony et al. 2006). These vectors have a multiple restriction site to clone promoters, therefore, the promoter pUbi1Zm, isolated from plasmid pAHC27 (Christensen and Quail 1996), was inserted into *PstI* restriction site to drive the expression of the *gus* gene.

For bombardment, explants were positioned in a 1-cm-circle defined in the center of the 6 cm diameter Petri dish containing M1.3 medium with 0.7 % Phytagel (bombardment medium). Plasmidial DNA (8 μg) was precipitated with tungsten microparticles (M10). The physical parameters were: helium pressure of 900 psi (unless otherwise specified), target distance of 6 cm and 27 lb Hg vacuum, one or two shots per plate.

Tissue culture media M1.2, M1.3, MS3 and PMM were described in Cabral et al. (2011), NBBAP, NBreg, DD1 were described in Cabral et al. (2015) (Table I). Culture room temperature was kept at 25 ± 2°C, transformation controls (bombardment without plasmid) and regeneration

controls (culture without selection pressure) were performed for all experiments.

For PCR and DNA gel blot analysis of PCR, DNA was extracted from calluses, cell suspension and young leaves from hygromycin-resistant plants. Explants were macerated in a 1.5 mL buffer Tris HCl pH 8 0.1 M, NaCl 0.25 M, EDTA 25 mM and SDS 0.5 %, at room temperature. The samples were centrifuged for 5 min at 12,000 rpm. Supernatant was transferred to a new tube, and one volume of isopropanol was added, and incubated for 5 min at room temperature. The samples were then centrifuged for 5 min at 12,000 rpm, the supernatant discarded and the sediment resuspended in 20 μL of sterilized ultrapure water. PCR was performed using primers for the *gus* gene, to amplify a fragment of 420 bp (forward TTGGGCAGGCCAGCGTATCGT and reverse ATCACGCAGTTCAACGCTGAC); for *hptII* gene, the primers used amplify a fragment of 473 bp (forward TCCGCAAGTGCTTGACATTGG and reverse ATGTTGGCGACCTCGTATTGG). Products were visualized by electrophoresis in 1.0 % agarose gel, and then transferred by capillarity to a nylon membrane (Hybond-N^+ Amersham Pharmacia Biotech) (Sambrook et al. 1989). The membrane was hybridized with [α^{32}P] dCTP labeled probes from *gus* or *htpII* genes.

GENETIC TRANSFORMATION USING FOUR TYPES OF EXPLANTS

Isolated embryo and seed

After peeling and disinfesting seeds according to Cabral et al. (2011), embryos were excised. These were cultured on somatic embryo induction medium (M1.2), in the dark, for one or three days, 110 embryos per treatment, two replications each treatment. To evaluate the *gus* expression stability, the embryos were bombarded and histochemically evaluated after 1 d (control of bombardment), 10 d, and 20 d. To establish the best time of induction prior to bombardment and type of explant, both, peeled seeds and isolated embryos were evaluated. The explants were plated on somatic embryogenesis induction medium M1.2 for 1, 3, 5 or 7 d of culture before bombardment, in a total of 75 explants per treatment. After the induction period, the explants were placed in Petri dishes with M1.2 medium and bombarded with plasmid pAct1-D. After 48 h a GUS histochemical assay was performed (Jefferson 1987).

