6

Genetic Transformation of Immature Sorghum Inflorescence via Microprojectile Bombardment

Rosangela L. Brandão, Newton Portilho Carneiro, Antônio C. de Oliveira, Gracielle T. C. P. Coelho and Andréa Almeida Carneiro*
Embrapa Maize and Sorghum
Brazil

1. Introduction

Sorghum bicolor is one of the most important cereals in the world after rice, maize, wheat and barley. In 2010, more than 60 million tons were produced from approximately 50 million ha around the world. It is an important crop in the arid and semi-arid regions of the world and it is primarily used in Brazil as a supply for an increasing livestock market. However, the Brazilian sorghum productivity is low (1,500 to 2,500 kg/ha) and extremely variable along the years, typical of a culture sowed in marginal climate conditions and mainly without the use of high input technologies. Conventional breeding programs have already done a great deal of research to increase the sorghum productivity, though in some fields, the gains obtained by these programs are reaching stationary levels due to the lack of genetic variability (Nwanze *et al.*, 1995). Alternatively, recombinant DNA technology and the generation of transgenic plants can increment conventional breeding programs through the amplification of the gene pool that can be used to improve sorghum environmental fitness and nutritional qualities.

However, unlike others Poaceae, sorghum transformation has been a challenge mainly due to recalcitrance in tissue culture and long periods of selection required for the recovery and regeneration of putative transgenic plants (Casas *et al.*, 1993, Zhao *et al.*, 2000; Jeoung *et al.*, 2002; Howe *et al.*, 2005). Since the earliest 90's, laboratories around the world have generated improvements in sorghum regeneration and transformation that are ensuing in more consistent protocols. Transgenic sorghum plants have been generated via biolistic (Casas *et al.*, 1993; Casas *et al.*, 1997; Zhu *et al.*, 1998; Able *et al.*, 2001; Emani *et al.*, 2002; Jeoung *et al.*, 2002; Devi and Sticklen, 2003; Tadesse *et al.*, 2003; Girijashankar *et al.*, 2005) or *Agrobacterium* mediated transformation (Zhao *et al.*, 2000; Carvalho *et al.*, 2004; Gao *et al.*, 2005; Howe *et al.*, 2006; Van Nguyen *et al.*, 2007; Nguyen et al., 2007; Gurel et al., 2009; Lu et al. 2009; Mall et al., 2011).

Even though the efficiency of sorghum transformation using the microprojectile bombardment had been improved, by all this studies, since the initial experiments (from 0,3 to 1,3%), it is still low if compared with the efficiency of sorghum transformation mediated by *Agrobacterium tumefaciens* from 2.1% – 4.5% (Zhao et al., 2000; Gao et al., 2005; Howe et

*Corresponding Author

al., 2006). So, there is still need for more improvements in the microprojectile bombardment, once this technique can be used for genotypes and explants not susceptible to the transformation mediated by *Agrobacterium tumefaciens*.

2. Genetic transformation of *Sorghum bicolor*

2.1 *In vitro* regeneration of transgenic sorghum cells

Sorghum tissue culture is reported to be highly recalcitrant mainly because the release of toxic phenolics compounds in culture media, lack of regeneration in long term *in vitro* cultures, and high degree of genotype dependence. Consequently, cell transformation followed by plant regeneration remains extremely complicated in sorghum transgenic technology. So, the establishment of sorghum regeneration systems from somatic cells constitutes a prerequisite of extreme importance within the process of transgenic sorghum plants production.

The regeneration of sorghum *in vitro* has been achieved from a variety of tissues, such as immature embryos (Bhat and Kuruvinashetti 1995; Bai et al. 1995; Elkonin and Pakhomova 1996), immature inflorescences (Kaeppler and Pedersen 1997), young leaves (Han et al. 1997) and shoot tips (Nahadi and de-Wet 1995; Patil and Kuruvinashetti 1998; Shyamala and Devi, 2003). Immature zygotic embryos and inflorescences are the explants with higher embryogenic competence and frequently used to regenerate various cultivars of sorghum. Gupta and co-workers (2006) compared the regeneration in tissue culture of immature embryos and immature inflorescences from five genotypes of *S. sudanense* (SDSL 98984, SDSL 98988, SDSL 981125, SDSL 981142 and SDSL 981144) and three genotypes of *S. bicolor* (2219B, GD 68727 and 981013). They indicated that the regeneration potential of immature inflorescences was much superior to that of immature embryos and their performance was almost equivalent across the genotypes tested. The superiority of immature inflorescences can be due to its higher proportion of meristematic tissues (floral meristems, rachis, rachillae, and primordial of various floral organs) in comparison to immature embryos (mainly scutellum) according to authors.

However, the main explants used in the transformation of sorghum are immature zygotic embryos between 1.5 and 2.0 mm in length (Casas et al, 1993; Emani et al, 2002; Jeoung et al, 2002; Gao et al, 2005, Howe et al, 2006; Gurel et al, 2009). One of the constrains in working with immature embryos is the intensive labor to generate large quantities of explants to be used in the transformation procedures. In this sense, immature inflorescences are easier to isolate, show very good regeneration rates in tissue culture and, morphogenetic competence over a wider size range (1–5 cm) than immature embryos. Besides, it is faster to grow donor plants for the production of immature inflorescence than for immature embryo (Cai and Butler, 1990; Kaepler and Pedersen, 1997; Jogeswar et al 2007; Brandão et al. 2007).

