J. Rubb. Res., **18(3)**, 171–188

Somatic Embryo, an Alternative Target Tissue for Agrobacterium-Mediated Transformation in Hevea brasiliensis

T.D. HUANG*, J. LI*, Y.T. LI*, H.S. HUANG* AND Y.W. HUA*#

Traditionally, embryogenic callus has been used as the only receptor source of genetic transformation in H. brasiliensis*. In this study, we showed that somatic embryos were a viable alternative to embryogenic callus. The appropriate concentration of both decontamination and selection antibiotics was firstly screened. Visual and cytological observations found both epidermal and subepidermal cotyledon cells could be infected, indicating that somatic embryos were susceptible to antibiotics and Agrobacterium. Subsequently, five isolated consecutive transformation experiments were carried out to produce the transgenic plants using a total of 1158 somatic embryos, of which 47 (4.06 %) GUS-positive embryos were produced after cell differentiation and embryogenesis. Six were successfully multiplied and produced 33 plants. PCR and Southern blot analysis confirmed that the T-DNA was integrated into the recovered plants. Finally, three additional cycles of secondary embryogenesis were carried out for transgenic embryo proliferation efficiency assessment. The rate of proliferation tended to slightly increase with each proliferation generation, and one initial transgenic embryo-derived GUS-negative embryo were observed in the fifth multiplication cycle, indicating that transgenic plants could be propagated through secondary embryogenesis and some initial transgenic embryos might be chimeric. This is the first attempt on producing transgenic plants using somatic embryos as the target tissue in* H. brasiliensis*. It is demonstrated that somatic embryos can be used as an alternative target tissue for Agrobacterium-mediated transformation in* Hevea brasiliensis*.*

Keywords: *Hevea brasiliensis*; somatic embryo; genetic transformation; *Agrobacterium tumefaciens*; secondary embryogenesis

Commercial natural rubber is mainly produced from latex synthesised in specialised cells called laticifers in the bark of *Hevea brasiliensis* (Euphorbiaceae). It is a heterozygous perennial tree crop native to the rain forests of the tropical region of the great Amazonian basin of South America[2]. At present, conventional breeding is a primary approach for the improvement of *H. brasiliensis*, but its long breeding cycle (25–30 years) limits genetic recombination[3].

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Genetic transformation can improve plants quickly and efficiently, and transformation systems have been established in many plants, including *H. brasiliensis*. The first transgenic rubber tree was developed with the incorporation of the GUS (β-*glucuronidase*) gene through particle bombardment[4]. Subsequently, several successful cases have been reported, including transformation of the superoxide dismutase (SOD) gene for improvement of tapping panel dryness (TPD)[5–8], the HMGR1 gene for enhancement of latex yield[9], and production of recombinant proteins like secreted human serum albumin (HSA)[10], a mature immunoglobulin single chain variable fragment (ScFv)[11] and the TB antigen protein[12]. However, all of the *H. brasiliensis* transgenic plants in previous study were produced using embryogenic callus as the target tissue of transformation, that is to say, embryogenic callus, whatever anther, inner integument, or leaf-derived, was the only receptor source of successfully producing transgenic plants in *H. brasiliensis*. Embryogenic callus, when used as the target tissue of transformation, can lead to low plant regeneration, which is not efficacious.

Somatic embryos could be an effective substitution for the target tissue of transformation, because they can undergo secondary somatic embryogenesis. Secondary somatic embryogenesis is the phenomenon whereby new somatic embryos are initiated from a somatic embryo without fusion of gametes, due to its characteristics: the epidermal origin, homogeneity and faster regeneration, secondary embryos have become a great target tissue for transformation[13–15]. Secondary somatic embryogenesis has been successfully used for transformation of plants such as rapeseed[16], alfalfa[17] and cork oak tree[14]. Additionally, secondary embryogenesis has been established in *H. brasiliensis*[18–20]. However, somatic embryos have not previously been used successfully as a target tissue for transformation. Herein, to broaden the resource of the target tissue, in this study, we investigated the antibiotics sensitivity of untransformed somatic embryos, characterised the *Agrobacterium* infection on somatic embryos, evaluated the transformation efficiency and multiplication of transformants, and successfully produced transgenic plants. Our results demonstrated that the somatic embryo is an alternative target tissue for *Agrobacterium*-mediated transformation in *H. brasiliensis*.

MATERIALS AND METHODS

Propagation of Somatic Embryos for Transformation

H. brasiliensis somatic embryos of the clone CATAS 87-6-62 were proliferated as the target receptor of *Agrobacterium* infection by cyclic secondary embryogenesis according to Hua *et al*.[20] In summary, anthers at the uninuclear stage were collected and cultured on callus and embryo induction media (MSC and MSE) in turn to produce primary somatic embryos, and then the primary embryos were proliferated by secondary embryogenesis in a cyclic manner. Mature cotyledonary embryos with the size of 1.5 cm were selected for transformation.

For callus induction, embryos and plant regeneration, the cultures were incubated in darkness at 22ºC, 25ºC and 28ºC, respectively. A photoperiod of 16 h was used for plant regeneration. MSC and MSE media were supplemented with appropriate ticarcillin (Duchefa, Netherlands) and kanamycin (BBI, Canada) levels to control the growth of *Agrobacterium* and to select kanamycin-resistant embryos. All callus and embryo induction media (MSC and MSE) and plant regeneration media (MSR) were the same with those of Hua *et al*.[20]

Selection of Appropriate Concentrations of the Antibiotics

Appropriate concentrations of selection and decontamination antibiotics were tested prior to the transformation experiment, using kanamycin and ticarcillin with various concentrations on untransformed embryos. Embryos were cut into 3 × 3 mm fragments and put into callus induction medium (MSC) supplemented with kanamycin (0, 50, 75, 100, 125 mg L^{-1}) or ticarcillin (0, 100, 200, 300, 400, 500 mg L^{-1}). After 20 days, the cultures were transferred to embryo induction medium (MSE) supplemented the same antibiotics and concentrations with the MSC medium. Two months later, the number of embryos was counted. Each experiment was replicated three times.

Transformation Vector and Bacterial Strain

The binary vector pCAMBIA 2301 carrying a selectable marker gene (*npt*II) and reporter gene (*uid*A) (CAMBIA, canberra, Australia) was used in this study. The selectable marker and reporter gene are under the control of the CaMV35S promoter. The reporter gene has an intron which prevents the expression of the reporter gene in *Agrobacterium*. The vector was introduced into *Agrobacterium tumefaciens* strain EHA105 by the freeze–thaw method[21]. The *Agrobacterium* culture was incubated overnight at 28ºC in liquid LB medium (BBI, Canada) containing 50 mg L^{-1} rifamycin and streptomycin (BBI, Canada) and 100 mg L^{-1} kanamycin on a rotary shaker with agitation (250 rpm). Prior to transformation, the bacterial cells were pelleted by centrifugation at 4000 rpm for 10 min and re-suspended in 20 mL liquid MSC medium[20] with 30 g L^{-1} sucrose but without Fe^{2+}, Ca^{2+} and any antibiotics. This prepared Agrobacterium culture (OD_{600nm} = 0.5) was then used for inoculation of somatic embryos.