Embryogenic callus

Calluses resulting from seeds cultivated in embryogenesis induction M1.3 medium (Table I; Cabral et al. 2011, 2015) for 7, 15 or 30 d, were transferred to bombardment medium in Petri dishes, and maintained in the dark, in culture room for 24 h before bombardment with pAHUG plasmid. A minimum of 390 calluses were bombarded for each treatment. After bombardment, the calluses were kept for 24 h in the dark. Half of the calluses was submitted to GUS histochemical assay while the other half was transferred either to the induction medium M1.3 with hygromycin (5 mg/L) and kept in the dark for 20 d, or to the regeneration medium, directly to the light. After this period, they were transferred to regeneration medium MS3 with hygromycin (10 mg/L) and kept at 12 h photoperiod, for 30 d. The resultant selected embryogenic calluses which regenerated 2-cm-long shoots, resistant to hygromycin, were transferred to plant maintenance medium (PMM), for 30 d supplemented with hygromycin (20 mg/L). Regenerated plantlets were transferred to 1:1 (v/v) substrate:vermiculite in the greenhouse. DNA was extracted from leaves for PCR analysis. Putative transgenic plants were transferred to soil and another PCR reaction was performed to confirm the presence of the transgene.

Cell suspensions

Cell suspensions (CS) induced in M1.3 or NBBAP pH 4 (Table I) were placed in filter paper to

TABLE I
Culture media used for *B. brizantha* somatic embryogenesis induction, plant regeneration and rooting.

Medium	Culture Medium Composition	Reference
M1.2	MS basal medium (Murashige and Skoog 1962), 100 mg/L casein hydrolysate, 4 mg/L 2,4-D	Silveira et al. 2003
M1.3	MS basal medium (Murashige and Skoog 1962), 300 mg/L casein hydrolysate, 3 mg/L 2,4-D, 0.2 mg/L BAP, pH 4	Cabral et al. 2011
MS3	MS basal medium (Murashige and Skoog 1962), 300 mg/L casein hydrolysate, 0.5 mg/L NAA, 1 mg/L BAP, 2.5 mg/L kinetin, pH 4	Cabral et al. 2011
PMM	MS salts (major salts strength), MS vitamins, casein hydrolysate 100 mg l-1, sucrose 20 g/L, NAA 0.2 mg/L, 0.5 mg/L kinetin, 0.2 mg/L GA3	Cabral et al. 2011
NB	N6 major salts, B5 minor salts and vitamins, Inositol 100 mg/L, proline 500 mg/L, glutamine 500 mg/L, casein hydrolysate 300 mg/L, 2.5 mg/L 2,4-D, sucrose 3 %, phytagel 0.3 %, pH 5.8	Sallaud et al. 2003
NBBAP	NB medium supplemented with 0.2 mg/L BAP, either at pH 4 or pH 5.8	Cabral et al. 2015
NBreg	NB medium supplemented with 3 mg/L BAP, 0.5 mg/L NAA, phytagel 0.6 %, pH 5.8	Sallaud et al. 2003
PRM	NB medium supplemented with 5 mg/L BAP, 1 mg/L NAA, 2 mg/L ABA instead of 2,4-D, agarose type I 0.7 % pH 5.8	Sallaud et al. 2003
DD1	MS basal medium (Murashige and Skoog 1962), 10 mg/L ascorbic acid, 1.1 mg/L 2,4-D, 0.2 mg/L Zeatin, sucrose 3 % pH 5.8	Cabral et al. 2015

For all media pH was adjusted to 5.8 with 1N KOH or to 4.0 with 1N HCl prior to autoclaving. Agar concentration was 7 g/L, except for pH 4, which was 14 g/L. Medium was autoclaved at 121ºC, for 20 min. When ABA and GA3 were supplemented media were filter sterilized.

drain the excess of medium. Approximately 200 to 300 mg of cell aggregates were spread on the bombardment medium, in a diameter of 1 cm avoiding the center of the Petri dish, and left for 4 to 24 h at room temperature. Plasmids pAHC27, pAct1D, pGPro1pUbi1, and pGPro2pUbi1 were bombarded at 900 psi and the last two were also bombarded at 1,200 psi. After bombardment, the cells were incubated in the dark for 24 or 48 h. Part of the cells was collected for GUS histochemical analysis, the other part was transferred to liquid NBBAP pH 4, or liquid or solid M1.3, with or without hygromycin (10 mg/L), for two weeks.

