Plant Cell Rep (2003) 22:201–209
DOI 10.1007/s00299-003-0666-x

GENETIC TRANSFORMATION AND HYBRIDIZATION

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Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene

Received: 12 December 2002 / Revised: 20 May 2003 / Accepted: 21 May 2003 / Published online: 9 July 2003

Abstract *Agrobacterium tumefaciens*-mediated genetic transformation and the regeneration of transgenic plants was achieved in *Hevea brasiliensis*. Immature anther-derived calli were used to develop transgenic plants. These calli were co-cultured with *A. tumefaciens* harboring a plasmid vector containing the *H. brasiliensis* superoxide dismutase gene (*HbSOD)* under the control of the CaMV 35S promoter. The β-glucuronidase gene (*uidA*) was used for screening and the neomycin phosphotransferase gene (*nptII*) was used for selection of the transformed calli. Factors such as co-cultivation time, co-cultivation media and kanamycin concentration were assessed to establish optimal conditions for the selection of transformed callus lines. Transformed calli surviving on medium containing 300 mg l^{-1} kanamycin showed a strong GUS-positive reaction. Somatic embryos were then regenerated from these transgenic calli on MS2 medium containing 2.0 mg l^{-1} spermine and 0.1 mg l^{-1} abscisic acid. Mature embryos were germinated and developed into plantlets on MS4 medium supplemented with 0.2 mg l^{-1} gibberellic acid, 0.2 mg l^{-1} kinetin (KIN) and 0.1 mg l^{-1} indole-3-acetic acid. A transformation frequency of 4% was achieved. The morphology of the transgenic plants was similar to that of untransformed plants. Histochemical GUS assay revealed the expression of the *uidA* gene in embryos as well as leaves of transgenic plants. The presence of the *uidA, nptII* and *HbSOD* genes in the *Hevea* genome was confirmed by polymerase chain reaction amplification and genomic Southern blot hybridization analyses.

Keywords *Agrobacterium tumefaciens* · Genetic transformation · *Hevea brasiliensis* · Somatic embryogenesis · Transgenic plants

Communicated by L. Peña

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Introduction

Hevea brasiliensis is one of the most important sources of natural rubber. The rubber tree *(Hevea brasiliensis* Muell. Arg.) is a heterozygous, woody perennial with a long juvenile period of about 6 years; consequently, genetic improvement has been slow because of the number of years required for a full evaluation of new genotypes. As it is a perennial tree crop with a long breeding cycle, the integration of specific desired characters through conventional breeding is both time-consuming and labor-intensive. Genetic engineering is certainly a more promising method for crop improvement when specific genetic changes need to be made in a short time period without loss of genetic integrity. Genetic transformation, however, requires the insertion of foreign DNA into the plant genome, its expression in the transformed cells and an efficient protocol by which to regenerate plants. Although the transfer of DNA into plant cells via *Agrobacterium* and other methods is now routine for many plant species, the coupling of transformation with the selection of transformed cells and the regeneration of transgenic plants is still difficult in many economically important tree species, including *Hevea.*

Tapping panel dryness (also known as brown bast syndrome) is considered to be a serious physiological disorder caused by oxidative stress in rubber trees that are frequently tapped for the extraction of natural rubber in the form of latex. Lutoids (latex-producing tissues) from dry trees exhibit abnormally high levels of NAD(P)H oxidase activity, which leads to the formation of reactive oxygen species like superoxide (toxic forms of oxygen) that damage lutoid membranes, thereby compromising their integrity and resulting in the cessation of latex production (Chrestin 1989). Superoxide dismutase (SOD), which dismutates two superoxide radicals to produce H_2O_2 and O_2, are metallo enzymes that have been shown to provide a defense against oxidative stress in plants. SOD is present in latex and assists in the maintenance of lutoid membrane integrity (Chrestin 1989). An enhancement of the plant's tolerance to oxidative stress would improve its ability to survive combinations of stresses like extreme temperature, drought, high light intensities, ambient ozone, sulfur dioxide, pathogens, etc. (Bowler et al. 1992). A number of cDNA gene constructs have been introduced into alfalfa to alter SOD expression, and detailed analyses were able to identify the transgenic plants displaying altered levels of SOD activity, enhanced stress tolerance and improved biomass production in field trials (Mckersie et al. 1999, 2000). The general conclusion drawn from these experiments was that the expression of an additional SOD isozyme is related to improved stress tolerance and plant vigor (Samis et al. 2002). In *Hevea,* an increase in the level of the scavenging activity of such toxic oxygen free radicle species by the incorporation of a SOD transgene under the control of the constitutive CaMV 35S promoter could provide better protection in plant cells against lutoid damage.

The genetic manipulation of *Hevea* species using *Agrobacterium* has been attempted in the recent past. Tumors were induced on stems of in vitro- and in vivo-propagated rubber seedlings infected with a strain of tumor-forming *Agrobacterium tumefaciens*, and transgenic plants were obtained (Arokiaraj and Rahaman 1991; Arokiaraj et al. 1996, 1998). Transgenic plants have also been produced using particle bombardment (Arokiaraj et al. 1994), however, the transformation efficiency was generally low. Montoro et al. (2000) studied the response of exogenous calcium on *Agrobacterium*-mediated gene transfer in *Hevea* friable calli but did not obtain transgenic plants.

