Romanian Biotechnological Letters *Vol. 18, No.1, 2013*

ORIGINAL PAPER

Genetic transformation and regeneration of Hevea brasiliensis transgenic plant with GAI gene by microparticle bombardment

Received for publication, November 29, 2012
Accepted, December 15, 2012

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Abstract

A typhoon is a major natural disaster in the cultivation areas of Hevea brasiliensis in China. In this study, we report a method for the transformation and regeneration of H. brasiliensis transgenic plants expressing the Arabidopsis gibberellic acid insensitive (GAI) gene to induce the dwarfing phenotype. A pBI121vector comprising CaMV35S promoter, kanamycin resistance gene (nptII), beta-glucuronidase (GUS) reporter gene, and GAI gene was used. The embryoid induction rate was 3.21% using a 40-day-old calli as target with 1100 psi helium pressure and 9 cm target distance. Kanamycin-selected calli and embryos were positive for GUS activity in histochemical assays. A transgenic line and 21 sub-plantlets were positive for GAI gene amplification using the polymerase chain reaction. The integration of the GAI gene was confirmed by Southern blot and showed a single copy insertion. These results illustrate the advantages of using microparticle bombardment for stable plant transformation in H. brasiliensis with possible applications in other plant species.

Keywords Genetic transformation, *Hevea brasiliensis,* Microparticle bombardment, *GAI* gene

Introduction

The rubber tree (*Hevea brasiliensis*) is an important perennial tropical crop that accounts for one third of the total rubber production worldwide. Over the past decades, the yield of rubber trees has significantly increased because of the cultivation of high-yielding clones and the use of ethephon, a mimic of the plant hormone ethylene. However, the production of natural rubber is seriously affected by typhoon, which is a major natural disaster in *H. brasiliensis* cultivation areas in China. Artificial pruning of dwarf *H. brasiliensis* effectively decreases damage caused by typhoons in the production areas.

However, typhoons still cause significant reduction in the rubber production during the initial years after pruning. Hence, the cultivation of dwarf *H. brasiliensis* cultivar is important.

The conventional breeding technique is slow and time consuming because of the long breeding cycle and highly heterozygous nature of *H. brasiliensis*. Genetic engineering can be used to produce desirable agronomic traits quickly and efficiently as well as introduce genes that can encode high-value recombinant proteins. *Agrobacterium tumefaciens*-mediated genetic transformation techniques were developed in several *Hevea* clones, namely, GL1, RRII 105, and PB 260 (JAYASHREE & al. 2003[1]; MONTORO & al. 2003[2]; RATTANA & al. 2001[3]; MONTORO & al. 2000[4]). To date, overexpression of endogenous genes involved in reactive oxygen species scavenging systems, such as MnSOD，a foreign gene encoding a human serum albumin, has been reported (JAYASHREE & al. 2003[1]; SOBHA & al. 2003[5]; AROKIARAJ & al. 2002[6]). *Hevea* transgenic plant lines that overexpress *H. brasiliensis* cytosolic *HbCuZnSOD* gene were successfully established and regenerated (LECLERCQ & al. 2012[7]). Targeting transgene expression in latex cells was also attempted using the promoter from gene *HEV2.1*, which encodes the major latex Hevein protein (MONTORO & al. 2008[8]). The stability of transgene expression was tested by fluorimetric *GUS* activity in Hevea transgenic *in vitro* plants and their subsequent budded sub-lines (LARDET & al. 2011[9]).

However, *Agrobacterium*-mediated transformation suffers from the limitation of host range specificity, comparatively low transformation frequency, and unwanted occurrence of vector backbone transfer (ALTPETER & al. 2005[10]). Microparticle bombardment is considered the most universal genetic transformation method due to its relative independence of biological target. It is considered applicable to genetically modify a wide range of explants with high regeneration ability; reducing time, labor and costs involved in the production of transgenic plants. So, in this study, an effective protocol by microparticle bombardment to generate stable transformants of *H. brasiliensis* was developed. The dwarf types of the *H. brasiliensis* do not exist naturally. GAI is one of the negative regulators affecting GA response in *Arabidopsis thaliana*, and its overexpression causes dwarfing of plants (PENG & al. 1997[11], FU & al. 2001[12]). Therefore, the gene coding for *GAI* was selected in this study for the genetic transformation of *H. brasiliensis* to develop transgenic plants with increased tolerance to typhoon damage.