Even though outstanding studies aiming to identify sorghum genotypes able to produce high quality callus from immature inflorescence have been conducted, the efficiency to produce transgenic sorghum plants using this type of explant is still very low.

2.2 Genetic transformation of *Sorghum bicolor* via microprojectile bombardment

The bombardment of plant cells with the DNA of interest is a direct method of transformation designed (Taylor and Fauquet, 2002) in the late 80's to manipulate the

genome of plants recalcitrant to transformation via *Agrobacterium*, including the cereals (Klein et al 1987; Taylor and Fauquet, 2002). In the transformation via particle bombardment, microprojectiles of metal physically covered with the gene of interest are launched toward the target cells, using equipment known as "gene gun" (Sandford et al. 1987). The velocity of these particles is fast enough (1500 km/h) to penetrate the cell wall of a target tissue and does not cause cell death. The precipitated DNA on the microprojectiles is released progressively into the cell after the blast, and integrated into the genome (Taylor and Fauquet, 2002). The acceleration of microprojectiles is obtained by a high voltage electrical discharge or compressed gas (helium). The metal particles used must be non-toxic, non-reactive, and lower than the diameter of the target cell. The most commonly used are gold or tungsten. Several physical parameters correlated with the biolistic equipment such as pressure, distance of the macro and micro-carrier flight, and vacuum, must be optimized for successful transformation. In addition to these parameters, the biology of the plant material and the gene of interest (GOI) should also be studied in preliminary experiments (Sandford et al., 1993). Since the 90's, the biolistic was used to transform a wide variety of plants, including sorghum.

Some advantages of the microprojectile bombardment are related to its efficiency in the transformation of monocots, the use of simple vectors, easier to handle, as well as the possibility of inserting more than one GOI into cells efficiently (Chen et al. 1998; Wu et al. 2002). Although considered a very efficient method in cereals, one drawbacks of this technique is the occurrence of multiple copies of the GOI in the transgenic plant and complex integration patterns (Wang and Frame, 2004).

The biolistic has proved to be an efficient method for introducing new features in sorghum and a few transformation protocols are already available (Casas et al. 1993; Casas et al. 1997; Zhu et al. 1998; Able et al. 2001; Devi and Sticklen, 2002 ; Emani et al. 2002; Tadesse et al., 2003; Girijashankar et al., 2005). The optimization of physical and biological parameters was the subject of most of the work published about sorghum transformation via bombardment. The pioneer work was done by Casas and collaborators between 1993 and 1997. Initially, using anthocyanin (*R* and *C1* genes) and β-glucuronidase (*uidA* gene) as reporter genes, they established an ideal pressure and microprojectile flying distance to transform immature sorghum embryos via bombardment. In this work it was also shown that the bialaphos was suitable to select transgenic cells of sorghum. Major problems in the protocol were the low efficiency (0,3%) and long time to select transgenic callus (7 months). In the next work (1997), the group tried to improve the transformation efficiency by using immature sorghum inflorescence, an explant with higher morphogenetic potential compared to immature embryos. At this time a greater number of transgenic plants were obtained but the overall efficiency of the process was low and the selection protocol generated many escapes.

Optimization of physical and biological parameters to produce transgenic sorghum plants was also the purpose of the work by Able et al. (2001) and Tadesse et al. (2003). Able and co-workers (2001) analyzed the transient expression of the reporter genes GUS and GFP over different physical bombardment parameters to identify the best conditions to generate transgenic sorghum plants using the particle inflow gun (PIG). Three transgenics events were confirmed by molecular analyses. Tadesse and associates (2003) also used reporter

genes to test different acceleration pressures, target distances, gap widths and macroprojectile travel distances to bombard immature and mature embryos, shoot tips and embryogenic calli. The strength of four different promoters (*ubi1*, *act1D*, *adh1* and *CaMV35S*) was also analyzed in transient assays. The optimization of the transformation conditions generated a protocol with an efficiency of 0.5 to 1.3% of transgenic sorghum production from shoots tips and immature embryos, respectively.

Currently few sorghum events expressing genes with agronomical interest were developed by the laboratories working with transformation of this specie via microprojectile bombardment. The gene *chi II*, encoding rice chitinase under the control of the constitutive CaMV35S promoter, has been transferred to sorghum for resistance to stalk rot (*Fusarium thapsinum*) by Zhu et al. (1998). Chitinases are proteins produced by plants as defense against pathogen attack; they function by degrading the fungal cell wall. Their work was done with calli developed from immature zygotic embryos as target tissue for microprojectile bombardment and, six independent events that were bialaphos-resistant and containing the chitinase gene were reported.

Another chitinase, the gene *ECH2*, and the *bar* gene were used to produce disease and herbicide resistant transgenic plants, respectively. Devi and Sticklen (2003) transformed shoots clumps, originated from mature sorghum seeds cultivated in presence of N^6-benzyladenine (BA), via microjectile bombardment with these genes. Shoot clumps were used with the purpose to develop a transformation protocol using an explant easier to be obtained throughout the year. Only five different events were generated, but the proficiency of sorghum shoot meristems for regeneration and transformation was demonstrated.