The objective of the investigation reported here was to transform a constitutive version of the superoxide dismutase gene, *HbSOD*, into *Hevea brasiliensis* via *Agrobacterium*-mediated genetic transformation, consequently developing transgenic plants. Histochemical and molecular evidence is presented that confirms the stable integration of the *uidA* [codes β-glucuronidase, GUS], *nptII* (neomycin phosphotransferase II) and *HbSOD* genes in regenerated *Hevea* plants.

Materials and methods

Plant material

Two-month-old immature anther calli of *Hevea brasiliensis* (a popular Indian clone RRII 105) were used in the transformation experiments. *Hevea* callus was initiated from immature anthers of clone RRII 105 in a modified MS (Murashige and Skoog 1962) medium (MS1) in which the NH_4NO_3 concentration was lowered (1.0 g l^{-1}) and 5% (w/v) sucrose, 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg l^{-1} KIN were added (Table 1).

Plasmid vector construction and bacterial growth

The plasmid vector used was pDU96.2144, which contains *uidA* as a reporter gene and *nptII* as a selectable marker gene plus the *HbSOD* gene under the control of a CaMV 35S promoter. The 702-nucleotide *HbSOD* cDNA was obtained by reverse transcription (RT)-polymerase chain reaction (PCR) from mRNA isolated from stem tissue of *H. brasiliensis* using primer sequences corresponding to a previously published sequence of *HbSOD* (Miao and Gaynor 1993). The RT-PCR products were cloned and confirmed by DNA sequence analysis. The *HbSOD* coding sequence was inserted into the binary vector pDU92.3103 (Tao et al. 1995) at the unique *Bam*HI site between the CaMV 35S promoter and 3′ polyadenylation sequences, thereby creating binary vector pDU96.2144 (Fig. 1). Binary vector pDU96.2144 was subsequently inserted into disarmed *Agrobacterium* strain EHA101 to create a functional vector for the transformation experiments. The EHA101 strain carrying the plasmid vector pDU96.2144 was grown in AELB

Table 1 Composition of the media[a] used for efficient callus induction, embryo formation, maturation and germination in *Hevea brasiliensis*

Name of Constituents	Concentration (in milligrams per liter unless indicated otherwise)			
	MS1	MS2	MS3	MS4
NH_4NO_3	1,000	500	500	500
KNO_3	1,600	800	800	800
MgSO4 (anhy)	180	90	90	90
$CaCl_2$ (anhy)	333	166	166	166
KH_2PO_4	170	85	85	85
Minor	MS	MS	MS	MS
Vitamins	B5	B5	B5	B5
FeNaEDTA	37.5	55.0	55.0	37.5
Myo inositol	100	100	100	100
Glutamic acid	–	150	150	150
Adenine hemi-sulphate	–	50	50	–
Casein Hydrolysate	–	200	300	400
Malt extract	–	150	100	50
Banana powder	–	150	150	200
Coconut water	5%	10%	10%	5%
Spermine	–	2.0	2.0	–
Sucrose	50 g/l	50 g/l	50 g/l	30 g/l
Phytagel	0.25%	0.4%	0.4%	0.2%
Abscissic acid	–	0.1	0.1	–
2,4-Dichlorophenoxyacetic acid	2.0	–	–	–
Benzyladenine	–	0.2	0.3	–
Kinetin	0.5	–	–	0.2
Gibberellic acid	–	0.5	0.3	0.2
Indole-3-acetic acid	–	–	0.1	–
pH	5.7	5.7	5.7	5.7

[a] MS1, Callus induction and proliferation medium; MS2, embryo induction medium; MS3, embryo maturation medium; MS4, germination medium

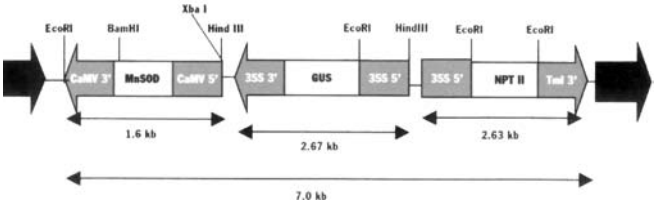

Fig. 1 Schematic representation of the T-DNA from pDU96.2144 showing the restriction sites of the superoxide dismutase transgene [*Hb(Mn)SOD*] and the *uidA* and *nptII* marker transgenes

medium (Dandekar et al. 1989) overnight at 28°C in the presence of 50 mg l^{-1} kanamycin and 20 mg l^{-1} gentamycin until an A_{600} of 0.5. The bacterial cell density was adjusted to 5×10^8 cells ml^{-1} in this medium and used for the transformation experiments.

Co-cultivation and selection of transformed callus lines

Approximately 2 g of calli were pre-cultured on callus proliferation medium (MS1) prior to infection with *Agrobacterium* (Table 1). These calli were immersed for 10 min in a culture of *Agrobacterium* cells grown overnight in liquid AELB medium. After infection, the calli were blotted dry on sterile filter paper and plated on co-cultivation medium (MS1 + acetosyringone, betaine HCl and proline). The effect of co-cultivation period on transformation frequency was evaluated by culturing *Agrobacterium*-infected calli for 1, 2, 3, 4 and 5 days in the co-cultivation medium. The effect of varying levels of acetosyringone (10, 15, 20, 25 m*M*), betaine HCl (10, 15, 20, 25 m*M*) and proline (9.5, 11.5, 13.5, 15.5 m*M*) on transformation frequency was also evaluated.