Materials and methods

Plant material and plasmid vector

H. brasiliensis clones, Haiken2, were planted at the experimental farm of the Chinese Academy of Tropical Agriculture Sciences (Danzhou, China). The study used a pBI121 vector carrying the *GUS* reporter gene, an *npt*II gene as the selectable marker gene for kanamycin resistance, and a *GAI* gene under the control of CaMV 35S promoter.

Callus tissue induction

H. brasiliensis calli were developed from immature anthers. Male flower buds were collected, sterilized with 70% (v/v) alcohol for 30 s, and then sterilized with 0.2% (v/v) mercuric chloride solution containing a few drops of Tween 20 for 10 min. The buds were washed five times with sterile distilled water. The anthers excised from the buds were

inoculated on Murashige and Skoog (MS) medium modified with 0.5 mg l^{-1} folic acid, 0.5 mg l^{-1} d-biotin, 150 mg l^{-1} glutamic acid, 5% (v/v) coconut milk, 7% (w/v) sucrose, 1.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 1 mg l^{-1} 6-furifury laminopurine (Kt), 0.5 mg l^{-1} naphthaleneacetic (NAA), and 0.25% (w/v) phytagel (WANG & al. 1980[13]). Compact calli were obtained under complete darkness in 5 wk to 6 wk after explant inoculation and then used for transformation.

Microparticle bombardment transformation

Microprojectile program was prepared according to the method of Becker (BECKER & al. 2000[14]). Compact calli were obtained under complete darkness in 5 wk to 6 wk and precultured for 2 d in an embryo induction medium with half-strength majorelement of MS medium, which contained 200 mg l^{-1} hydrolyzed tyrosine, 0.1 mg l^{-1} abscisic acid, 1 mg l^{-1} 6-benzyl aminopurine (6-BA), 1 mg l^{-1} Kt, 0.3 mg l^{-1} NAA, 0.5 mg l^{-1} gibberellic (GA_3), 1 g l^{-1} activated charcoal, 4% (w/v) maltose, 3% (w/v) sucrose, and 0.5% (w/v) phytagel (WANG & al. 1980[13]).

Bombardment was performed using a plasmid at 1100 psi with two different distances of 6 cm and 9 cm. For the bombardment, the plasmid was attached to a gold microparticle by the addition of 2.5 M calcium chloride and 0.1 M espermidine. A total of 0.8 μg DNA was used in each bombardment.

Co-cultivation and selection

The bombarded calli that proliferated for 7 d to 10 d in the embryo induction medium were transferred to the same medium supplemented with 50 mg l^{-1} kanamycin. The putative transgenic calli were subcultured once in the embryo growth medium with half-strength MS medium (1 mg l^{-1} 6-BA, 1 mg l^{-1} Kt, 0.3 mg l^{-1} NAA, 0.5 mg l^{-1} GA_3, 1 gl^{-1} activated charcoal, 3% (w/v) sucrose, and 0.5% (w/v) phytagel) until maturity(WANG & al. 1980[13]). Nontransgenic calli were maintained as control. For plant regeneration, mature somatic embryos were transferred to half-strength MS medium containing 0.5 mg l^{-1} 6-BA , 1 mg l^{-1} GA_3 and 50 mg l^{-1} kanamycin. When the plantlets grew to 6 cm to 8 cm, the leaves were removed and cut into stem segments of 1 cm to 2 cm with axillary bud or apical bud. The stem segments were inoculated in the MS medium with 2 mg l^{-1} 6-BA and 50 mg l^{-1} kanamycin (CHEN & al. 1998[15]). The plantlets were cut into 3 cm stem segments after subculturing for four times and inoculated in half-strength MS medium with 0.1 mg l^{-1} 3-indolylbutyrie acid(IBA) and 50 mg l^{-1} kanamycin until the plantlets have grown with roots. The mature transgenic plants were transplanted in pots. The cultures for callus proliferation and embryo induction were kept under complete darkness at 25 °C ± 2 °C, whereas the cultures for plant regeneration were maintained at 25 °C ± 2 °C under 12 h photoperiod.

GUS histochemical assay

Resistant callus tissues and somatic embryos were histochemically tested for β-glucuronidase activity (JEFFERSON & al. 1987[16]). The materials for staining were incubated overnight at 37 °C in 2 mM X-Gluc (5-bromo-4 chloro-3-indolyl b-d glucuronide) in a pH 7.0 phosphate buffer (10 mM Ethylene Diamine Tetraacetic Acid, 0.5 mM potassium ferricyanide, and 0.1% (v/v) Triton X-100) and then examined under a binocular microscope. *GUS*-positive tissues were used for the establishment and molecular characterization of the putative transgenic callus lines.