The *cry1Ac* gene from *Bacillus thuringiensis* under the control of the wound-inducible promoter from the maize protease inhibitor gene (*mpiC1*) was inserted in the genome of three independent transgenic sorghum events. Shoot apices were bombarded and subcultured in a MS medium supplemented with benzylaminopurine (BAP) and naphthalene acetic acid (NAA). Leaf damage by the spotted stem borer (*Chilo partellus*) was reduced up to 60% in the sorghum transgenic plants generated (Girijashankar et al., 2005).

To withstand toxic aluminum concentrations present in acidic soils, sorghum was genetically modified to express the *ALMT1* (Sasaki et al. 2004) gene from wheat (Brandão, 2007). The *ALMT1* gene codes a malate transporter Al^{+3} activated protein that is highly expressed in wheat root apices of aluminum tolerant cultivars. Transgenic sorghum plants grown in hydroponic culture under stress of Al^{+3}, showed a higher level of aluminum tolerance when compared with isogenic non-transgenic control plants.

3. Genetic transformation of immature sorghum inflorescence

Here, we report improvements made in the transformation process via microprojectile bombardment that enable us to obtain a protocol where putative transgenic plants can be produced with an efficiency ranging from 1.01 to 3.33% using immature inflorescences of sorghum.

3.1 Material and methods

3.1.1 Plant material and explants preparation

Seeds from nine *Sorghum bicolor* (Moench L.) accessions were obtained from the Embrapa Maize and Sorghum National Research Center – Brazil. Shoots were collected at different developmental stages (3 to 5 cm in length), from field plants prior to the appearance of the flag leaf (Figure 1). The outermost leaf blades were removed and shoots rinsed with 70% ethanol and sterile distilled water. After that, immature inflorescences were dissected, chopped in approximately 5 mm long segments and cultivated on callus induction medium (CIM) as described by Tadesse et al. (2003), with minor modifications [MS salts (Murashige and Skoog, 1962), 1 $mg.L^{-1}$ thiamine HCl, 7.5 $mg.L^{-1}$ glycine, 100 $mg.L^{-1}$ DL-asparagine, 100 $mg.L^{-1}$ myo-inosital, 0.2 $mg.L^{-1}$ kinetin, 2.5 $mg.L^{-1}$ 2,4-D and 30 $g.L^{-1}$ sucrose]. Medium pH was adjusted to 5.8 with 1 N potassium hydroxide prior to autoclaving.Cultures were incubated at 25^{o}C in dark for three to four weeks.

For the biolistic experiments 30 calli pieces of approximately 3 mm diameter (Figure 2B) were uniformly distributed within a 35 mm diameter circle of 60 x 15 mm Petri dishes containing CIM media in which a higher osmotic value was achieved by the addition of 12% sucrose.

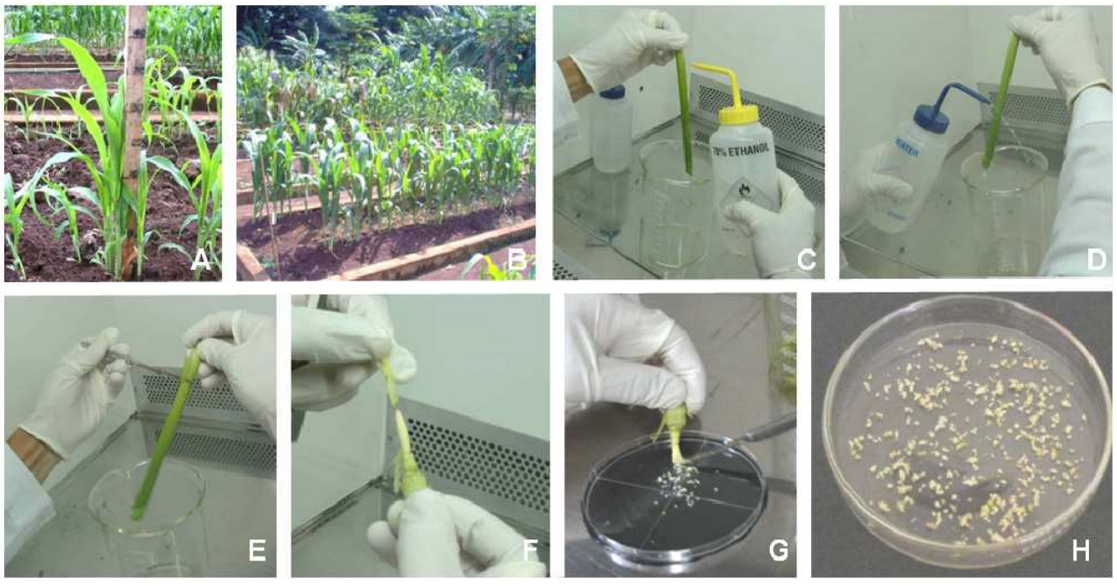

Fig. 1. Harvest and cultivation of immature sorghum inflorescence. (A and B) Sorghum plant used to isolate immature inflorescence; (C) Rinsing of sorghum shoots, without the outermost leaves, with 70% ethanol; (D) Rinsing of shoots with sterile distilled water; (E and F) Dissection of immature inflorescence; (G) Cutting the immature inflorescence in 5mm pieces; (H) Culturing inflorescence pieces.