CS cultivated in liquid media were then transferred to pre-regeneration DD1 or PRM liquid medium containing 10, 20 or 30 mg/L hygromycin, under orbital agitation of 100 rpm in the dark. After one week, cells were transferred to regeneration medium MS3, liquid or solid, containing hygromycin (20 mg/L), at 16 h photoperiod and 50 µmol m-2 s-1 intensity light for 3 d, when they were transferred to 75 µmol m-2 s-1 light intensity.

CS from solid M1.3 were cultivated for 20 d in the dark and then transferred to regeneration media MS3 or NBreg, supplemented with hygromycin (20 mg/L) for 15 d. Regenerated shoots were transferred to PMM medium, without hygromycin, in magenta boxes, for 20 d. Shoots were then individually transferred to test tubes with PMM medium. After one month, the plantlets were acclimatized in 1:1 (v/v) substrate:vermiculite and thereafter transferred to soil in the greenhouse.

RESULTS AND DISCUSSION

ISOLATED EMBRYOS AND SEEDS

The highest number of explants expressing *gus* was observed in embryos bombarded after 3 d in induction medium M1.2 (Table II, Fig. 1a). In general, the expression of *gus* decreased at 10 d (Fig. 1b) and 20 d after bombardment (Fig. 1c), comparing to the 1 d control, embryos induced

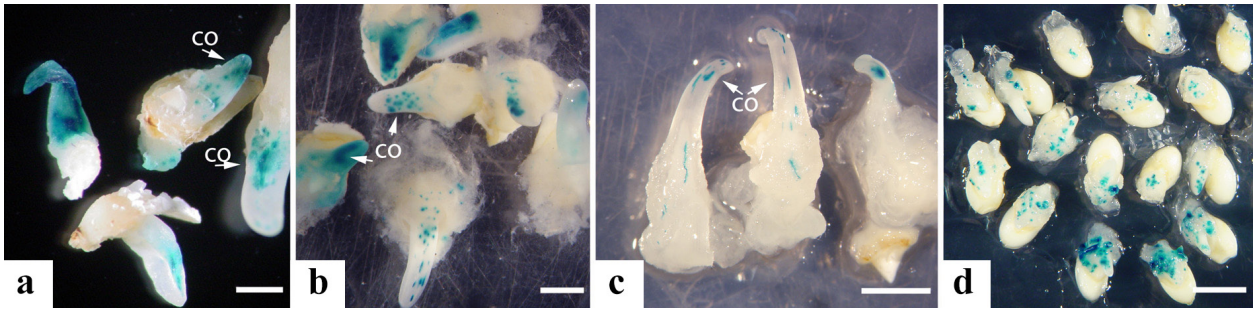

Figure 1 - Expression of gene *gus* in *Brachiaria brizantha* cv. Marandu isolated embryos and seeds after bombardment with pAct1-D: (**a**) embryos cultured for 3 d in induction medium and 48 h after bombardment; (**b**) embryos induced for 3 d before bombardment and cultivated for 10 d after bombardment; (**c**) embryos 20 d after bombardment; (**d**) dehulled seeds 48 h after bombardment. co = coleoptile. Bars: (**a**) 0.5 cm; (**b**, **c**) 1 cm; (**d**) 2 cm.

TABLE II
Expression of *gus* in isolated embryos of *Brachiaria brizantha* cv. Marandu after bombardment, according to different periods of time in induction medium prior to bombardment.

Days in induction medium	Days of culture in induction medium after bombardment		
	1 d	10 d	20 d
	% of explants expressing *gus*	% of explants expressing *gus*	% of explants expressing *gus*
1 d	62	20	10
3 d	89	83	40

for 1 d being more affected. The reduction of expression after bombardment possibly indicates low plasmid integration into the plant genome, but also a low proliferation of cells stably expressing *gus*. Coleoptile cells elongate during development, giving a linear pattern of expression (Fig. 1c). The expression of *gus* was more evident in coleoptile cells (Fig. 1a-c) rather than in friable callus cells, in which somatic embryo development preferentially occurs (Cabral et al. 2011).