After co-cultivation, the callus lines were sub-cultured on selection medium, MS1 + 0.5 mg l^{-1} cefotaxime + different concentrations of kanamycin sulphate (0, 50, 100, 150, 200, 250, 300 and 350 mg l^{-1}) for 2 months (with two subcultures per month). The cultures were maintained at 25±2°C in the dark. Each treatment contained 25 callus lines and was repeated twice. After four subcultures, cefotaxime was omitted from the selection medium, and the screening for putatively transformed callus lines in the presence of kanamycin was continued. After 8 weeks of subculture, the kanamycin-resistant callus lines were subjected to GUS assay (repeated three times with ten samples/repetition). Transformation frequency was calculated using the formula

$$\text{Transformationfrequency} = \frac{\text{Totalno.ofGUSpositivecalluslines}}{\text{Totalno.ofcalluslinescultured/plate}} \times 100$$

The transformed callus lines were sub-cultured into fresh medium for embryo induction.

Factors influencing somatic embryogenesis and plant regeneration

The effects of abscisic acid (ABA), Phytagel, polyamines and organic supplements on somatic embryogenesis and plant regeneration were studied, and the optimal concentrations were standardized. Putatively transformed callus lines growing on selection medium were transferred onto embryo induction medium (MS2) (Table 1) supplemented with different concentrations of ABA (0.05, 0.1, 0.2 and 0.3 mg l^{-1}) and Phytagel (0.2%, 0.3%, 0.4% and 0.5%, w/v) and sub-cultured to fresh medium with the same formulation at 2-week intervals. Data on embryo induction was recorded after 4 weeks of culture. For studying the effect of polyamines on embryo induction, the transformed callus was plated on MS2 medium containing each of the three polyamines (putrescine, spermine and spermidine) at five concentrations (1, 2, 3, 4 and 5 mg l^{-1}) for 4 weeks. Transformed embryo formation frequency was calculated using the formula

$$\text{Transformedembryo formationfrequency} = \frac{\text{Totalno.oftransformedembryos}}{\text{Totalno.transformedcalluslinescultured}} \times 100$$

Putatively transformed embryogenic callus lines were also cultured on MS2 medium containing various concentrations of four organic supplements: casein hydrolysate (100, 200, 300 and 400 mg l^{-1}), malt extract (50, 100, 150 and 200 mg l^{-1}), coconut water (5, 10, 15 and 20%) and banana powder (100, 150, 200 and 250 mg l^{-1}) for 4 weeks. Subculture was performed at 2-week intervals into fresh medium of the same formulation. Embryos obtained were transferred to MS3 (Table 1) medium supplemented with different levels of organic supplements for maturation. Each treatment contained 20 callus lines and was repeated five times. The cultures were maintained at 25±2°C under a 16/8-h (light/dark) photoperiod. Mature bipolar embryos containing two cotyledons were placed on MS4 medium (Table 1) supplemented with growth regulators and different levels of organic supplements, as mentioned above. This experiment was repeated five times with 20 embryos each. Plantlets formed in the MS4 medium were transferred to sterile 1/2-strength MS liquid medium for 2 weeks. They were then transferred to small polybags containing autoclaved soil rite and kept under controlled conditions. For hardening, the humidity was gradually decreased, and the acclimatized plants were transferred to large polybags. Data on embryo induction, maturation and germination were recorded.

Histochemical analysis of uidA gene expression

The GUS histochemical assay was conducted according to Jefferson et al. (1987). Putatively transformed callus lines, embryos and shoots regenerating from the kanamycin-resistant callus clumps were used for GUS assays. For staining, the materials were incubated overnight at 37°C in 2 m*M* X-Gluc (5-bromo-4 chloro-3-indolyl β-D glucuronide) in a pH 7.0 phosphate buffer containing 10 m*M* EDTA, 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide and 0.1% (v/v) Triton X-100 and then viewed under a binocular microscope. In the case of shoots, chlorophyll was bleached by soaking the plant materials in 95% (v/v) ethanol after X-Gluc staining.

Amplification of uidA, nptII and HbSOD genes by PCR

GUS-positive plantlets were screened for the presence of the *uidA, nptII* and *HbSOD* genes by PCR using specific primers. DNA was extracted from leaves according to Dellaporta et al. (1983). For detecting the presence of the *uidA* gene, we used the forward primer 5′-TAG AGA TAA CCT TCA CCC GG-3′ and the reverse primer 5′-CGC GAA AAC TGT GGA ATT GA-3′, corresponding to the *uidA*-coding region. For detecting the *nptII* gene, we used the forward primer 5′-GAG GCT ATT CGG CTA TGA CT-3′ and the reverse primer 5′-AAT CTC GTG ATG GCA GGT TG-3′, specific for the *nptII* coding region. Specific DNA primers—forward (5′-ATG GCT CTG CGA CT CTA GTG ACC C-3′) and reverse (5′-CTA AGA AGA AGG GCA TTC TTT GGC AT-3′)—were also used to amplify the *HbSOD* transgene. Plasmid DNA was used as a positive control, whereas untransformed plant DNA was used as a negative control. Reactions were carried out using 50 ng of DNA, 100 m*M* of each dATP, dTTP, dGTP, dCTP, 250 n*M* of each primer, 0.5 U *Taq* DNA polymerase and 1.5 m*M* $MgCl_2$ in a final volume of 20 μl. The reaction mixture for PCR was incubated in a thermal cycler (Perkin Elmer 480, Foster City, Calif.), and the PCR conditions for both the *uidA* and *nptII* amplifications were 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with a final extension step of 72°C for 7 min. The PCR conditions for amplifying DNA from the *HbSOD* gene were: an initial denaturation of DNA at 94°C for 4 min, then 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, followed by a final extension step of 72°C for 7 min. The amplified PCR products were visualized on a 1.2% agarose gel stained with ethidium

bromide using TBE (0.5×) as a running buffer (Sambrook et al. 1989).