3.1.2 Plasmid constructs

The genetic cassettes p35S::*C1* and p35S::*Bperu* (Goff *et al.* 1990), used in the transient transformation experiments, were kindly provided by Dr. Vicki Chandler from the Department of Plant Science, University of Arizona, Tucson, Arizona. These plasmids

contain the CaMV35S promoter, directing the expression of *B-peru* (1.9Kb) and *C1* (1.1 Kb) cDNAs, maize *Adh1* intron and nopaline synthase terminator. For the stable transformation experiments, the plasmid pCAMBIA3301 (Cambia, Canberra, Australia) which contains β-glucuronidase (GUS) reporter gene (Jefferson et al., 1987) and the *bar* gene (De Block et al., 1987) that encodes phosphinothricin acetyltransferase (PAT), both driven by the CaMV35S promoter was used.

3.1.3 Particle bombardment

Embryogenic calli were bombarded with tungsten microprojectiles using a biolistic particle helium acceleration device (Biomics – Brasília / Brazil). For the transient experiments 3 μL of each plasmid (stock 1 μg/μL), p35S::*C1* and p35S::*Bperu*, were co-precipitated with tungsten particles, while for the stable transformation 10 μL pCAMBIA3301 plasmid (stock 1 μg/μL) were used. To precipitate DNA onto the microparticles, plasmid DNA were mixed with 50μL (60 mg.mL^{-1}) tungsten particle M10 (Sylvania, GTE Chemicals/ Towanda – USA) under low agitation. Next, 50μL of 2.5M $CaCl_2$ and 20μL of 0.1M spermidine were sequentially added and homogenized. The mixture was kept for three minutes under low agitation and for an additional three minutes without agitation. Particles coated with DNA were centrifuged for five seconds and the supernatant was removed. DNA-coated particles were washed carefully once with 70% ethanol, twice with 100% ethanol, and suspended in 100% ethanol. Eight microliters of DNA-coated particle were deposited at the center of sterile macro-carries membrane (Ficael, São Paulo, SP).

Treatments	Osmotic Media (hours)	Helium Pressure (psi)	Microcarrier Flying Distance (cm)
1	0	1 000	3
2	0	1 000	6
3	0	1 000	9
4	4	1 000	3
5	4	1 000	6
6	4	1 000	9
7	24	1 000	3
8	24	1 000	6
9	24	1 000	9
10	0	1 200	3
11	0	1 200	6
12	0	1 200	9
13	4	1 200	3
14	4	1 200	6
15	4	1 200	9
16	24	1 200	3
17	24	1 200	6
18	24	1 200	9

Table 1. Conditions tested in transient transformation experiments.

Eighteen treatments (Table 1) were designed to test the permanence of explants on osmoticum prior to bombardment (0, 4 and 24 hs), pressure of the accelerating helium pulse (1000 and 1200 psi), and microprojectile flying distance (3, 6 and 9 cm), in transient sorghum transformation. The distance between the high pressure chamber and the macro-carrier membrane (8 mm), the distance between the macro-carrier membrane and the retention screen (17 mm) and the vacuum pressure (27 mmHg) were maintained constant. For each treatment three plates containing 30 calli pieces were bombarded once.

3.1.4 Expression analysis

A*nthocyanin:* For the anthocyanin expression studies, bombarded calli were incubated at 27^{o}C for 2 d in the darkness. The number of anthocyanin spots was scored under a stereoscope (Zeiss Stemi SV11, Germany).

GUS: GUS expression was detected after explants were incubated at 37^{o}C for 20 h in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D glucuronide (X-Gluc, Sigma Chem. Co., São Paulo, Brazil), 50 mM phosphate buffer pH 6.8, 20% methanol, 1% Triton X-100 (Rueb and Hensgens, 1998). Chlorophyll was extracted from leaf tissue in 70% ethanol for 30-60 min.

PAT: Seeds from four independent transgenic T_0 events tested positive by PCR and PAT activity were propagated, and the T_1 plants screened for PAT and GUS enzyme activity inheritance. From each T_0 line 50 seeds were propagated in greenhouse. Seedlings with four leaves were sprayed with 0.6% aqueous solution of the commercial herbicide Finale® (Bayer, São Paulo, Brazil) to confirm expression of the *bar* gene. Control non-transgenic plants or segregating seedlings showed symptoms in 2–5 d and died within 2 weeks. Leaf material from PPT-resistant plants was GUS stained as described above. Chi-square goodness of fit was used to test the significance of observed versus expected ratios.

3.1.5 Selection procedures

Explants were cultured on solid CIM media at 25^{o}C in the dark for one week and transferred to selective SE media (modified CIM supplemented with 0.5 mg.L^{-1} kinetin and without DL-asparagine) containing 15 µL.L^{-1} of the herbicide Finale® (3 mg.L^{-1} 4-hidroxi(methyl) phosphynol-DL-homoalanine ammonium salt) for one week. After that, the explants were transferred to a media containing 30 and 45 µL.L^{-1} herbicide every week. Growing calli were cultured for one more week in a SE media supplemented with 45 µL.L^{-1} herbicide, and subsequently transferred to a callus maturation media RM [MS salts and vitamins (Murashige and Skoog, 1962), 60 g.L^{-1} sucrose, 100 mg. L^{-1} myo-inositol, 0.2 mg.L^{-1} NAA, 3 g/L phytagel, pH 5.8) supplemented with 30 µL.L^{-1} herbicide and cultured in the dark at 25^{o}C for somatic embryo maturation. Approximately 2 to 4 weeks later, mature somatic embryos showing a white and opaque coloration were transferred to Magenta boxes (Sigma, São Paulo, Brazil) containing germination media composed by MS media without plant growth regulators, supplemented with 15 µL.L^{-1} herbicide and placed in a lighted (16 h / 60 µmol m^{-2} s^{-1}) growth room. Germinated plantlets (4-6 cm) were cultured in soil, for the first week under a plastic lid, in a greenhouse.