Table III shows the percentage of *gus* expression in isolated embryos and peeled seeds subjected to different periods of culture in somatic embryogenesis induction medium and bombardment with plasmid pAct1-D. The percentage of isolated embryos showing *gus* expression increased with the days in culture before bombardment reaching up to 39 % with 5 d decreasing at 7 d in culture. The percentage of seeds with *gus* expression also increased with the period in culture in M1.2 medium before bombardment, being 37 % with 1 d, up to 78 % with 7 d. Besides being easier to manipulate, the higher percentage of seeds expressing *gus* (Fig. 1d) when compared to isolated embryos, makes seeds a preferred explant for testing vectors and constructions via transient expression after bombardment.

EMBRYOGENIC CALLUSES

Calluses cultivated for 7, 15 or 30 d before bombardment showed *gus* expression 24 h after bombardment with the plasmid pAHUG (Fig. 2a). Calluses transferred directly to regeneration medium, promptly differentiated somatic embryos that turned out to be chimeric for *gus* expression (Fig. 2b). Calluses that were initially transferred to induction medium supplemented with hygromycin (5 mg/L) proliferated into embryogenic calluses. After multiplying and transferring to regeneration medium with a higher hygromycin concentration (10 mg/L), these calluses produced a few shoots after 30 d (Fig. 2c). The percentage of shoots resistant to hygromycin obtained from calluses cultivated for 7 d in induction medium prior to bombardment with pAHUG was 1.5 %. This percentage increased when calluses were cultivated for 15 d (3 %) and