PCR assay

DNA from the leaves of putative transgenic and control plants was extracted as previously described in (DOYLE & al. 1990[17]). Plasmid DNA was isolated from *Escherichia coli* after the alkaline lysis method. Forward (5'-ATG AAG AGA GAT CAT CAT CAT CAT-3') and reverse (5'-CTA ATT GGT GGA GAG TTT CCA AGC-3') primers were employed to detect the *GAI* gene. Polymerase chain reactions (PCRs) were performed in 20 ml volumes containing 100 mM dNTPs, 250 nM of each primer, 1.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase with 20 ng template DNA in a thermal cycler. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, with final elongation at 72 °C for 10 min. Amplified DNA fragments were electrophoresed in 1.0% agarose gels stained with ethidium bromide and visualized under UV light.

Southern blot analysis of the putative transgenic plant

DNA from a putative transgenic plant showing positive signals in histochemical staining and PCR was used for Southern hybridization analysis. A total of 10 μg of genomic DNA was digested with *Bam*HI/*Xba*I to detect the fragments of the predicted size, and meanwhile digested with *Bam*HI or *Xba*I to confirm the integration into the host genome and the number of insertions. The fragments were separated in 1.0% (w/v) agarose gel and transferred onto nylon membranes (Hybond N+, Amersham Pharmacia, UK). A PCR-amplified 1.5 kb internal fragment of the *GAI* gene of the vector was used as the probe, which was labeled with Digoxigenin-11-dUTP using DIG-High prime labeling kit (Roche Diagnostics). Hybridization was performed overnight at 40 °C. The membranes were then washed twice at room temperature in 2×SSC and 0.1% (w/v) SDS, followed by two high-stringent washes for 15 min each at 65 °C in 0.5×SSC and 0.1% (w/v) SDS. Blots were exposed to X-ray film with intensifying screens.

Results

Establishment and selection of transgenic lines of *H. brasiliensis*

Bombardment was performed using 40-day-old calli as target at 1100 psi with two different distances of 6 cm and 9 cm (Figure 1a). The bombarded callus tissues that proliferated for 7 d in the embryo induction medium were transferred to the same medium supplemented with 50 mg l^{-1} kanamycin. Somatic embryos emerged after 4 wk and were transferred to half-strength MS medium with kanamycin selection (Figures 1b and 1c). Resistant callus tissues and somatic embryo were histochemically tested for *GUS* activity. The regenerated shoots were subcultured (Figure 1d) and successfully developed into plantlets (Figure 1e), which were checked using PCR and Southern hybridization for the number of T-DNA inserts. The mature transgenic plants were transplanted in pots (Figure 1f).

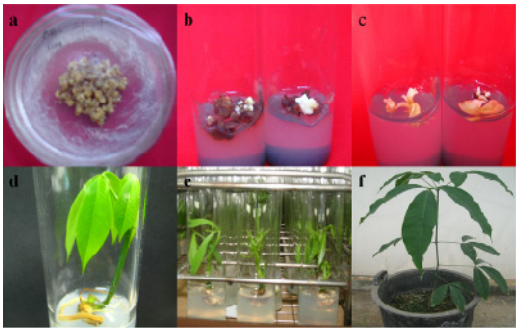

Figure 1. Different stages of putative transgenic plant regeneration in *H. brasiliensis*. a: *H. brasiliensis* anther callus prepared for transformation by microparticle bombardment; b: embryo induced from callus after transformation; c: development of embryoid; d: plantlet induced from embryoid; e: plantlets with kanamycin resistance and *GUS*-positive propagated in tube; f: transgenic plants were transplanted in the pot.

The effect of shooting distance on genetic transformation

A total of 560 good callus tissues with two-day pre-cultivation were bombarded. The 1100 psi helium pressure interacting with two target distances (9 cm and 6 cm) were performed. After 30 d, the embryoid induction rate reached 3.21% when shot at a 9 cm distance. However, the embryo can hardly be induced when shot at a 6 cm distance (Table 1).

Table 1. Efficiency of shooting distance on genetic transformation

Shooting distance (cm)	Number of callus tissues	Number of embryoid	Embryoid induction rate (%)
9	280	9	3.21
6	280	2	0.7

GUS assay

Two weeks after microparticle bombardment, resistant callus tissues were histochemically tested for *GUS* activity. Nontransgenic calli were maintained as control. The bombarded callus tissues were observed under the microscope. The putative transgenic callus tissues showed *GUS* activity as determined from the blue stains observed in the histochemical test, indicating the occurrence of transformation (Figures 2a and 2b).