Southern blot hybridization analysis

To detect the integration of the *uidA* and *nptII* genes, *Hevea* genomic DNA was digested with *Hin*dIII, *Bam*HI and *Xba*I, separated by agarose gel electrophoresis (10 μg DNA per lane; 1% agarose) and transferred onto nylon membranes (Hybond N+, Amersham-Pharmacia, UK) by capillary blotting. For *HbSOD* gene detection, total genomic DNA was isolated from independent transgenic plants along with a control. Then 10-μg DNA samples were digested with *Hin*dIII and *Bam*HI or with *Hin*dIII, separated and blotted in the same way. Radioactively labeled *uidA, nptII* and *HbSOD* gene probes (internal fragments of *uidA, nptII* and *HbSOD* amplified by PCR) were synthesized with α-[P]^{32}dATP (BARC, Mumbai, India, 14.8×10(13) Becq) using a random primer labeling kit (Amersham-Pharmacia) and separated for unincorporated nucleotides as recommended by the manufacturer. Radiolabeled *uidA, nptII* and *HbSOD* gene probes were added to the blots, and hybridization was carried out in 6× SSC, 5× Denhardt's, 0.5% sodium dodecyl sulfate at 65°C in a rotary hybridization oven (Amersham-Pharmacia) for 16 h. Following hybridization, the filters were washed twice at low stringency at room temperature (2× SSC + 0.1% SDS for 10 min and 1× SSC + 0.1% SDS for 15 min) and twice at high stringency at 65°C (0.5× SSC + 0.1% SDS for 30 min and 0.1× SSC + 0.1% SDS for 30 min), followed by signal detection. The labeled blots were then exposed to X-ray film (X-Omat, Kodak) with intensifying screens at −80°C (Sambrook et al. 1989).

Results and discussion

Factors affecting transformation frequency

The different parameters affecting the transformation frequency of *H. brasiliensis*, such as the effect of acetosyringone, proline and betaine HCl in the co-cultivation media and the duration of co-cultivation, were evaluated. The influence of the length of the co-cultivation period on the frequency of transformed callus lines is illustrated in the Fig. 2. No transformation was observed when the callus was transferred to selection medium immediately after infection with *Agrobacterium* (without co-cultivation). The highest transformation frequency (4%) was observed following a 3-day co-cultivation. Prolonging the co-cultivation period beyond 3 days resulted in a profuse overgrowth of the bacteria, which eventually suppressed the growth of the callus. The influence of the duration of the co-cultivation period on transformation frequency has been reported in many crops. In pear and citrus, a prolonged co-cultivation period of more than 3 days was used to develop transgenic plants (Mourgues et al. 1996; Cervera et al. 1998). In the present study, a 3-day co-cultivation period was found to be optimum for maximum transformation frequency. Similar results were also reported earlier in other species (Hu et al. 2002; Khanna et al. 2003).

The effect of kanamycin concentrations (0–350 mg l^{-1}) was evaluated with respect to obtaining the maximum percentage of transformed callus (Fig. 3). Callus lines growing on kanamycin medium were used for GUS assay