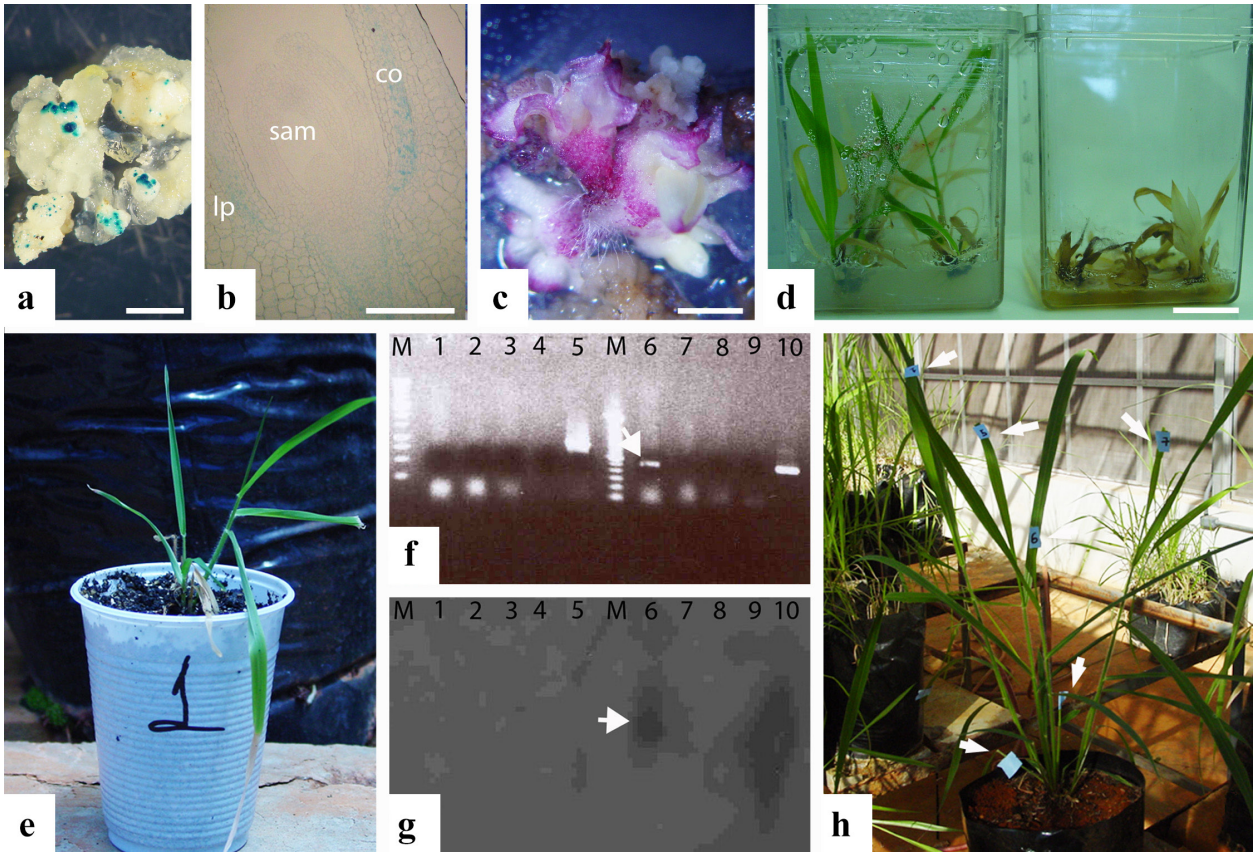

Figure 2 - Transformation of *Brachiaria brizantha* cv. Marandu embryogenic calluses via biolistics, and molecular analyses of putative transformed plants: (**a**) *gus* expression in embryogenic calluses induced for 30 d on M1.3 medium and bombarded with pAHUG; (**b**) longitudinal section of a somatic embryo developed from embryogenic callus bombarded with pAHUG, and cultivated in regeneration medium, showing *gus* expression in the coleoptile and leaf primordium, and absence of expression in the shoot apical meristem; (**c**) germinating somatic embryos in MS3 medium with hygromycin showing anthocyanin pigments; (**d**) hygromycin resistant plantlets (left) and untransformed control (right) in medium containing 20 mg/L hygromycin; (**e**) acclimatized hygromycin resistant plantlet; (**f**) detection of *gus* (1-5) and *hptII* (6-10), by PCR; pAHUG (5 and 10) as positive control; putative transformed plants (1-4 and 6-9); column 6 is a plant showing an amplified band corresponding to *hptII* (arrow), M = marker; (**g**) DNA gel blot with positive signal for *hptII* in column 6 (arrow); (**h**) two-month-old plant, arrow indicate sampling areas for PCR. co = coleoptile; lp = leaf primordium; sam = shoot apical meristem; pe = proembryos. Bars: (**e**) 1 cm; (**f**) 0.2 mm; (**g**, **h**) 2 cm.

TABLE III
Expression of *gus* in isolated embryos and seeds of *Brachiaria brizantha* cv. Marandu, 75 explants per treatment cultivated before bombardment on induction medium M1.2 for different periods of time, evaluated 48 h after bombardment with pAct1-D.

Days of cultivation prior to bombardment (d)	Embryos expressing *gus* (%)	Seeds expressing *gus* (%)
1	12	37
3	17	47
5	39	61
7	27	78

30 d (8.5 %). Regeneration of putative transgenic plantlets was observed only from calluses induced for 30 d before bombardment (Fig. 2d). Only one of the regenerated plants survived acclimatization (Fig. 2e). For this plant, GUS histochemical assay was negative. However, PCR analysis showed a fragment of 473 bp of the *hptII* gene, with the specificity confirmed by PCR DNA gel blot analysis, and no amplification corresponding to *gus* (Fig. 2f, g). The transgenic plant was transferred to soil and after two months (Fig. 2h) a PCR performed with DNA extracted from fragments of

seven individual leaves did not amplify the *hptII*. It is possible that the plant was a chimera and during the development, the transgenic tillers were lost.