After 6 wk, the putative transgenic embryos showed *GUS* activity as determined from the blue stains observed in the histochemical test, indicating that the expressed *GUS* gene was relatively stable after transformation (Figures 2c and 2d).

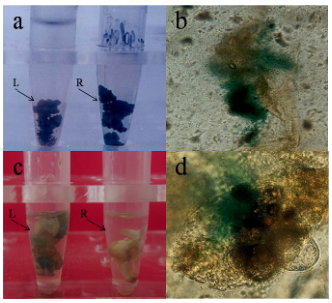

Figure 2. *GUS* expression in *H.brasiliensis*. a: *GUS* expression in *H. brasiliensis* (L: control and R: putative transgenic callus); b: slide of putative transgenic callus with *GUS* expression; c: *GUS* expression in *H. brasiliensis* embryoid (L: putative transgenic embryoid and R: control); d: slide of putative transgenic embryoid with *GUS* expression

PCR analysis

DNA isolated from the putative transgenic plant along with a nontransgenic plant as negative control was used in the PCR analysis. The plasmid vector was used as the positive control, whereas the untransformed plant and H_2O were used as blank control. The presence of *GAI* gene was confirmed by the amplification of a 1.5 kb single product in the transgenic clone and in the control plasmid. No amplification was detected in the untransformed plant (Figure 3A).

The *H. brasiliensis* transgenic plant was cut into stem segments with buds and subcultured four times to obtain 21 sub-plantlets. DNA isolated from the putative transgenic plantlets along with a nontransgenic plant as negative control were used in the PCR analysis with primers designed for the amplification of the *GAI* gene. The plasmid vector was used as the positive control. The presence of *GAI* gene was confirmed by the amplification of 1.5 kb single product in the 21 sub-plantlets and in the control plasmid. No amplification was detected in the untransformed plant (Figure 3B).

Southern blot

The integration of the T-DNA region into the genome of the putative transgenic plant was confirmed by Southern hybridization analysis. The digoxigenin-11-dUTP-labeled *GAI* gene probe generated a band of a predicted size (1.5 kb) with *Bam*HI/*Xba*I double digestion in the putative transgenic plant and in the positive control (vector plasmid). Hybridization of *Bam*HI or *Xba*I digested with *GAI* probe produced bands with sizes of more than 2.0 kb, representing the DNA fragments containing the *GAI* gene and a part of the plant genomic DNA. The different hybridization patterns observed for the putative transgenic plant indicate random integration and a single copy insertion of the DNA into the genome of the plant. Hybridization was not detected in the nontransgenic negative control (Figure 3C).

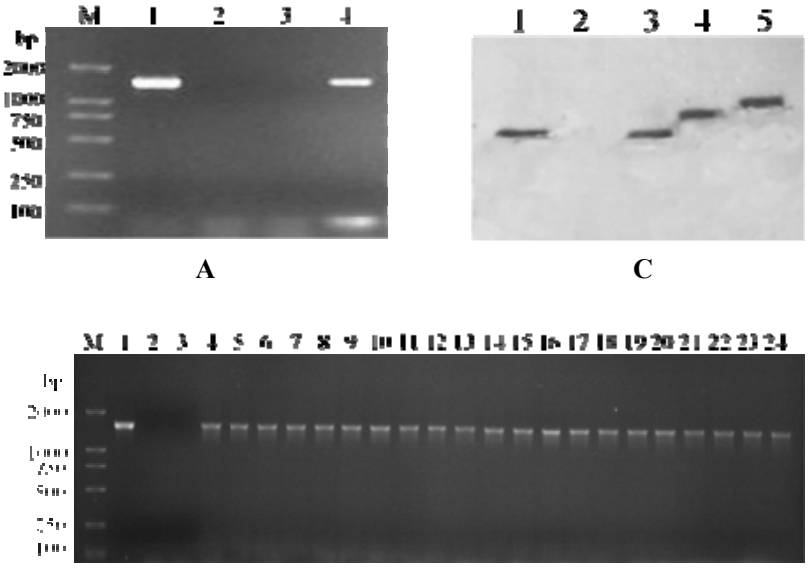

Figure 3.PCR and Southern blot analyses of the *H. brasiliensis* plants bombarded with *GAI* gene. A. Results of the PCR analyses of an anti-kanamycin plantlet. M: marker, DL2000; 1: positive control; 2: blank control; 3: negative control; 4: anti-kanamycin plantlet;
B. Results of the PCR analyses of anti-kanamycin plantlets subcultured in the 4th generation. M: marker, DL2000; 1: positive control; 2: blank control; 3: negative control; 4 to 24: putative transgenetic plantlets. C. Results from the Southern blot analysis.1: positive control; 2: nontransgenic plantlet DNA *Bam*HI/*Xba*I double digestion; 3: putative transgenic plantlet DNA *Bam*HI/*Xba*I double digestion; 4: putative transgenic plantlet DNA *Xba*I digestion; 5: putative transgenic plantlet DNA *Bam*HI digestion.