Transformation

Mature cotyledonary embryos with the size of about 1.5 cm (*Figures 3A–C*) were collected and submerged in 20 mL *Agrobacterium* solution (as above) with the addition of 100 μM As for 6 minutes. After blotting them dry on sterile filter paper to remove excess *Agrobacterium* solution, the embryos were co-cultured at 22ºC in darkness in MSC medium supplemented with 10 mg L^{-1} silver nitrate for three days. The embryos were then washed with sterile water thrice and ticarcillin solution once, in turn, with gentle agitation, then blotting dry on sterile filter paper, and cultured at 22ºC in darkness on MSC medium supplemented with an appropriate concentration of ticarcillin for three days for recovery.

Selection, Proliferation and Plant Regeneration

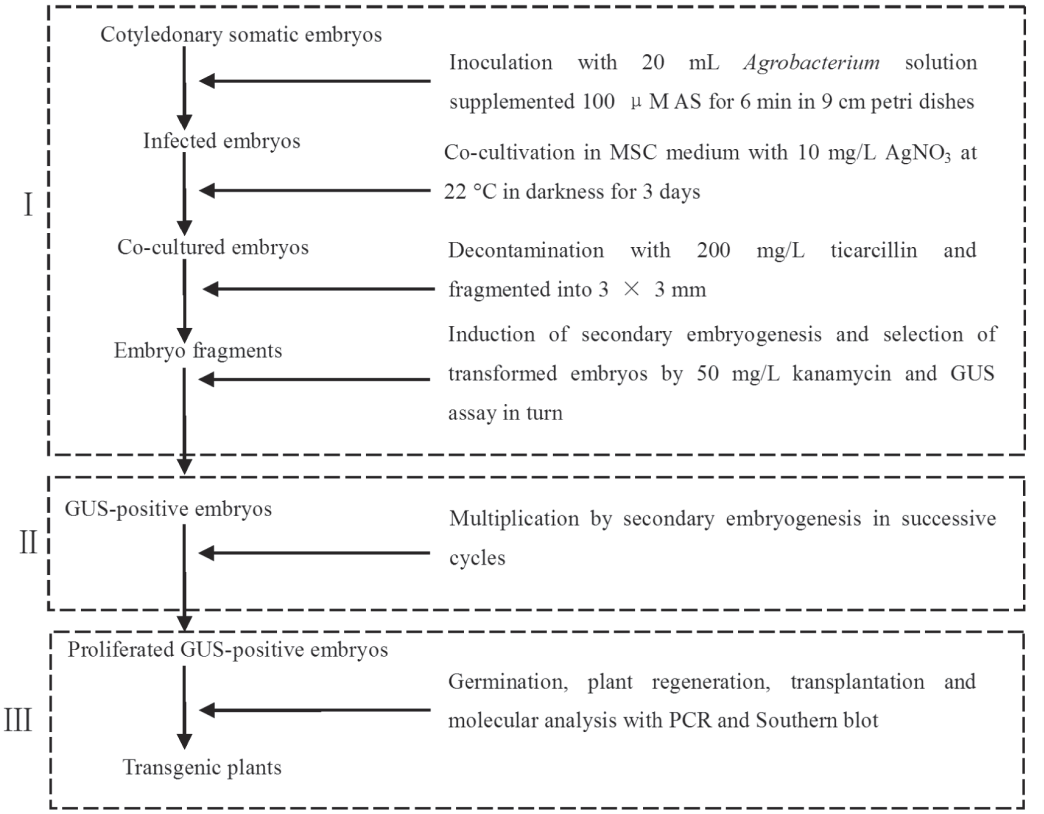
After the three day recovery period, the infected embryos were cut into 3 × 3 mm fragments and cultured on callus induction medium (MSC) to select embryos resistant to kanamycin. After 25 days, the cultures were transferred onto embryo induction medium (MSE) and subcultured at 20-day intervals until kanamycin-resistant embryos formed. Kanamycin-resistant embryos with the size of 1.5 cm (*Figure 3G*) were collected and a single fragment with a size of 3 × 3 mm was excised from the cotyledon of the embryos for GUS assays (*Figures 3G*, *H*). The remaining GUS-positive embryos (P_0) were cut into 3 × 3 mm fragments and cultured on callus induction medium (MSC) for 20 days, and then transferred onto embryo induction medium (MSE), to increase the number of embryos. Two cycles of multiplication were carried out (P_1, P_2). After increasing the number of the GUS-positive embryos, the embryos of 1.5 cm size were incubated in 30 × 250 mm test tubes with 50 mL regeneration medium (MSR)

without antibiotics for plant regeneration. The entire transformation procedure is summarised in *Figure 1*.

GUS Assay

GUS assays[22] were performed by immersing plant materials overnight at 37ºC in a GUS assay buffer (0.1% Triton X-100, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 20% methanol and 1 mM X-Gluc (BBI, Canada)), which was prepared with 10 mM EDTA and 50 mM phosphate buffer (PH 7.0). Methanol was added to the reaction mixture to suppress endogenous GUS-like activity.

Cytological Observation of Agrobacterium Infection. After co-cultivation, the embryos



Transgenic plants were generated through the following steps: (I) Transformation (II) Propagation of the transgenic embryos by repetitive secondary embryogenesis (III) Regeneration of transgenic embryos into plants.

Figure 1. Scheme for Agrobacterium tumefaciens-mediated somatic embryo transformation in Hevea brasiliensis.

were washed with sterile water thrice, after blotting dry on filter paper, put into GUS assay solution, and incubated overnight at 37ºC. The embryos with blue staining were collected and fixed in 80% ethanol for 8 h, dehydrated with a series of 90% and 100% ethanol rinses and embedded in paraffin[23]. Paraffin sections were cut 10 µm thick with a microtome. After being covered, the slides were examined under a Leica light microscope and photos were taken using a Leica CCD DMF 500 (Leica, Germany).

Acclimatisation and Transplantation. Regenerated plantlets were taken out of the test tubes with forceps and the roots were washed with water to remove the medium, followed by transferring into a sand bed in a greenhouse and maintaining for 3–4 weeks at 80–85% relative humidity (RH) at 30ºC. Subsequently, the plants were exposed to external conditions over a period of another 3–4 weeks. Finally, hardened plantlets were transplanted into 35 × 18 cm polybags containing a mixture of soil and coconut chaff (3:1 v/v).

Molecular Analysis. Total genomic DNA was isolated from young leaves of putative transgenic plantlets and an untransformed plant using the HP Plant DNA Kit (OMEGA, USA). Plasmid DNA was extracted from total DNA according to the plasmid mini kit I (OMEGA). The pCAMBIA 2301 plasmid DNA was served as a positive control and the DNA from untransformed plantlets was used as a negative control in both PCR and Southern blot analysis.

PCR analysis was employed to detect *uid*A sequence in putative transgenic plantlets. The PCR mixture (15 µL) contained 2 µL 50 ng μL^{-1} genomic DNA, 1.5 µL 10× Taq Buffer, 1.2 µL 25 mM Mg^{2+}, 0.2 µL 10 mM dNTPs, 0.15 µL 5 U μL^{-1} Taq (Fermentas, USA) and 0.3 µL 10 µmol L^{-1} each primer (*Table 1*). PCR was performed in a thermal cycler (Eppendorf Mastercycler gradient, Germany) with the following conditions: 1 cycle of 94ºC for 3 min, followed by 35 cycles of 94ºC for 30s, 58ºC for 40s and 72ºC for 90s with a final extension step of 72ºC for 10 minutes. Amplicons were visualised on a 1.0% agar gel stained with Goldenview I (Solarbio, China) and photographed under UV light using Unipro (Unitec, USA).