CELL SUSPENSIONS (CS)

CS plated 24 h before bombardment got shrunk and brownish after bombardment, and did not show *gu*s expression. Cells bombarded 4 h after introduction in bombardment medium showed best results, in terms of viability and gene expression. Cell aggregates bombarded with plasmids pGPro1pUbi1 and pGPro2pUbi1 showed transient *gus* expression in a higher percentage when pressure condition was 1,200 psi compared to 900 psi (Table IV). Nevertheless, at 1,200 psi they died after one month of culturing.

Only the cell aggregates kept for two weeks in NBBAP containing hygromycin (10 mg/mL), and transferred to DD1 supplemented with hygromycin (10 mg/L) or PRM with hygromycin (30 mg/L) proliferated new embryogenic calluses resistant to the antibiotic. These embryogenic cell agregates kept their proliferative capacity after successive subcultures, during 10 months, and showed stable *gus* expression (Fig. 3a, b). The *gus* and *hptII* genes were detected by PCR (Fig. 3c, d), and confirmed by DNA gel blot analysis of the amplified bands (Fig. 3e, f), ratifying stable transformation. These stably transformed embryogenic cell aggregates did not regenerate shoots and at this point, the calluses had a vitrified aspect, possibly due to several subcultures.

Cell suspensions bombarded with pAct1D and pAHC27 also showed *gus* expression (Table IV; Fig. 4a, b). CS bombarded with pAHC27 resulted in the regeneration of three multiple shoots, in a medium without selective agent, and when isolated two shoots showed *gus* expression (Fig. 4c, d).

The competence to regenerate transgenic plants is generally genotype-dependent, a limiting step in plant biotechnology (Harwood 2012, Tie et al. 2012). In *Brachiaria,* Ishigaki et al. (2012) reported transgenic plants from the diploid and from the colchicine-tetraploidized calluses of *B. ruziziensis*. They recovered two transgenic plants that presented a normal phenotype, however, no seeds were produced by the diploid one, and a fertile transgenic plant was obtained only from an artificially tetraploidized callus (Ishigaki et al. 2012). In this work, we showed that *Brachiaria brizantha* cv Marandu, an apomictic tetraploid, is competent to regenerate transgenic plants using either embryogenic calluses or cell suspension systems when bombarded and selected in hygromicin, besides the production of some escapes. Nowadays, we have been using these systems with different selections to obtain a

TABLE IV
Expression of *gus* in *Brachiaria brizantha* cell suspensions bombarded with different plasmids using different helium pressures.

Plasmid	Hellium pressure (PSI)	Total number of bombarded Petri dishes	Total number of Cell aggregates*	
			bombarded	expressing *gus* after bombardment (%)
pGPro1pUbi1	900	12	231	27 (12)
	1200	12	307	97 (32)
pGPro2pUbi1	900	12	194	8 (4)
	1200	12	384	154 (40)
pAHC27	900	38	788	123 (16)
pAct1D	900	18	408	93 (23)

*Average of two experiments, one shot per Petri dish.