Discussion

The current genetic transformation systems in *H. brasiliensis* are based on the *Agrobacterium*-mediated transformation method (BLANC & al. 2006[18]; MONTORO & al. 2008[8]; LARDET & al. 2009[19]; LECLERCQ & al. 2012[7]; LARDET & al. 2011[9]), in which *GUS* or *GFP* can be used as a reporter gene. In the present study, *GUS* was used as the reporter gene with kanamycin selection. The bombarded callus tissues proliferated for 7 d in the embryo induction medium without antibiotic. This procedure is beneficial to the recovery of callus tissues growth. Similarly, paromomycin was added after the third subculture in the transgenic callus for the *HEV2.1* gene promoter (MONTORO & al. 2008[8]). Histochemical analyses of *GUS* activity in the transgenic callus tissues and somatic embryos revealed that the *GAI* gene was expressed stably in calli and embryos. Transgene expression was assessed by *GUS* activity *in vitro* of plants from transgenic lines and their sublines were obtained by budding (LARDET & al. 2011[9]).

Gene delivery by microparticle bombardment is regarded as the most efficient and consistent method for transferring foreign DNA. Unlike the *Agrobacterium*-mediated transformation method, which is a biological process, microparticle bombardment is a

physical process of gene delivery, and any plant tissue can be theoretically transformed by this method (SHARMA & al. 2005[20]). In microparticle bombardment experiments, the different physical and biological parameters, such as the mechanism of delivery, osmoticum, velocity of microparticle delivery, DNA microparticle concentration and precipitation procedures, and the amenability of target tissues for transformation should be carefully selected. The distance between the macrocarrier and target cell was one of the key factors in this method. In this study, The range from macrocarrier to target tissue was found to have a significant effect on the embryoid induction rate. The target distance of 6 cm greatly damaged the bombarded small tissues and embryos can hardly be induced. At a distance of 9 cm, the embryoid induction rate reached 3.21%. Therefore, 9 cm was a suitable parameter for *H. brasiliensis* transformation. In a previous study, the same distance has been shown to enable good targeting (SUWANAKETCHANATIT & al. 2007[21]), which was in agreement with the present data.

Southern blot analysis of the putative transgenic *H. brasiliensis* plant showed only one band with variable size. In the transformation via microparticle bombardment, multiple copies usually exist in the transgenic plants (KLIMASZEWSKA & al. 1997[22]; TAKAHASHI & al. 1998[23]; WALTER & al. 1998[24]). Klimaszewska (1997) obtained two transgenic *Larix laricina* lines with five or six copies of the bombarded DNA. Conversely, high-frequency signal-copy integration (35 among the 50 total transgenic plants) was reported in the transgenic loblolly pine (TANG, & al. 2003[25]). Klimaszewska & al. (2003[26]) suggested that different species and genotypes can have different abilities of integrating DNA. In addition, the possibility of the effects of other factors, such as vectors and condition of explants, could not be excluded.

Conclusion

To the best of our knowledge, this study is the first to report on the genetic transformation of *H. brasiliensis* callus with gene coding for *GAI* and the regeneration of transgenic plants by microparticle bombardment method. The embryoid induction rate was 3.21% using 40-day-old calli as target, 1100 psi helium pressure, and 9 cm target distance. Kanamycin-selected calli and embryos were positive for *GUS* activity in histochemical assays. A transgenic line and 21 sub-plantlets were confirmed by PCR and Southern blot analyses. The use of this transformation system in *H. brasiliensis* permits the introduction of genes that regulate morphologically and economically important traits, such as genes that improve production and increase disease resistance.

Acknowledgements

This study was supported by the National Nonprofit Institute Research Grant of CATAS-ITBB (ITBB110203) and the National Natural Science Foundation of China (30660098). We deeply thank Dr. Kui Hong for the careful modification of this manuscript.

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