Southern blot analysis was performed according to the manufacturer's instructions (Roche, Germany). Ten micrograms of genomic DNA was digested overnight with *Hind* III (NEB, England) at 37ºC, separated by electrophoresis in a 0.8% (w/v) agarose gel, and transferred onto a nylon membrane (Millipore, USA). DNA probes with sizes of 1200 bp corresponding to *uid*A, was labeled with digoxigenin by the PCR method according to the PCR DIG Probe Synthesis Kit (Roche, Germany); the primers used to amplify the probe were the same as those in PCR analysis. The hybridisation was performed in a hybridisation chamber (Multidizer, USA) at 50ºC for 16 hours. The hybridised membrane was washed and subjected to chemiluminescent development according to the DIG High Prime DNA

TABLE 1. PRIMERS FOR PCR AND SOUTHERN ANALYSIS OF TRANSGENIC PLANTLETS

Target genes	Primers	Sequences
*uid*A*	P1	5'-GGTGGGAAAGCGCGTTACAAG-3'
	P2	5'-GTTTACGCGTTGCTTCCGCCA-3'

* The *uid*A has an intron.

Labeling and Detection Starter Kit II (Roche), and then exposed to film (Kodak Biomax Light 1, Japan).

Statistical Analysis. In antibiotics selection experiments, the percentages of embryo sections producing secondary embryos, resistant secondary embryos and dicotyledonary embryos were calculated. The percentages were calculated by taking the number of embryo sections producing secondary embryos, resistant secondary embryos and resistant dicotyledonary embryos and dividing them by the total number of total embryo sections × 100. Stable transformation efficiency (%) was calculated as the number of GUS staining events recovered per 100 infected embryos. The GUS-positive embryos were defined as the dicotyledonary embryos which stained a uniform deep blue. The blotted and light blue embryos, likely caused by background, were not counted. A significance test was carried out with the SAS software, version 8.0.

RESULTS

Effects of Kanamycin and Ticarcillin on Embryo Formation

The effects of kanamycin and ticarcillin on somatic embryo formation were evaluated. Somatic embryogenesis was significantly suppressed by the addition of kanamycin into the callus and embryo induction media, and the number of embryos declined with increasing kanamycin concentration (*Figure 2, Table 2*). The percentage of cotyledonary embryos sharply declined from 54.49% (without kanamycin) to 8.80% (50 mg L^{-1} kanamycin), while there was no significant difference between media with 50 or 75 mg L^{-1} kanamycin added. Therefore, 50 mg L^{-1} kanamycin was chosen for selection of putative transformants. In contrast, the cotyledonary embryo number increased and then decreased with increasing ticarcillin concentration. The cotyledonary embryo number reached a maximum (46.74%) at 200 mg L^{-1} ticarcillin (*Figure 2, Table 2*),

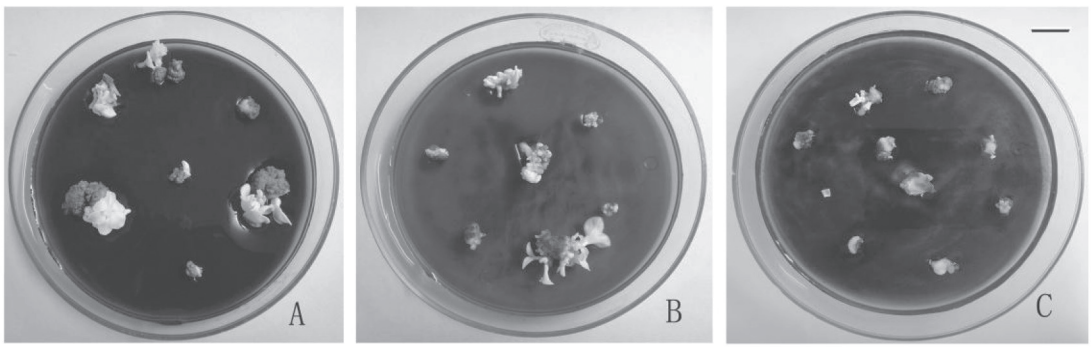


Figure 2. Effects of antibiotics on development of secondary somatic embryos.
(a) Embryos developed on MSE medium without antibiotics (b) With 200 mg/L ticarcillin
(c) With 100 mg/L kanamycin. Scale bar = 1.0 cm.

TABLE 2. EFFECTS OF VARIOUS CONCENTRATIONS OF KANAMYCIN AND TICARCILLIN ON SECONDARY EMBRYOGENESIS

Treatments	Levels	% embryo sections producing secondary embryos[1]	% resistant secondary embryos[2]	%resistant dicotyledonary secondary embryos[3]
Kanamycin	0 mg l^{-1}	52.54±31.77^{a}	223.37±126.85^{a}	54.49±35.00^{a}
	50 mg l^{-1}	39.99±3.74^{ab}	108.11±37.36^{ab}	8.80±10.79^{b}
	75 mg l^{-1}	21.78±8.86^{ab}	52.73±35.53^{b}	8.63±10.28^{b}
	100 mg l^{-1}	6.78±5.52^{b}	19.86±21.92^{b}	1.08±1.86^{b}
	125 mg l^{-1}	10.51±3.99^{ab}	21.54±2.18^{b}	0.00±0.00^{b}
Ticarcillin	0 mg l^{-1}	39.79±11.54^{b}	62.60±25.98^{a}	18.60±1.82^{b}
	100 mg l^{-1}	61.05±6.04^{a}	93.97±8.53^{a}	43.04±14.38^{a}
	200 mg l^{-1}	58.09±12.61^{ab}	98.24±13.97^{a}	46.74±5.84^{a}
	300 mg l^{-1}	41.52±9.04^{ab}	69.43±35.07^{a}	32.55±11.89^{ab}
	400 mg l^{-1}	45.24±10.91^{ab}	73.21±27.49^{a}	30.36±15.87^{ab}

1 No. embryo sections producing secondary embryos/total embryo sections × 100
2 No. resistant secondary embryos/total embryo sections × 100
3 No. resistant dicotyledonary secondary embryos/total embryo sections × 100
The letters show significant difference ($P<0.05$)

which was higher than that of no addition of ticarcillin (18.60 %), indicating that addition of an appropriate amount of ticarcillin into the callus and embryo induction media can control *Agrobacterium* growth and was also helpful for embryo formation. Thus, 200 mg L^{-1} ticarcillin was used to control the growth of *Agrobacterium*.

Characterisation of Agrobacterium Infection of Somatic Embryos

To characterise the infection of somatic embryos by *Agrobacterium*, 30 cotyledonary embryos with the size of 1.5 cm were inoculated with *Agrobacterium* in three isolated experiments, and the infection levels of somatic embryos were evaluated by transient GUS expression. In the results, 9.33 ± 1.15 somatic embryos per 10 embryos had blue foci and each embryo had 16.00 ± 6.00 blue spots on average. The blue foci were mainly distributed along the cotyledon, rarely at the base of the embryos, and the blue foci were mainly a deep blue colour (*Figures 3A, B*). Additionally, cytological observations showed that the intensity of the blue was correlated to cell layers stained by GUS; the more cell layers stained, the deeper the colour of the blue foci was (*Figures 3D, E*). Indigo dye precipitates were observed in the cytoplasm (*Figure 3F*), indicating that both the epidermal and subepidermal layer cells of cotyledons of the somatic embryos were transformed by *Agrobacterium* and that the T-DNA of the vector pCAMBIA2301 was successfully integrated into the cells of somatic embryos.