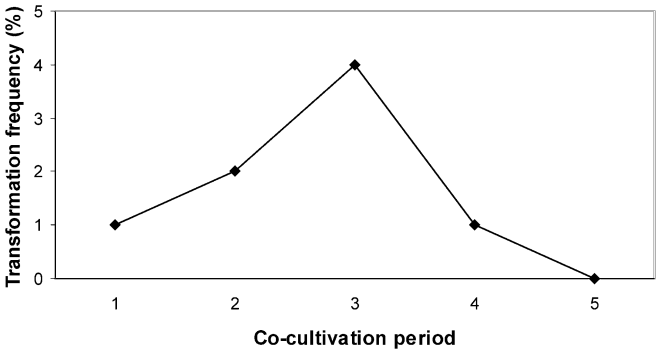

Fig. 2 Influence of co-cultivation period (days) on transformation frequency

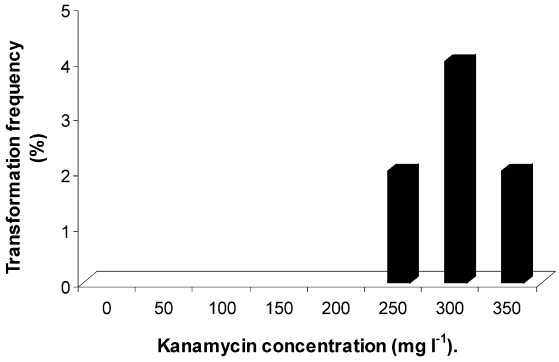

Fig. 3 Effect of kanamycin concentration on transformation frequency

after 8 weeks of culture. Transformed cell lines were obtained in medium containing 250–350 mg l^{-1} kanamycin, with the maximum transformation frequency (4%) observed at 300 mg l^{-1} kanamycin. When the kanamycin concentration in the medium was increased beyond 350 mg l^{-1}, a decrease in transformation frequency was observed. The occurrence of escapes is a major problem in perennial tree crops such as citrus, (Moore et al. 1992; Pena et al. 1995; Gutierrez et al. 1997; Cervera et al. 1998), which suggests to some researchers that growth on media supplemented with only 100 mg l^{-1} kanamycin may not be a reliable indicator of transformation. In contrast, De Bondt et al. (1994) reported that 2% of the leaf explants developed transgenic shoots using 50 mg l^{-1} kanamycin as the selective antibiotic in apple. However, in our experiment, profuse growth of non-transgenic calli was observed from 0–200 mg l^{-1} kanamycin, which indicated that kanamycin concentrations up to 200 mg $^{-1}$ were ineffective for selecting transformed cell lines. The growth of non-transgenic calli was completely arrested at 300 mg l^{-1} kanamycin, and at this concentration only transgenic calli were proliferated. Callus growth was markedly reduced at 350 mg l^{-1} kanamycin. When the kanamycin concentration was 400 mg l^{-1} or higher, callus normally swelled, partially turned brown and failed to proliferate further.

In this experiment, certain components of the co-cultivation medium also enhanced the frequency of transformation. Among the different concentrations of acetosyringone, betaine HCl and proline used in the co-cultivation media, the addition of 20 m*M* acetosyringone, 15 m*M* betaine HCl and 11.55 m*M* proline increased the transformation frequency (data not shown). The beneficial role of acetosyringone has been demonstrated in the genetic transformation of some woody fruit species, such as apple (James et al. 1993), kiwifruit (Janssen and Gardner 1993) and citrus (Cervera et al. 1998). James et al. (1992) studied the influence of acetosyringone and betaine HCl on the efficiency of gene transfer to apple explants and found that the addition of these compounds significantly enhanced transformation frequency.

Factors influencing somatic embryogenesis and plant regeneration

The influence of ABA, Phytagel concentration, organic supplements and polyamines on somatic embryogenesis and plant regeneration was assessed. The capacity of *Agrobacterium*-infected callus to produce embryos was tested in medium containing 0.05, 0.1, 0.2 or 0.3 mg l^{-1} ABA and solidified with different concentrations of Phytagel (0.2%, 0.3%, 0.4% or 0.5%). As shown in Table 2, the medium containing a combination of 0.1 mg l^{-1} ABA and 0.4% Phytagel produced the highest frequency of transformed embryos (54%). Among the different ABA concentrations tested, the maximum frequency of transformed embryo formation was obtained with 0.1 mg l^{-1} followed by 0.20 mg l^{-1}, 0.30 mg l^{-1} and 0.05 mg l^{-1}. Cailloux et al. (1996) reported that a higher concentration of ABA (2.64 mg l^{-1}) promoted long-term somatic embryogenesis and the maturation of somatic embryos in *H. brasiliensis*. In contrast to this observation, in our experiments, when the ABA concentration was increased beyond 0.2 mg l^{-1}, the frequency of embryogenesis sharply declined.

The effect of the three polyamines (putrescine, spermine and spermidine) on embryogenesis was compared, and results are shown in Table 3. Among the three polyamines used, the maximum percentage of embryogenesis (58%) was observed with 2 mg l^{-1} spermine, followed by 1 mg l^{-1} spermidine. Of the five concentrations of spermidine used, the highest percentage of somatic embryogenesis (40%) was noticed with 1 mg l^{-1}. The percentage of embryogenesis decreased with higher concentrations of both these polyamines (spermine and spermidine). Putrescine did not significantly influence somatic embryogenesis. Earlier work with embryogenic callus of *Panax ginseng* had shown that the exogenous application of polyamines in the initiation phase increased embryo production (Kevers et al. 2000).

The efficacy of four organic supplements on embryogenesis was also evaluated. Among the organic supplements, casein hydrolysate was found to be the best for embryo induction (69.4%), while the highest frequency of embryo maturation (48%) was observed with 150 mg l^{-1} banana powder. Casein hydrolysate (400 mg l^{-1}) and malt extract (50 mg l^{-1}) were found to be equally effective for embryo germination (7%). A germination percentage of 7% was also obtained when the medium was supplemented with 200 mg l^{-1} banana powder (Table 4).

The above-mentioned experimental results indicate that a suitable transformation protocol for *Hevea* consist of a 3-day co-cultivation with *Agrobacterium* in the presence of 20 m*M* acetosyringone, 15 m*M* betaine HCl and 11.55 m*M* proline followed by selection on medium containing 300 mg l^{-1} kanamycin. Media supplemented with ABA, polyamines and organic supplements and with the optimal agar concentration favored embryogenesis and the regeneration of transgenic plants. A 4% transformation frequency was observed on media containing 300 mg l^{-1} kanamycin. Upon subsequent subculture into fresh media, we obtained 69.4% embryo induction, 48% embryo maturation and 7% embryo germination. The germinated plantlets were subjected to GUS expression assays, and all were found to be GUS-positive. Putative transgenic plants were successfully transferred to the glasshouse in polybags and kept for further evaluation (Fig. 4).