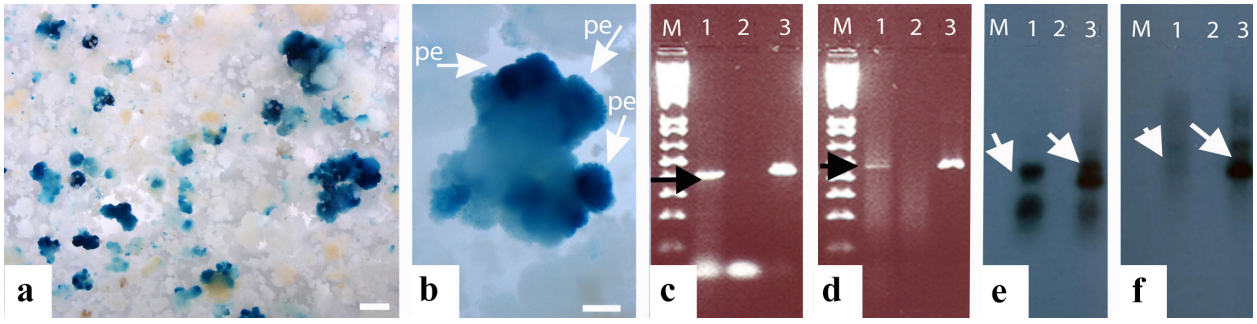

Figure 3 - Cell suspension of *Brachiaria brizantha* transformed by biolistic and molecular analysis of putative transformants. (**a-b**) cell suspension obtained in NBBAP (pH 4) after bombardment with pGPro1pUbi1; (**a**) stable *gus* expression in embryogenic calluses after cultivation for 10 m in regeneration medium (RM) in the presence of hygromycin (20 mg/L); (**b**) detail of (**a**) with proembryos expressing *gus*; (**c-d**) detection of *gus* (**c**) and *hptII* (**d**) by PCR: column 1, DNA from hygromycin resistant calluses (arrows), column 2 negative control (water), column 3 plasmid pGPro1pUbi1 used as positive control; (**e-f**) PCR DNA gel blot hybridized with a *gus* probe (**e**, arrows) and with *hptII* probe (**f**, arrows). M, molecular weight marker (1 kb). pe = proembryos. Bars: (**a**) 1 cm; (**b**) 0.2 cm.

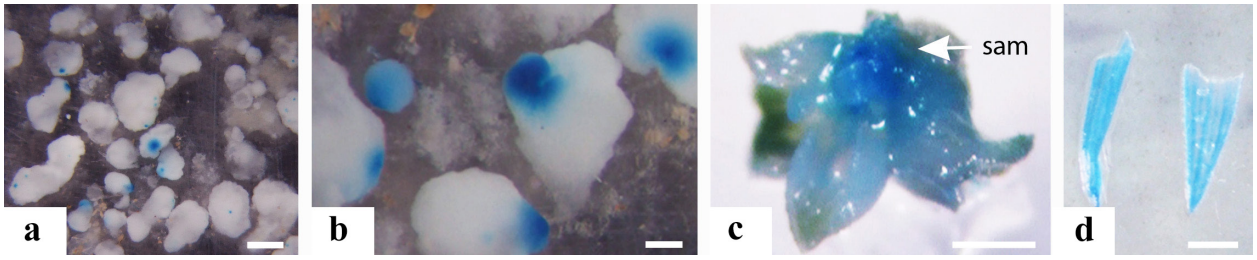

Figure 4 - Cell suspension of *Brachiaria brizantha* transformed by biolistic. (**a, b**) *gus* expression after cell suspension bombardment with pAHC27, 24 h after bombardment; (**c**) *gus* expression in shoot apical meristem; (**d**) leaf fragments expressing *gus*. sam = shoot apical meristem. Bars: (**a**, **c**, **d**) 1 cm; (**b**) 0.2 cm.

high throughput method to produce *B. brizantha* transgenic plants.

The present work provides valuable information towards the development of a methodology to obtain transgenic plants of *B. brizantha,* which is still considered as a recalcitrant monocot species.

CONCLUSIONS

In conclusion, all the explants tested and earlier successfully reported as efficient for somatic embryogenesis were suitable for transient gene expression after bombardment, showing *gus* expression and resistance to hygromycin. *B. brizantha* mature seeds cultured for seven days in somatic embryo induction medium, embryogenic calluses cultured for 15 or 30 days, as well as cell suspensions, proved to be the best explants to test vectors and constructions via transient gene expression after particle bombardment. The use of embryogenic calluses and cell suspensions as targets, allowed for the recovery of transgenic plants. However, the combined action of the marker gene *hptII* expression and selection in hygromycin seems to favor regeneration from untransformed cells, suggesting that other selective agents should be tested for more effectiveness in selecting the transformed cells.

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