3.1.6 Plant DNA extraction, polymerase chain reaction (PCR) and Southern blot hybridization analysis

Total genomic DNA was isolated from leaf tissue of primary transformants using a CTAB protocol described by Saghai-Maroof *et al.* (1984). The presence of *bar* and *uid*A genes were detected, initially, by the polymerase chain reaction (PCR). The 407 bp coding region of *bar* gene was amplified using primers (AGA AAC CAC GTC ATG CC and TGC ACC ATC GTC AAC CAC). The 406 bp coding region of *uid*A gene was amplified using the primers (TCG TGC TGC GTT TCG ATG and GCA TCA CGC AGT TCA ACG). Each 25 uL amplification reactions containing 50 ng of template DNA, 5 µM each primer, 500 µM dNTP mixture, 2.5 µl TAQ DNA polymerase reaction buffer and 1 unit Taq DNA polymerase (Invitrogen, São Paulo, Brazil) were carried out using a thermal cycler (Eppendorff Mastercycler, Hamburg, Germany) under the following conditions: 94°C for 5 min; 30 cycles 94°C for 60 s; 55°C for 30s, and a final extension at 72°C for 10 min. The amplified products were separated by electrophoresis on a 1.2% agarose gel and visualized with ethidium bromide.

For Southern blot analysis 10 µg of total genomic DNA from each T_0 plant were completely digested with *Xho*I at 37°C overnight. Digested DNA fragments were separated by electrophoresis in 0.8% agarose gel, and then transferred to a Hybond-N^+ nylon membrane (Amersham, São Paulo, Brazil) according to Sambrook *et al.* (1989). The blot was hybridized with a P^{32} labeled *bar* gene coding region. Negative control samples consisted of non-transgenic genomic DNA. After overnight hybridization at 65°C, the membranes were washed in 2X SSC, 0.1% SDS at room temperature and in 1X SSC, 0.1% SDS at 65°C and exposed to Kodak™ XAR-5 film at 75°C for 3 d.

3.1.7 Statistical data analysis

The data obtained from the transient experiment was collected in Microsoft Excel (Version 5).The experimental design was based on randomized blocks, factorial 2x3x3 (2 pressure of the accelerating helium pulse, 3 time of explants on osmoticum and 3 microprojectile flying distance), with triplicates, totalizing 54 experimental units. The data were subjected to ANOVA and means compared by Tukey test ($p< 0.05$), using the statistical program SISVAR 4.0 (Ferreira, 2000).

4. Results

4.1 Explant preparation and selection of transformed calli *in vitro*

Nine *Sorghum bicolor* accessions were selected and screened for the quality of callus produced four weeks after cultivation of immature inflorescence on callus induction media (CIM). Embryogenic calli were produced at different efficiency levels by all of the accessions (data not shown). For this study, the genotype CMSXS102B, which gave the highest percentage of immature inflorescence sections producing embryogenic callus (85%), was used.

Embryogenic calli from immature inflorescence of sorghum were bombarded three to four weeks after cultivation in CIM media (Fig. 2*B*), with the plasmids containing the *B-peru* and *C1* or *bar* and GUS genes for transient and stable transformation experiments, respectively. Within this cultivation period, explants started to expand and enlarge at the cut edges.

Initially, the bombarded embryogenic calli had a compact appearance and then they developed into more friable structures in the next weeks of cultivation. After bombardment, calli were transferred back to CIM media without selection pressure for one week, and then moved to selection medium.