The Transformation Efficiency of Somatic Embryos

To evaluate the transformation efficiency by stable GUS expression, five independent transformation experiments were carried out (*Table 3*). A total of 1158 cotyledonary embryos

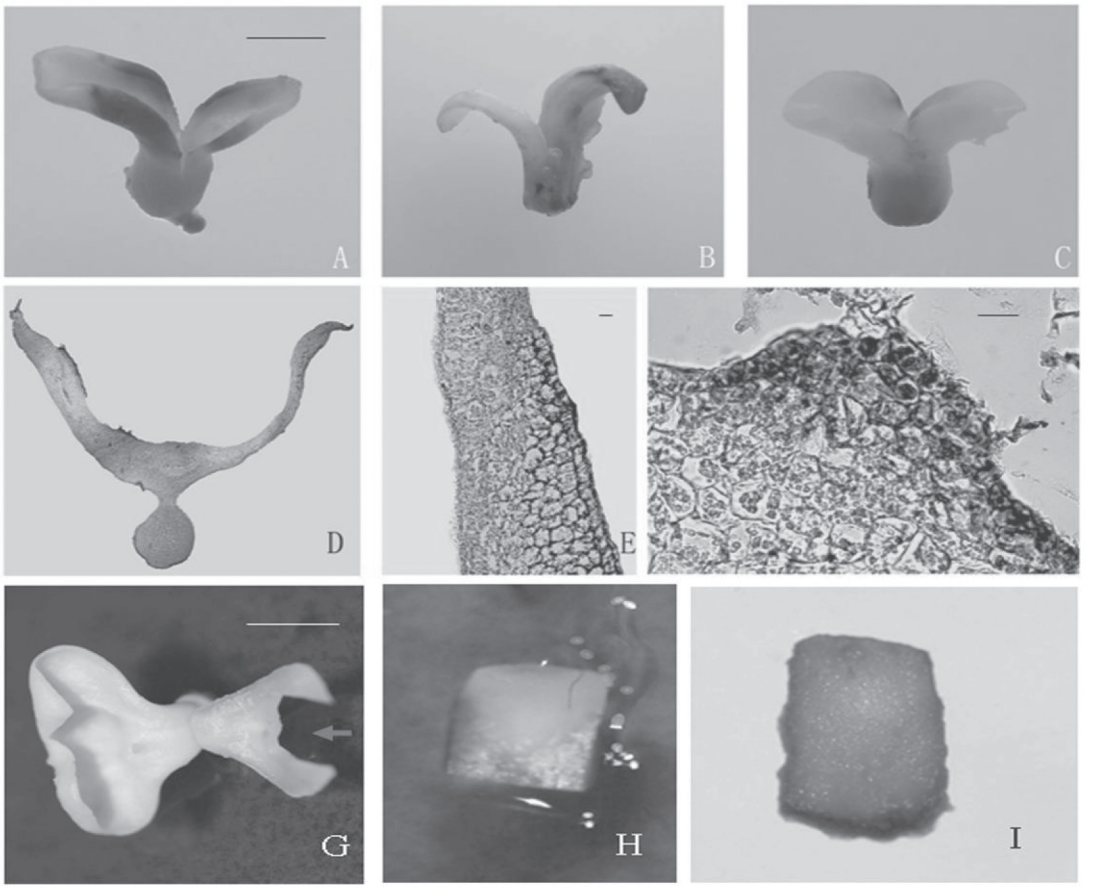


Figure 3. GUS transient and stable expression. (a–f) GUS transient expression. Blue foci were mainly distributed along the surface (b) and the edge (a, c) of the cotyledon (c). Both epidermal (e) and subepidermal (d) layers of the cotyledon were infected by Agrobacterium and the GUS gene was expressed in the cytoplasm (f) (deep blue cells). (g-i) selection of GUS-positive embryos: 3 × 3 mm fragments (h) were isolated from kanamycin-resistant embryos for GUS assay, the arrow shows the position of an embryo fragment (g). After GUS assay (i), the remaining GUS-positive embryos (g) were propagated by secondary embryogenesis. Scale bars = 0.5 cm (a–d, g-h) and 10 μm (e, f).

were infected and 2445 kanamycin-resistant embryos were regenerated, therefore 2.11 (2445/1158) kanamycin-resistant embryos were produced per embryo by transformation. Additionally, 47 resistant embryos (P_0, initial and non-propagated transgenic embryos) were GUS-positive, showing that the T-DNA was successfully integrated into the nuclear genome of rubber tree. The rate of transformation ranged from 1.94 to 6.03% in the five transformation experiments, with an average of 4.06 % (47/1158).

TABLE 3. EVALUATION OF TRANSFORMATION EFFICIENCY IN FIVE ISOLATED TRANSFORMATION EXPERIMENTS

Experiments	Infected embryos Nos.	Regenerated embryos resistant to Kan Nos.	GUS-positive embryo Nos.	Rate (%)
1*	232	115	14	6.03[a]
2	230	610	11	4.78[b]
3	248	832	13	5.24[c]
4	206	638	4	1.94[d]
5	242	250	5	2.07[e]
Total	1158	2445	47	4.06

*The GUS-positive embryos were used for evaluation of the stability of GUS activity after multiplication, plant regeneration and molecular analysis. The letters show significant difference ($P<0.05$).

Transgenic Plant Regeneration and Transplantation

Fourteen P_0 embryos produced in the first transformation experiment were propagated by secondary embryogenesis for two cycles (P_1, P_2). The two cycles of secondary embryogenesis created 7 lines (L10, L12, L13, L14, L20, L27, and L68). Seven were successfully propagated and produced 114 P_2 cotyledonary embryos (*Table 4*), of which 51 P_2 embryos derived from 6 P_0 embryos were converted into 33 plantlets, in which 19 plantlets from 6 independent transformed embryos (58%) survived after transplantation into polybags (*Figure 4, Table 4*), indicating that transgenic plants could be obtained after transplantation, as long as GUS-positive embryos could be propagated through secondary embryogenesis.

Propagation of GUS-Positive Embryos through Secondary Embryogenesis

In order to evaluate the propagation efficiency of the GUS-positive embryos, three additional generations of proliferations (P_3-P_5) were carried out through successive secondary embryogenesis. Each cycle of proliferation tended to slightly increase with multiplication cycles (*Table 5*). For example, when Line 10 went through its proliferation cycles, P_3 increased by the rate of 3.3, while P_5 increased by 5.3. This increase in the rate of proliferation was uniform throughout the lines that were produced showing that transgenic embryos could be propagated through successive secondary embryogenesis.

After each generation of multiplication, mature embryos used for next multiplication were collected for GUS assay. All of the embryos from Lines 10, 12 and 13 were GUS-positive, but two out of 18 embryos from Line 20 after the fifth generation of multiplication were GUS-negative. We propagated these two embryos for one more multiplication and monitored the stability of GUS activity. We found that two embryos from one negative embryo were GUS-negative, while five embryos from the other negative embryo showed different situation, two were GUS-negative, three were GUS-positive, indicating that the initial embryo of Line 20 might have been chimeric.