PCR analysis of putatively transformed tissue

Three transgenic plantlets that gave a positive staining in the histochemical GUS assay were selected for PCR

Table 2 Effect of different concentrations of abscissic acid and Phytagel on the induction[a] of somatic embryos from putative transgenic callus of *H. brasiliensis*. For each treatment, 20 replicates were used. The experiment was repeated five times

Phytagel (%)	Abscissic acid (mg l^{-1})			
	0.05	0.1	0.2	0.3
0.2	0	0	0	0
0.3	0	21.8±1.92	20.4±2.07	0
0.4	18±1.58	54±1.58	34±2.73	34±2.73
0.5	13±1.51	42.8±1.48	16.6±1.51	12±1.58

[a] Embryo induction frequency is expressed as the mean ± standard deviation of the percentages obtained in the five repetitions of the experiment

Table 3 Efficacy of polyamines on the development[a] of somatic embryos from putative transgenic cell lines of *H. brasiliensis*. For each treatment, 20 callus groups were used. The experiment was repeated five times

Polyamines	Concentration (mg l^{-1})				
	1.0	2.0	3.0	4.0	5.0
Putrescine	4±1	3±0.7	1±0.7	0	0
Spermine	30±3.4	58±2.8	28±1.58	10±1.58	4±1
Spermidine	40±2.54	32±1.87	23±2.34	6±1.58	1±0.7

[a] Values are expressed as the mean ± standard deviation of the percentage of somatic embryos obtained from transgenic callus in five repetitions of the experiments

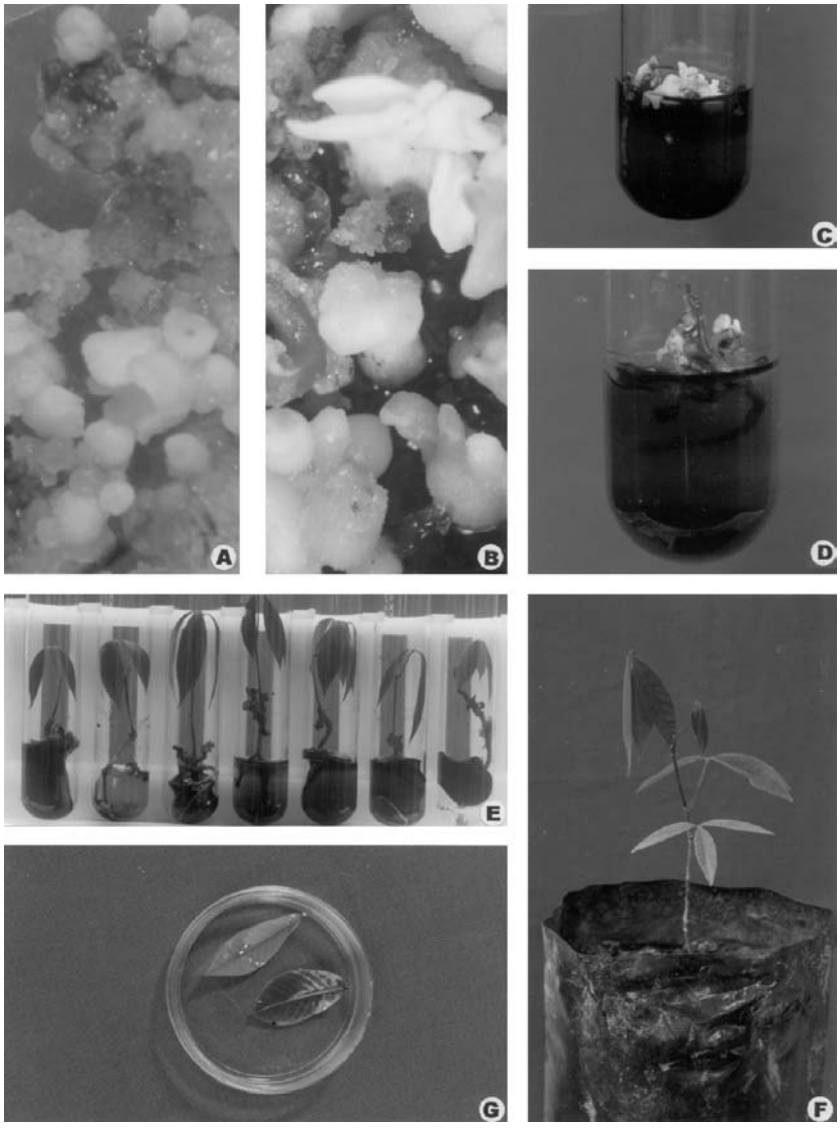

Fig. 4A–G Somatic embryogenesis and development of transgenic plants of *Hevea brasiliensis*. **A** Emerging globular embryos, **B** cotyledonary embryos, **C** mature embryos kept for germination, **D** a germinated embryo showing bipolar differentiation, **E** plantlets in culture tubes, **F** acclimatized transgenic plant in polybag, **G** transgenic leaf showing GUS expression along with a control leaf

Table 4 Effect of various organic supplements on embryo induction, maturation and germination in *H. brasiliensis*

Organic supplement	Concentration	Embryo Induction[a] (%)	Embryo maturation[b] (%)	Embryo germination[c] (%)
Casein Hydrolysate (mg l^{-1})	100	40±2.7[d]	12±1.58	2±1
	200	69.4±2.7	28±1.58	3±1
	300	23±2.1	38±1.58	5±1.58
	400	15±1.6	19±1.58	7±1.58
Malt extract (mg l^{-1})	50	12±1.9	9±2.23	7±1.58
	100	27±1.6	22±2.34	3±1.22
	150	33±1.58	12±2.1	1±0.7
	200	22±4.2	10±1.58	1±0.7
Banana powder (mg l^{-1})	100	43±2.54	36±2.34	2±1.22
	150	54±1.58	48±2.9	3±1.58
	200	49±2.5	40±1.58	6±1
	250	38±3.8	30±2.54	1±1
Coconut water (%)	5	38±2.23	23±1.58	5±1.58
	10	45±2.5	35±2.7	2±0.7
	15	30±1.58	25±1.58	2±1
	20	28±1.58	30±3.8	1±0.7