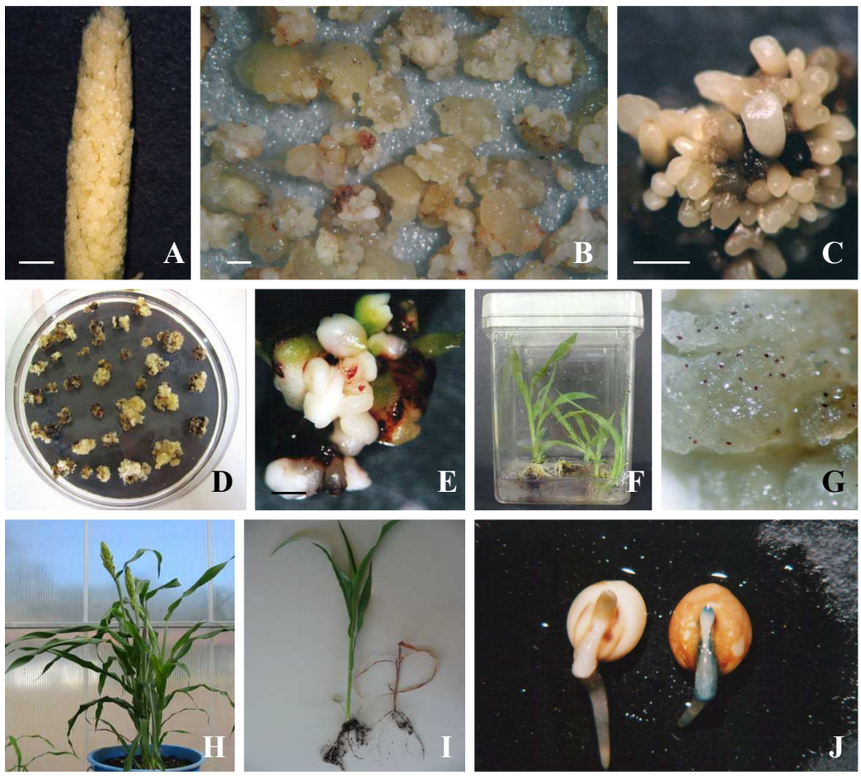

Fig. 2. Genetic transformation of calli from immature sorghum inflorescence. (A) Immature sorghum inflorescence; (B) Calli used for the particle bombardment; (C) Embryogenic callus induced from immature inflorescence; (D) Herbicide resistant calli after 6 weeks particle bombardment; (E) Maturation of calli after selection; (F) Regeneration of herbicide resistant callus, (G) Transient expression of anthocyanin; (H) Putative transgenic *Sorghum* plants in greenhouse; (I) Transformed (left) and wild type (right) sorghum plants weeks after application of herbicide Finale®; (J) GUS expression of wild type (left) and transgenic (right) germinated seeds. *Bar* = 1mm.

Growth of bombarded callus was slightly inhibited and some of them turned brown on selection medium supplemented with 3.0 mg.L^{-1} of PPT, compared to non-bombarded ones. After one week of cultivation the concentration of PPT on selection medium was increased to 6 mg.L^{-1} and most of the bombarded calli turned black. In order to reduce escapes within chimerical clusters, surviving bombarded clumps were carefully divided and cultured, at one week intervals, onto selection medium supplemented with 9 mg.L-1 PPT, during four weeks. At this herbicide concentration complete inhibition of non-transgenic calli growth was observed, most calli turned dark, necrotic and died (Fig. 2*D*). After six weeks of selection, surviving calli were transferred onto maturation media supplemented with 6

mg.L^{-1} PPT. On this medium, as soon as the yellowish calli become white and opaque, between 2 to 4 weeks of cultivation, they were transferred onto germination medium (Figure 2E). A concentration of 3 mg.L^{-1} PPT was used for the differentiation and germination of mature calli that occurs around 20 d of cultivation. Control non-bombarded explants did not survive on selection medium containing 6 or 9 mg.L^{-1} PPT. This selection procedure has been used successfully in different transformation experiments with calli derived from immature inflorescence of sorghum. The overall time for selection and regeneration of putative transgenic plants using this protocol is around 16 weeks.

4.2 Transient and stable transformation

The optimization of DNA delivery parameters was initially performed by using the transient expression of maize *R* and *C1* transcriptional activators. All bombardments were carried out with plasmidial DNA from the same stock, and the number of anthocyanin spots ranged from 3 to 348 depending on the particle bombardment conditions used (Fig. 2G).

Statistical analyses of the transient anthocyanin expression (Table 2) identified interactions among the different factors studied. Embryogenic calli submitted to 1000 psi of helium accelerating pressure, cultivated during 4 h in a higher osmotic medium and positioned at 3 cm from the micro-carrier launch platform (Treatment 4) presented a larger number of cells expressing anthocyanin than when the explants were positioned at 6 or 9 cm (Treatment 5 and 6). Without the pre-cultivation of explants in an osmotic medium, there were no differences among the positions (3, 6 or 9 cm) of explants (Treatments 1 to 3) and the overall amount of anthocyanin spots was lower. At 1200 psi acceleration pressure, treatments 10 to 18, the best results obtained were when calli were cultivated in osmotic medium for 24 h and positioned at 3 or 6 cm from the micro-carrier launch platform (Treatments 16 and 17).

Time in osmotic medium	**TARGET DISTANCE** **Pressure 1000 psi**		
	3 cm	6 cm	9 cm
Without pre-treatment	(1) 30,283 aA	(2) 47,246 aA	(3) 37,013 aA
4 hours	(4) 91,356 bB	(5) 52,786 aAB	(6) 31,170 aA
24 hours	(7) 74,930 aA	(8) 79,240 aA	(9) 42,813 aA
Time in osmotic medium		**Pressure 1200 psi**	
Without pre-treatment	(10) 43,183 aA	(11) 41,686 aA	(12) 44,626 aA
4 hours	(13) 44,736 aA	(14) 74, 263 aA	(15) 44,370 aA
24 hours	(16) 88,576 bB	(17) 59,086 aB	(18) 18,343 aA

Note. Means followed by the same small letter in the vertical and capital letter in the horizontal are not significantly different at 5% level according to Tukey's multiple range test. Numbers in parentheses represent the different treatments.

Table 2. Mean number of anthocyanin spots induced transiently by p35S::*C1* and p35S::*Bperu* constructs in embryogenic callus of the sorghum.

The highest number of cells expressing the anthocyanin genes was obtained with embryogenic calli cultivated in osmotic medium during 4 h before the bombardment, positioned at 3 cm distant from the microcarrier release platform and using 1000 psi

accelerating pressure. Therefore, these conditions were used in six independent experiments to test stable transformation of embryogenic calli obtained from immature sorghum inflorescences, with a cassette containing the *bar* and *iud*A genes. The transformation efficiency for these sets of experiments ranged from 1.01% to 3.33 % (Table 3).