Molecular Analysis of Transgenic Plants

PCR and Southern blot analysis were carried out to detect the sequences of *uid*A and *npt*II to further validate whether foreign

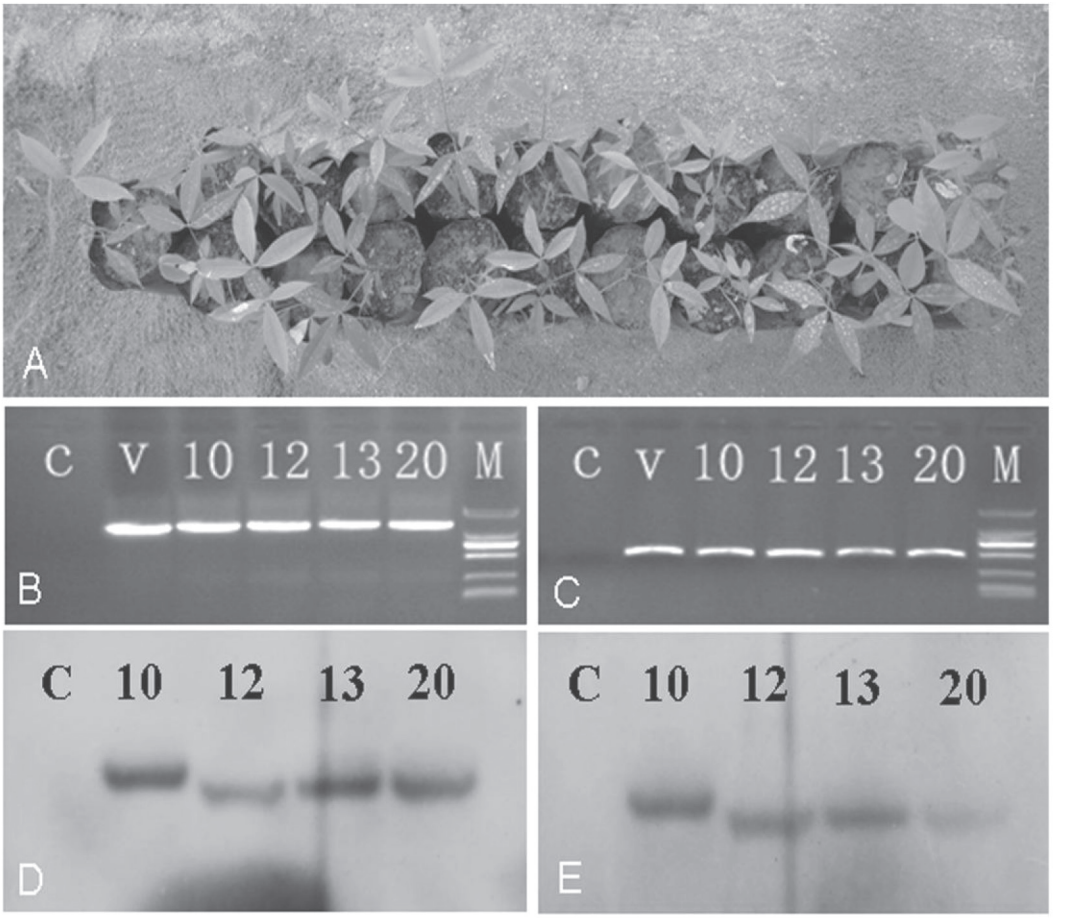


Figure 4. Plantlets regenerated from transgenic embryos and detection of uidA and nptII by PCR and Southern blot analysis. (a) Plantlets regenerated from GUS-positive embryos; b and c, detection of uidA (b) and nptII (c) in regenerated plantlets by PCR analysis; d and e, detection of uidA (d) and nptII (e) in regenerated plantlets by Southern blot analysis. In b–e, 'C' refers to non-transformed plants, 'V' refers to plasmid DNA of the transformation vector, 'M' refers to the 100 bp marker ladder, and '10', '12', '13' and '20' refer to transformed plant lines produced in the first transformation experiment.

DNAs have integrated into the genome of transgenic plantlets. Primers were designed to amplify DNA fragments of 453 bp and 1.2 kb, respectively. Because only one plantlet was obtained from Lines 27 and 68, only Lines 10, 12, 13 and 20 were analysed by PCR and Southern blot analysis. For PCR analysis, primers were designed to amplify portion of the *uid*A gene with a fragment size of 1.2 kb. Strong bands corresponding to *uid*A was detected in the four transgenic plant lines and the plasmid DNA (positive control) by PCR, but not in the non-transformed control (*Figures 4B, C*), indicating that the T-DNA was integrated into the nuclear genome of the four transgenic plant lines.

TABLE 4. PLANT REGENERATION AND TRANSPLANTATION OF INDIVIDUAL TRANSGENIC EMBRYOS DERIVED FROM THE FIRST TRANSFORMATION EXPERIMENT AFTER TWO-CYCLE MULTIPLICATIONS BY SECONDARY EMBRYOGENESIS

References	Embryos Nos. after two-cycle multiplications	Embryos for plant regeneration Nos.	Regenerated plantlets Nos.	Survived plantlets Nos.
L10	21	7	5	4
L12	19	7	2	2
L13	21	2	2	2
L14*	1			
L20	30	20	15	9
L27	20	13	8	1
L68	2	2	1	1
Total	114	51	33	19

*Plant regeneration was not carried out because few embryos were produced through two cycles of secondary embryogenesis.

TABLE 5. THE RATE OF MULTIPLICATION AFTER THREE FURTHER GENERATIONS OF MULTIPLICATION

Generations	References	L10	L12	L13	L20
P_3	The initial embryo Nos.	9	7	23	14
	Mature cotyledonary embryo Nos.	30	17	115	46
	The rate of multiplication	3.3^{a}	2.43^{b}	5.0^{c}	3.29^{ac}
P_4	The initial embryo Nos.	23	21	24	18
	Mature cotyledonary embryo Nos.	76	22	165	89
	The rate of multiplication	3.3^{a}	1.0^{b}	6.9^{c}	4.9^{d}
P_5	The initial embryo Nos.	32	30	26	28
	Mature cotyledonary embryo Nos.	170	135	204	133
	The rate of multiplication	5.3^{a}	4.5^{b}	7.8^{c}	4.8^{bd}

Note: the rate of multiplication being compared between each line and the letters show significant difference ($P<0.05$).

The four transgenic plantlet lines were further analysed with *uid*A probe in a southern hybridisation. Distinct bands representing *uid*A gene was detected in the genomes of the transgenic plants. Additionally, the presence of a single band in each lane might suggest single integration event in these lines (*Figures 4D, E*). All those results indicated that the T-DNA of the vector pCAMBIA2301 was successfully transferred into rubber tree.