[a] Twenty callus lines were used, and each experiment was repeated five times
[b] Twenty embryogenic lines were used for each treatment, and the experiment was repeated five times
[c] Twenty matured embryos were cultured for each treatment, and the experiment was repeated five times
[d] Values are the mean ± standard deviation of the percentages obtained in five repetitions of the experiment

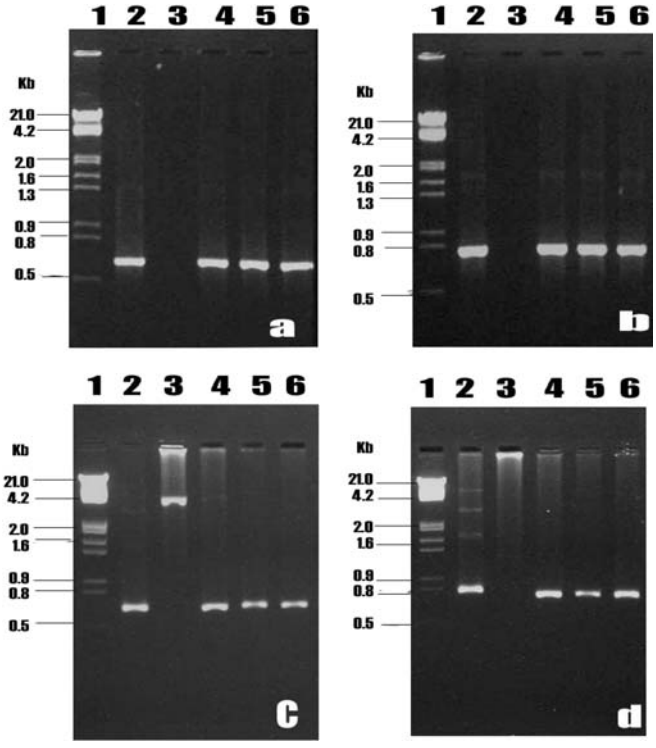

Fig. 5a–d PCR analysis for the detection of the *uidA*, *nptII* and *HbSOD* genes in *H. brasiliensis* putative transgenic plants. **a**, **b** PCR amplification using specific primers for the *uidA* (**a**) and *nptII* (**b**) genes. *Lanes*: *1* DNA size marker, *2* positive control (plasmid DNA), *3* negative control (untransformed plant), *4–6* putative transgenic plants. **c** PCR amplification using specific primers for the *HbSOD* gene. *Lanes*: *1*DNA size marker, *2* positive control (plasmid DNA), *3* negative control (untransformed plant), *4–6* putative transgenic plants. **d** PCR amplification using an upstream primer specific for the CaMV 35S promoter sequence and a downstream primer specific for the *HbSOD* gene. *Lanes*: *1* DNA size marker, *2* positive control (plasmid DNA), *3* negative control untransformed plant, *4–6* putative plants

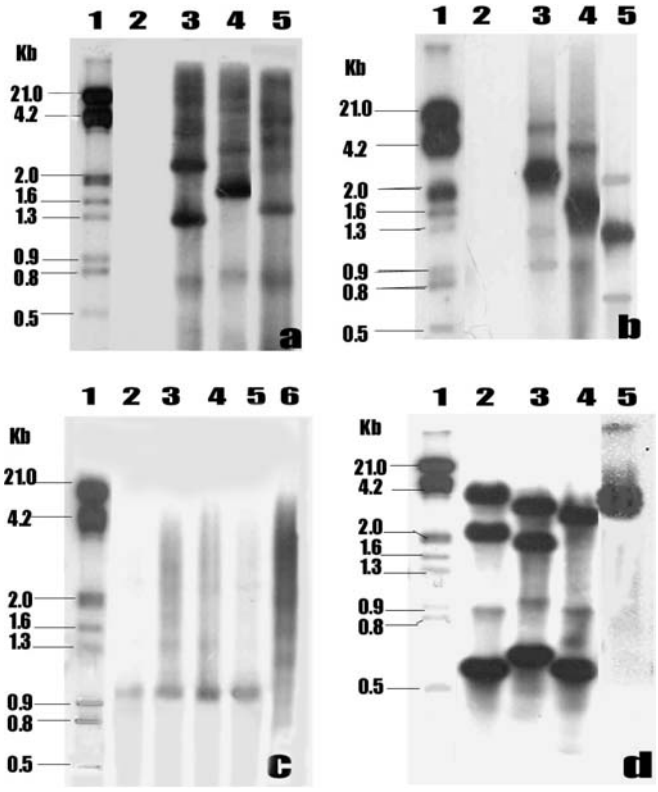

Fig. 6a–d Southern blot analysis of genomic DNA islolated from *H. brasiliensis* transgenic plants. **a**, **b** Total DNA (10 μg) was digested with *Hin*dIII (*lane 3*), *Bam*HI (*lane 4*) and *Xba*I (*lane 5*) enzymes and hybridized with a *uidA* probe (**a**) and a *nptII* probe (**b**). *Lanes*: *1* DNA marker, *2* untransformed plant (negative control), *3–5* GUS-positive plants. **c**, **d** Total genomic DNA (10 μg) was digested with *Hin*dIII and *Bam*HI (**c**) and with *Hin*dIII only (**d**) and hybridized with a *HbSOD* probe. **c** *Lanes*: *1* DNA marker, *2–5* four individual GUS-positive plants, *6* untransformed plant (negative control). **d** *Lanes*: *1* DNA marker, *2–4* three individual GUS-positive plants, *5* untransformed plant (negative control)