Experiment number	Number of transgenic events	Number of calli bombarded	% Efficiency
RLB3962008	1	93	1.07
RLB5420703	3	120	2.5
RLB5430703	2	60	3.33
RLB5222202	2	180	1.11
RLB020106	3	296	1.01
RLB120705	2	150	1.33

Table 3. Biolistic transformation of calli from immature sorghum inflorescence

4.3 Evaluation of transgenic material

To estimate the transgene copy number and the inheritance of the *bar* gene, T_1 progenies were tested for their tolerance to the herbicide Finale ®. Germinated T_1 transgenic and control seedlings at the stage of five leaves were sprayed with herbicide and scored for damage 7 and 14 d after the application. Transgenic seedlings segregated for the presence of the *bar* gene, there were plants with and without tissue damage, while all control non-transgenic plants presented necrosis and died (Fig. 2I).

Transgenic Lines Number	Herbicide-resistant plants	Herbicide-sensitive plants	Segregation ratio	Chi-square
1 (RLB3962008)	28	16	3:1	$\chi^2 = 3,03$; P>0,05
2 (RLB5222202)	07	01	3:1	$\chi^2 = 0,67$; P>0,05
3 (RLB5430703)	34	12	3:1	$\chi^2 = 0,029$; P>0,05
4 (RLB5420703)	37	8	3:1	$\chi^2 = 1,25$; P>0,05
5 (RLB2109056A)	23	25	1:1	$\chi^2 = 0,83$; P>0,05
6 (RLB2109053B)	28	20	1:1	$\chi^2 = 1,33$; P>0,05
Wild-type plant (CMSXS102B)	0	50	ND[a]	ND[a]

[a] – ND, Not determined

Table 4. Independent transgenic T_1 generation plants analyzed for the inheritance and segregation of herbicide resistance

Segregation data obtained from six T_1 progenies sprayed with herbicide is presented in Table 4. Among the progenies of self -pollinated T_0 transgenic plant lines, Chi-square tests showed a Mendelian segregation ratio of 3:1 in four lines. This ratio indicated that the *bar* gene was inserted in a single locus, efficiently inherited and transcribed in T_1 progeny plants. Two lines (Lines 5 and 6) showed a 1:1 ratio, suggesting semi-dominance.

Leaves of all T_0 transgenic events tested negative for the β-glucuronidase expression. However, GUS expression could be detected in germinated seeds (T_1) of event RLB5420703 (Fig. 2J).

The presence of *uid*A and *bar* genes in genomic DNA of all independent T_0 lines was confirmed by PCR analysis of genomic DNA. The results revealed the presence of 406 bp band of *uid*A and 407bp band of *bar* genes in all of the plants tested (Fig. 3A and B).

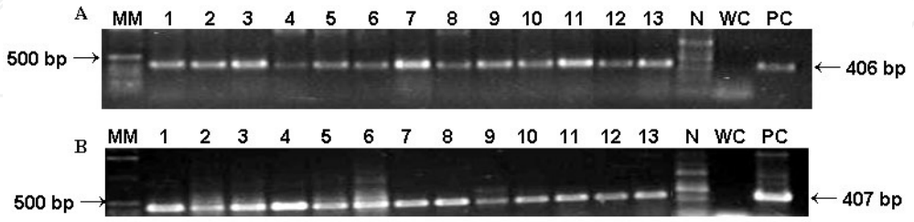

Fig. 3. PCR analysis of genomic DNA extracted from transgenic primary generation (T_0) of *Sorghum bicolor*. (A) PCR amplification of a *uid*A gene, showing the 406 bp fragment (lanes 1-13); (B) PCR amplification of *bar* gene, showing a 407 bp fragment (lanes 1-13). M: molecular weight marker, N: control wild type sorghum plants; WC: water control; PC: plasmid positive control.

The stable integration of the *bar* gene in the transgenic events were analyzed by Southern blotting of genomic DNA digested with *Xho*I, which cuts outside the *bar* coding sequence releasing a 564 bp fragment. No hybridization signal was present in the digested DNA from the untransformed plants (Fig. 4).