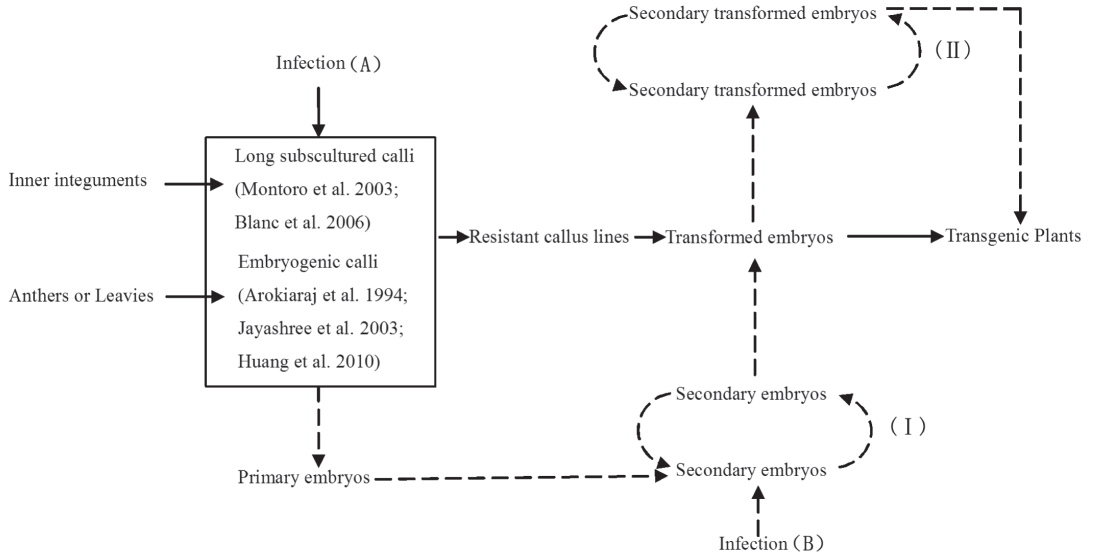
DISCUSSION

Somatic Embryo as the Target Tissue of Transformation in *H. brasiliensis*

The first transgenic rubber tree was produced by particle bombardment[4]. Subsequently, an *Agrobacterium*-mediated transformation system was developed[5,12,25–27], and is more popularly used now. The proven target material for *Agrobacterium* infection is, embryogenic callus, whatever anther, seed inner integument or leaf-derived, was the only source of transformation in *H. brasiliensis* in previous studies (*Figure 5*). However, the regeneration capacity of the embryogenic callus sharply decreases after infection by *Agrobacterium*, which is one of the key reason on low efficiency of genetic transformation in *H. brasiliensis*. A highly efficient somatic embryogenesis system is a prerequisite for the development of a highly efficient genetic transformation system. The establishment of secondary embryogenesis in *H. brasiliensis*[20] allowed us to investigate somatic embryos as the target material for *Agrobacterium* infection, and transgenic plants with *uid*A and *npt*II genes were successfully generated through the following three steps: transformation, proliferation of positive embryos and plant regeneration (*Figure 1*). The rate of stable transformation with 4.06% on average was achieved.

Compared to the previous embryogenic callus transformation in *H. brasiliensis*, somatic embryos as the target material for *Agrobacterium* infection can be stably maintained by secondary embryogenesis in a reproducible manner. Therefore, the transformation material can be available year round, avoiding the need to frequently collect explants (*e.g*. anthers, fruits, or leaves) in the field. Additionally, after transformation and production of resistant embryos, only small piece of tissue needs to be excised from each resistant embryo for GUS assays, and then the remaining GUS-positive fragments can be proliferated by secondary embryogenesis in successive cycles. Proliferation of GUS-positive embryos by successive cycles of secondary embryogenesis results in each transgenic embryo generating a transgenic embryo line with uniform genotype, after plant regeneration, allowing the avoidance of the loss of transformants during plant regeneration and transplantation (*Figure 5*). Of course, not all of transgenic embryos could be successfully propagated. About 42.9% (6/14) of transgenic embryos were successfully propagated by secondary embryogenesis using only two cycles, so the propagation rate needs to be improved.

Due to poor transformation efficiency, only a small number of genetically modified rubber trees have been previously able to be produced from the clones GL1, RRII 105, PB260 and CATAS 7-33-97[5,25,27–28]. The poor transformation efficiency of *H. brasiliensis* mainly results from poor regeneration capability of the transformed calli[28]. Blanc *et al*.[28] produced transgenic plants from 6 of 9 independent transgenic callus lines (66.7%), which is the highest efficiency reported for *H. brasiliensis*. In contrast, 6 of 7 transgenic embryo lines (85.7%) from our study produced transgenic plants, and because of the ability to proliferate the transgenic



The major difference with previous reports was that the somatic embryos (B) were used as the target material with Agrobacterium instead of calluses (A), resulting in the infection materials being available year round and transgenic plants being directly proliferated by repetitive secondary embryogenesis (I and II, respectively).

Figure 5. Comparison of transformation strategies in previous reports (solid lines) and this study (dotted lines).

embryos by secondary embryogenesis, there were enough individuals in each line so that each line had transgenic plants that survived after transplantation. In addition, the friable embryogenic calli are difficult to be produced[28].

Secondary Embryogenesis as an Effective Way to Propagate Transgenic Plants in *H. brasiliensis*

Transgenic plants have been widely produced and utilised in many plant species. Preliminary studies showed that the expression of three transgenes, *uid*A, *npt*II and ScFv4715, was relatively stable in transgenic *H. brasiliensis* after three successive vegetative propagation cycles by budding[10,25,29], but a lower level of GUS activity and greater variability were observed in fourth to seventh generation of budded plants compared with self-rooting transgenic plants[24], indicating somatic embryogenesis remains an effective way of propagating transgenic lines[24]. In this investigation, five propagation cycles were successfully produced, and number of embryos generated per cycle tended to progressively increase with each proliferation cycle (*Table 5*). Therefore, secondary embryogenesis system could be an effective way to propagate the transgenic plants.

Transformation of *H. brasiliensis* Somatic Embryos

Secondary embryos usually originate from epidermal but not mesophyll cells of somatic or zygotic embryos, and this characteristic is beneficial for infection by *Agrobacterium*[13]. Thus, many plant transformation systems choose somatic or zygotic embryos as target tissues for *Agrobacterium* infection[14,30–32]. In our investigation, *Agrobacterium* infection of *H. brasiliensis* somatic embryos was cytologically characterised, and we found that both epidermal and mesophyll cells were successfully infected and that the T-DNA was also transferred into the infected cells. However, *H. brasiliensis* secondary embryos mainly originate from the epidermal cells of somatic embryos (unpublished data), so infecting mesophyll cells will not help to improve the transformation efficiency. On the contrary, excessive infection might result in a decrease of the ability of epidermal cells to undergo embryogenesis, and reduce the transformation efficiency. The infection protocol used, such as concentration of the bacterial cells, duration of inoculation and pre-treatment of somatic embryos, could be optimised to balance the infection efficiency and vigour of the epidermal cells.

To date, *npt*II has been the only selectable marker gene used in *H. brasiliensis* transformation, including this study[7,27–28,33]. However, two kinds of selection antibiotics, paromomycin[28] and kanamycin[7,27,33] have been chosen to select transformants. Paromomycin had a higher selection efficiency than kanamycin; for example, 19 out of 24 resistant transgenic callus lines selected using paromomycin gave a positive GUS assay[28], whereas 6% and 8% of transgenic callus lines selected using kanamycin were positive in Arokiaraj *et al*.[33] and Sobha *et al*.[7] respectively. Notably, two selection strategies were used: selection with a gradually increasing concentration of antibiotics[28] and continual selection with the same concentration of antibiotics[7,27,33]. In our study, only 1.92% (47/2445) of resistant embryos was found to be transgenic using continual kanamycin selection with the same concentration during subculture. Therefore, it is necessary to optimise selection strategies further for somatic embryo transformation in *H. brasiliensis*.