detection of *uidA, nptII* and *HbSOD* gene sequences. PCR analysis was conducted on these three transgenic plants, one non-transgenic plant (negative control) and plasmid DNA as a positive control. Primers for the *uidA* and *nptII* genes were designed to amplify the DNA fragments of 650 bp and 800 bp, respectively. The presence of the *uidA* (Fig. 5a) and *nptII* (Fig. 5b) genes was confirmed in all three transgenic plants and in the plasmid DNA (positive control), whereas corresponding bands were not detected in the non-transformed control. When *HbSOD* gene-specific primers were used for PCR amplification, a 700-bp band was amplified in both the transgenic plants and the positive control, but this band was not amplified in the untransformed control (Fig. 5c). However, because the endogenous SOD gene was present in the untransformed *Hevea* plants, a 4.0-kb genomic DNA band was amplified during PCR analysis (lane 3). To rule out the possibility of endogenous genomic SOD gene amplification in the transgenic plants, we also used a gene primer from the CaMV 35S promoter sequence in a second PCR analysis. The results of this second PCR analysis were that a 800-bp of *HbSOD* gene amplification was obtained with both the transgenic plants and the positive control and no amplification was observed with the untransformed negative control plant (Fig. 5d), indicating that the newly introduced *HbSOD* transgene was stably integrated in the nuclear genome of *Hevea* transgenic plants.

Southern blot analysis of transgenic plants

Putative transgenic plants derived from transformed callus lines were selected for genomic Southern blot hybridization analysis to further confirm the integration of T-DNA in the nuclear genome of the transgenic plants. Genomic DNA was digested with the restriction enzymes *Hin*dIII, *Bam*HI, and *Xba*I. As shown in Fig. 6a, b, α-[^{32}P]-dATP-labeled *uidA* and *nptII* DNA probes were respectively hybridized to digested genomic DNA of regenerated plants (lanes 3–5). No hybridization could be detected for the negative control plant (non-transgenic)

(lane 2). DNA samples were digested with *Hin*dIII, which liberates the internal *uidA* gene cassette (3.3 kb), *Bam*HI and *Xba*I, which have a unique restriction site at the right border of the T-DNA. In *Hin*dIII-digested DNA sample, a hybridization band of 3.3 kb could be detected with the *uidA* probe. Both *Bam*HI- and *Xba*I-digested DNA samples showed different hybridization patterns with *uidA* and *nptII* probes. These single enzyme digests were expected to give mostly large fragments. However, we also detected numerous other smaller bands. This could be due to either incomplete digestion of the genomic DNA or the integration of numerous fragmented copies (perhaps more than one integration event) of the *uidA* and *nptII* genes in the plant genome. This type of insertion has also been described earlier in other crops (Cervera et al. 1998; Walter et al. 1998). The different hybridization patterns observed for the transgenic plants indicate random integration and multiple insertions of the T-DNA in the genome of these plants. To confirm the integration and presence of the *HbSOD* transgene in the putatively transgenic plants, we digested genomic DNA with *Hin*dIII and *Bam*HI, which liberates the *HbSOD* gene cassette (1.0 kb), and with only *Hin*dIII, which has a unique restriction site at the right border of the T-DNA. In the *Hin*dIII- and *Bam*HI-digested-DNA samples, hybridization bands of 1.0 kb could be obtained (Fig. 6c) only with the transgenic plants (lanes 2–5) and a much larger band, probably corresponding to the endogenous *SOD* gene, was detected in the untransformed plant (negative control, lane 6). To detect the *HbSOD* transgene copy number, we digested DNA samples with *Hin*dIII, and the three transgenic plants analyzed revealed two to three strong hybridization bands and one to three faint bands (Fig. 6d), indicating different T-DNA copy numbers. Again a large band was detected in the non-transgenic control, probably corresponding to the undigested endogenous *SOD* gene. These results provide strong evidence that the *uidA*, *nptII* and *HbSOD* genes were integrated in the transgenic *Hevea* plants, confirming the validity of this *Agrobacterium*-mediated gene transfer and regeneration system.

Conclusion

In summary, we have established an efficient *Agrobacterium*-mediated transformation and regeneration system and have identified factors that allow enhanced generation of transformed embryos and subsequent plant development. Thus, this gene transfer and whole-plant regeneration system could enable the development of elite tree clones with engineered traits of economic importance. It will be interesting to study the oxidative stress tolerance of the *HbSOD* transgenic plants in the field. However, our transgenic plants are still growing in polybags. After bud grafting and the production of considerable number of progenies, field experiments to study the stress tolerance of the plants can be conducted. Moreover, the oxidative stress could be induced with 6-year-old trees by tapping. To our knowledge, this is the first report on the production of transgenic plants with a superoxide dismutase gene (*HbSOD*) using an *Agrobacterium tumefaciens*-mediated gene transfer system for *Hevea brasiliensis*.

Acknowledgements We wish to thank Dr. N.M. Mathew, Director, for his constant encouragement during this research, and Dr. Leandro Pena for his valuable suggestions which enabled us to successfully complete this work.

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