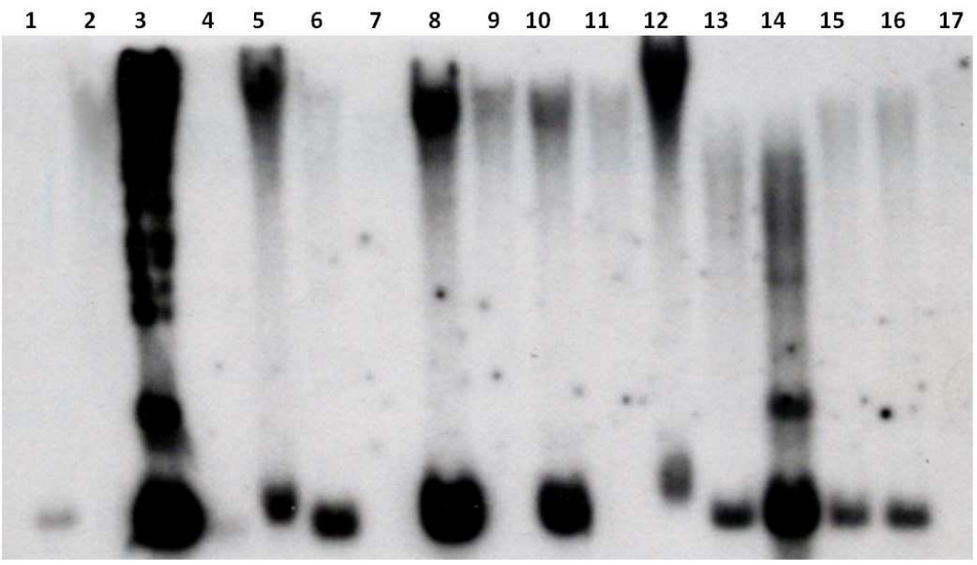

Fig. 4. Southern blot analysis of genomic DNA from transgenic *Sorghum bicolor* plants (cultivar CMSXS 102B). Genomic DNA was digested with *xho*I and hybridized with a radiolabelled 520 pb *bar* fragment. Lane 1: plasmid positive control; lanes 2-16: transformed plants ; lane 17: wild type *S. bicolor* total DNA.

5. Discussion

Immature inflorescence proved to be an excellent organ to increase considerably the quantity of tissue competent of embryogenic callus production. A large number of high quality calli is relatively easier and faster to produce from immature inflorescence.

Transient expression of anthocyanin allows us to detect in a rapid and precise manner the most efficient combination of biolistics parameters that rendered a higher transient expression. The frequency of transient activity expression as an indicator of stable transformation efficiency has already been used, successfully, by Christiansen *et al.* (2005) to optimize the transformation conditions of *Brachypodium distachyon*. They observed that treatments with a higher number of GUS spots where the ones that produced a larger number of stable transgenic events.

An important step in the transformation via biolistics is the wound suffered by the explant during the microparticle entry into the cell. Usually to minimize this type of problem and to increase the capacity for somatic embryogenesis and plant regeneration, the target cells are plasmolised by an osmotic treatment (Vain *et al.* 1993). In this study all treatments where sorghum explants were incubated in an osmotic media a few hours before bombardment produced a higher number of anthocyanin spots, confirming that plasmolysis of cells can reduce damage and increase the efficiency of bombardments.

Acceleration pressure and microcarrier flying distance are parameters that influence the ability to deliver DNA into various explants. Analyzing the transient expression of anthocyanin, it was observed that a helium gas pressure of 1000 psi combined with a distance of 3 cm rendered the higher number of anthocyanin spots. This combination of biolistic physical parameters when tested in stable transformation experiments showed an efficiency of up to 3.33% of transgenic sorghum events production. Even though, biolistic parameters should be optimized for each equipment and explant used, other authors found optimal bombardment conditions similar to our results. Casas *et al.* (1993) and Tadesse *et al.* (2003) were able to generate transgenic sorghum plants via biolistic using a macro-carrier flying distance of 6 cm and a pressure of 1100 psi with an efficiency ranging from 0,3% to 1,3%.

We introduced the *uidA* and *bar* genes under the control of the CaMV35S promoter into sorghum CMSXS102B genome. The transformed plants were determined by a combination of PCR and Southern blot analysis, together with assays demonstrating functional gene product activity, PAT and GUS. Histochemical GUS activity was absent in leaves of the T_0 plants, but could be detected in the T_1 germinating seeds of one of the events investigated in this study. The absence of GUS expression in transgenic sorghum has been reported by several investigators (Emani *et al.*, 2002; Carvalho *et al.*, 2004; Girijashankar *et al.*, 2005; Van Nguyen *et al.*, 2007). Factors such as methylation based silencing (Emani *et al.*, 2002), regulatory sequences present in the genetic cassette (Tadesse *et al.*, 2003; Carvalho *et al.*, 2004) and phenolic compounds typically present in the sorghum tissue culture (Carvalho *et al.*, 2004) might have contributed for the absence of GUS expression.

The analysis of PPT-resistance showed that the trait was expressed by all of the transgenic events recovered, probably because of the herbicide selection pressure. In addition, it was inherited by the T_1 progenies with a typical Mendelian segregation pattern in four out of six transgenic lines studied. Two lines showed a 1:1 segregation ratio; this type of transgene segregation had already been reported in wheat and maize (Cheng *et al.*, 1997; Ishida *et al.*

1996). This abnormal segregation pattern might be partially caused by gene silence or non-detectable gene expression in the transgenic plants (Cheng *et al*., 1997; Vaucheret *et al*., 1998).

We report a transformation methodology for calli derived from immature inflorescence of sorghum, via biolistics; these transformation conditions are already being used at Embrapa Maize and Sorghum to introduce genes of agronomical interest into the sorghum genome.

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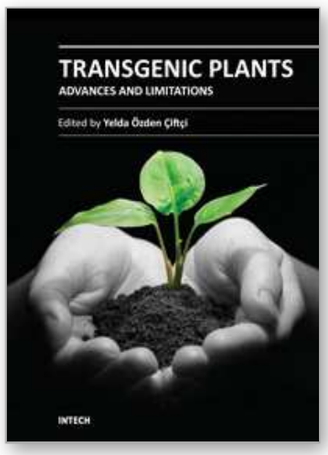

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Development of efficient transformation protocols is becoming a complementary strategy to conventional breeding techniques for the improvement of crops. Thus, Transgenic Plants - Advances and Limitations covers the recent advances carried on improvement of transformation methods together with assessment of the impact of genetically transformed crops on biosafety. Each chapter has been written by one or more experienced researchers in the field and then carefully edited to ensure throughness and consistency.

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