The Generation of Escape and Chimerism

Here, we found that only 1.92 % (47/2445) of resistant embryos was transgenic using continual kanamycin selection with the same concentration during subculture, and Blanc *et al*. also observed the production of non-transgenic tissues according to their GUS and paromomycin-based protocol[28]. The objective of transformation selection is to let the transgenic cells develop into plants and inhibit development of non transgenic cells, but high concentration of selection agent would inhibit development of both transgenic and non transgenic cells, so escape and selection should be balanced in plant transformation. In this investigation, lower concentration of kanamycin (50 mg L^{-1}) was chosen for selection of putative transformants, which enable transgenic tissues to survive in the brown non-resistant surrounding tissues[34].

In this investigation, five multiplication generations were successfully produced, but 2 out of 18 embryos from Line 20 were GUS-negative in the fifth generation, subsequent propagation and GUS assay showed that some embryos were GUS-positive but the others were negative, we inferred that the initial embryo of Line 20 should be chimeric. In apple and tomato, chimerism was detected as well under selective and non selective conditions with kanamycin[35,36]. The development of chimeric plants depends mostly on the transformation system which

is responsible for whether dedifferentiated or differentiated cells become transformed[36]. Our study showed that secondary embryos of rubber tree occurred at both epidermal and subepidemal cells but mainly at epidermal cells, and secondary embryos- derived epidermal cells originated from single cell (unpublished data). Here, the microscopic observation of transient expression of GUS showed that the epidermal and subepidermal cells could be infected by *Agrobacterium*. We inferred that chimeric transgenic embryos may derive from subepidermal cells which are multicellular origin and need to be verified in further experiments.

CONCLUSION

In this study, we showed that somatic embryos is a viable alternative to embryogenic callus in transformation of *Hevea* and stable transformation embryos was 4.06% (47/1158) on average. Additionally, 33 plants derived from six individual transgenic embryos were produced through somatic embryo transformation.

ACKNOWLEDGMENT

This research was supported by basic research funds for the Central Public Research Institute (NO. 1630022012006), the National Natural Sciences Foundation (NO. 30960322; 31200503), the 863 Program (2013AA102605) and the 973 program (2012CB723005).

Date of receipts: January 2015
Date of acceptance: July 2015

REFERENCES

1. MURASHIGE, T. AND SKOOG, F. (1962) A revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Physiol. Plant*, **15(3)**, 473–497.

2. PRIYADARSHAN, P.M. AND GONCALVES, P.S. (2003) *Hevea* Gene Pool for Breeding. *Genet. Resour. Crop Evol*., **50(1)**, 101–114.

3. CLÉMENT-DEMANGE, A., PRIYADARSHAN, P.M., HOA, T.T.T. AND VENKATACHALAM, P. (2007) *Hevea* Rubber Breeding and Genetics. *Plant Breed. Rev*., **29**, 177–283.

4. AROKIARAJ, P., JONES, H., CHEONG, K.F., COOMBER, S. AND CHARLWOOD, B.V. (1994) Gene insertion in *Hevea brasiliensis*. *Plant Cell Rep*., **13(8)**, 425–431.

5. JAYASHREE, R., REKHA, K., VENKATACHALAM, P., URATSU, S.L., DANDEKAR, A.M., KUMARI JAYASREE, P., KALA, R.G., PRIYA, P., SUSHMA KUMARI, S., SOBHA, S., ASHOKAN, M.P., SETHURAJ, M.R. AND THULASEEDHARAN, A. (2003) 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. *Plant Cell Rep*., **22(3)**, 201–209.

6. REKHA, K., JAYASHREE, R., KUMARI JAYASREE, P., VENKATACHALAM, P., JINU, C. AND THULASEEDHARAN, A. (2006) An Efficient Protocol for *Agrobacterium*-Mediated Genetic Transformation in Rubber Tree (*Hevea brasiliensis*). *Plant Biotechnol. Cell Mol. Biol*., **7(3–4)**, 155−158.

7. SOBHA, S., SUSHAMAKUMARI, S., THANSEEM, I., KUMARI JAYASREE, P., REKHA, K., JAYASHREE, R., KALA, R.G., ASOKAN, M.P., SETHURAJ, M.R., DANDEKAR, A.M. AND THULASEEDHARAN, A. (2003) Genetic Transformation of *Hevea brasiliensis* with the Gene Coding for Superoxide Dismutase with FMV 34S promoter. *Curr. Sci*., **85(12)**, 1767–1773.

8. LECLERCQ, J., MARTIN, F., SANIER, C., CLÉMENT-VIDAL, A., FABRE, D., OLIVER, G., LARDET, L., AYAR. A.,

PEYRAMARD, M. AND MONTORO, P. (2012) Over-Expression of a Cytosolic Isoform of the *HbCuZnSOD* Gene in *Hevea brasiliensis* Changes its Response to a Water Deficit. *Plant Mol. Biol*., **80(3)**, 255–72.

9. AROKIARAJ, P., JAAFAR, H., HAMZAH, S., YEANG, H.Y. AND WAN ABDUL RAHAMAN, W.Y. (1995) Enhancement of *Hevea* Potential by Genetic Transformation: HMGR Activity in Transformed Tissue. In: Proceedings of the International Rubber Research Development Board Symposium on Physiological and Molecular Aspects of Breeding of *Hevea brasiliensis*. November 6–7, Penang, Malaysia, 74–82.

10. AROKIARAJ, P., RUEKER, F., OBERY-MAYR, E., SHAMSUL BAHRI, A.R., HAFSAH, J., CARTER, D.C. AND YEANG, H.Y. (2002) Expression of Human Serum Albumin in Transgenic *Hevea brasiliensis*. *J. Rubb. Res*., **5(3)**,157–166.

11. YEANG, H.Y., AROKIARAJ, P., JAAFAR, H., ARIF, S.A.M., RAJAMANIKAM, S., CHAN, J.L., SHARIB, J., LEELAVATHI, R., SAMSIDAR, H. AND VAN DER LOGT, C.P.E. (2002) Expression of a Functional Recombinant Antibody Fragment in the Latex of Transgenic *Hevea brasiliensis*. *J. Rubb. Res*., **5(4)**, 215–225.

12. KALA, R.G., ANU, K.S., MANESH, K., SALEENA, A., JARYSREE, P.K., NARAYANAN, P.R., THOMAS, G. AND THULASEEDHARAN, A. (2006) *Agrobacterium* Mediated Genetic Transformation in *Hevea brasiliensis* form Recombinant Protein Production. *J. Plantation Crops*, **34(3)**, 582–586.

13. RAEMAKERS, C.J.J.M., JACOBSEN, E. AND VISSER, R.G.F. (1995) Secondary Somatic Embryogenesis and Applications in Plant Breeding. *Euphytica*, **81(1)**, 93–107.

14. ÁLVAREZ, R., ALONSO, P., CORTIZO, M., CELESTINO, C., HERNÁNDEZ, I., TORBIO, M. AND ORDÁS, R.J. (2004) Genetic Transformation of Selected Mature Cork Oak (*Quercus suber L*.) Trees. *Plant Cell Rep*., **23(4)**, 218–223.

15. KUMAR, V., SATYANARAYANA, K.V., SARALA ITTY, S., INDU, E.P., GIRIDHAR, P., CHANDRASHEKAR, A. AND RAVISHANKAR, G.A. (2006) Stable Transformation and Direct Regeneration in *Coffea canephora* P. ex. Fr. by *Agrobacterium rhizogenes* Mediated Transformation Without Hairy-Root Phenotype. *Plant Cell Rep*., **25(3)**, 214–222.

16. ABDOLLAHI, M.R., MOIENI, A., MOUSAVI, A. AND SALMANIAN, A.H. (2011) High Frequency Production of Rapeseed Transgenic Plants *via* Combination of Microprojectile Bombardment and Secondary Embryogenesis of Microspore-Derived Embryos. *Mol. Biol. Rep*., **38(2)**, 711–719.

17. NINKOVIĆ, S., MILJUS-DJUKIĆ, J. AND NESKOVIĆ, M. (1995) Genetic Transformation of Alfalfa Somatic Embryos and Their Clonal Propagation through Repetitive Somatic Embryogenesis. *Plant Cell Tissue Organ Cult*., **42(3)**, 255–260.

18. ASOKAN, M.P., KUMARI, P.J., THOMAS, V., SUSHAMA, S.K., KALA, R.G., JAYASREE, R., SETHURAJ, M.R. AND THULASEEDHARAN, A. (2002) Influence of 2, 4-D and Sucrose on Repetitive Embryogenesis in Rubber (*Hevea brasiliensis* Muell. Arg. cv GT1). *J. Tree Sci*., **21(1−2)**, 18–26.

19. KALA, R.G., KURUVILA, L., JAYASREE, P.K., KUMARI, S.S., JAYASHREE, R., REKHA, K., SOBHA, S. AND THULASEEDHARAN, A. (2008) Secondary Embryogenesis and Plant Regeneration From Leaf Derived Somatic Embryo of *Hevea brasiliensis*. *J. Plantation Crops*, **36(3)**, 218–222.

20. HUA, Y.W., HUANG, T.D. AND HUANG, H.S. (2010) Micropropagation of Self-Rooting

Juvenile Clones by Secondary Somatic Embryogenesis in *Hevea brasiliensis*. *Plant Breed*, **129(2)**, 202–207.

21. BURROW, M.D., CHLAN, C.A., SEN, P. AND MURAI, N. (1990) High Frequency Generation of Transgenic Tobacco Plants after Modified Leaf Disk Cocultivation with *Agrobacterium* tumefaciens. *Plant Mol. Biol. Rep*., **8(2)**, 124−139.

22. JEFFERSON, R.A. (1987) Assaying Chimeric Genes in Plants, the GUS Gene Fusion System. *Plant Mol. Biol. Rep*., **5(4)**, 387–405.

23. JOHANSEN, D.A. (1940) Plant Microtechniques. New York: McGraw-Hill.

24. LARDET, L., LECLERCQ, J., BÉNISTAN, E., DESSAILLY, F., OLIVER, G., MARTIN, F. AND MONTORO, P. (2011) Variation in GUS Activity in Vegetatively Propagated *Hevea brasiliensis* Transgenic Plants. *Plant Cell Rep*., **30(10)**, 1847–1856.

25. AROKIARAJ, P., JONES, H., JAAFAR, H., COOMBER, S. AND CHARLWOOD, B.V. (1997) *Agrobacterium*-Mediated Transformation of *Hevea* Anthers Callus and Their Regeneration Into Plantlets. *J. nat. Rubb. Res*., **11(2)**, 77–87.

26. MONTORO, P., TEINSEREE, N., RATTANA, W., KONGSAWADWORAKUL, P. AND MICHAUX-FERRIERE, N. (2000) Effect of Exogenous Calcium on *Agrobacterium* Mediated Gene Transfer in *Hevea brasiliensis* (Rubber Tree) Friable Calli. *Plant Cell Rep*., **19(9)**, 851–855.

27. HUANG, T.D., LI, Z., SUN, A.H., ZHOU, Q.N., HUA, Y.W. AND HUANG, H.S. (2010) Establishment of *Agrobacterium tumefaciens*-Mediated Anther Calli Transformation System in *Hevea brasiliensis*. *Acta. Agron. Sin*., **36**, 1691–1697 (in Chinese, with English abstract).

28. BLANC, Ġ., BAPTISTE, C., OLIVER, Ġ., MARTIN, F. AND MONTORO, P. (2006) Efficient *Agrobacterium tumefaciens*-Mediated Transformation of Embryogenic Calli and Regeneration of *Hevea brasiliensis* Müll Arg. Plants. *Plant Cell Rep*., **24(12)**, 724–733.

29. AROKIARAJ, P., SHAMSUL BAHRI, A.R. AND HAFSAH, J. (2003) Field Evaluation of Transgene Expression in Vegetative Generations and Embryo-Rescued Transgenic *Hevea brasiliensis*. In: Proceedings of the International Rubber Research Development Board Symposium 'Challenges for Natural Rubber in Globalization', Chiang Mai, Thailand, 15−16 Sept.

30. SINGH, N., MISHRA, A., JOSHI, M. AND JHA, B. (2010) Microprojectile Bombardment Mediated Genetic Transformation of Embryo Axes and Plant Regeneration in Cumin (*Cuminum cyminum* L.). *Plant Cell Tissue Organ Cult*., **103(1)**, 1–6.

31. LIU, W., LIANG, Z., SHAN, C., MARSOLAIS, F. AND TIAN, L. (2013) Genetic Transformation and Full Recovery of Alfalfa Plants *via* Secondary Somatic Embryogenesis. *In Vitro Cell Dev. Biol. Plant*, **49(1)**, 17–23.

32. MAXIMOVA, S., MILLER, C., DE MAYOLO, A., PISHAK, S., YOUNG, A. AND GUILTINAN, M.J. (2003) Stable Transformation of *Theobroma cacao* L. and Influence of Matrix Attachment Regions on GFP Expression. *Plant Cell Rep*., **21(9)**, 872–883.

33. AROKIARAJ, P., YEANG, H.Y., CHEONG, K.F., HAMZAH, S., JONES, H., COOMBER, S. AND CHARLWOOD, B.V. (1998) CaMV 35S Promoter Directs β-glucuronidase Expression in the Laticiferous System of Transgenic *Hevea brasiliensis* (Rubber Tree). *Plant Cell Rep*., **17(8)**, 621–625.

34. LECLERCQ, J., LARDET, L., MARTIN, F., CHAPUSET, T., OLIVER, G. AND MONTORO, P. (2010) The Green

Fluorescent Protein as an Efficient Selection Marker For *Agrobacterium tumefaciens*-mediated Transformation in *Hevea brasiliensis* (Müll. Arg). *Plant Cell Rep*., **29(5)**, 513–522.

35. LI, B., XIE, C. AND QIU, H. (2009) Production of Selectable-Marker-Free Transgenic Tobacco Plants Using a Non-Selection Approach: Chimerism or Escape, Transgene Inheritance, and Efficiency. *Plant Cell Rep*., **28(3)**, 373–386.

36. MALNOY, M., BORESJZA-WYSOCKA, E.E., NORELLI, J.L., FLAISMANN, M.A., GIDONI, D. AND ALDWINCKLE, H.S. (2010) Genetic Transformation of Apple (Malus x Domestica) Without Use of a Selectable Marker. *Tree Gen. Gen*., **6(3)**, 423–433.