Possible Technologies for reducing emissions from the Automobile sector

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Replacing synthetic rubbers with NR or modified forms along with judicious choice of compounding ingredients mainly fillers contributes a lot in reducing the green house gas (GHG) emissions in rubber industry. Production of synthetic rubbers is energy intensive and causes GHG emissions while natural rubber (NR) is produced from a renewable means that removes carbon dioxide from the atmosphere. Carbon black and silica are the two important reinforcing fillers used in rubber industry. Carbon black is obtained by burning hydrocarbon oils while silica is obtained from mineral sources by simpler and less emitting methods. By replacing carbon black with silica there can be considerable reduction in GHG emissions as the production of carbon black from fossil fuel generates about 2.18 tons of CO_2 per tone of carbon black produced.

The tyre industry demands properties like low heat build-up along with combination of excellent abrasion resistance, good road grip and low rolling resistance. Globally about 22 million tons of rubbers are produced per year and about 50 % of this is consumed in the tyre sector. Natural rubber is used specifically for truck tyres and aero tyres while styrene butadiene rubber (SBR) is predominantly used for car tyres. The major synthetic rubbers consumed by the tyre industry are SBR, polubutadiene rubber (BR) and halobutyl rubber, the latter being used mostly as inner lining of tubeless tyres. By chemical modification the mechanical properties of NR can be suitably changed and used as substitutes for synthetic rubbers in tyre sector. One of the promising rubbers of this group is epoxidised natural rubber (ENR). Due to the presence of epoxy group, ENR helps in better interaction with silica filler and can by used for preparing rubber compounds filled with silica even without the aid of silane coupling agents. ENR has the additional advantage of good oil resistance along with air impermeability characteristics.

Development in future tyres will be based on better fuel efficiency and performance. Reduced rolling resistance gives improvement in fuel efficiency leading to lesser emissions. As the tire rotates under the weight of the vehicle, it experiences repeated cycles of deformation and recovery, and it dissipates the hysteresis energy loss as heat. Hysteresis is the main cause of energy loss associated with rolling resistance. About eighty five percent of the automobile derived carbon dioxide is emitted during driving and reduction of fuel consumption by modification in tyre design and compounding in reducing CO_2 emission is very critical. When developing tire compounds for low rolling resistance, the material classes having the most effect such as polymers and fillers have to be carefully chosen so as to have less hysterisis, good rubber filler interaction and good filler dispersion that lead to improved mechanical properties. The reduction in weight of tyres also reduces fuel consumption.

Solution SBR is known to provide outstanding wet grip/rolling resistance for fuel efficient tyre treads based on silica and/or carbon black. Carbon black is predominantly used in tires, but the use of silica has become a primary alternative to improve rolling resistance. When silica is used to improve rolling resistance, it is

traditionally used with a silane coupling agent, which helps in bonding the silica to the polymer but this also makes the compound more expensive. By use of ENR there is better interaction with silica and can be used as substitute for solution SBR which gives low rolling resistance along with better wet traction and abrasion resistance. Better mechanical properties along with good cure characteristics are obtained when up to twenty percent by weight of SBR is replaced by ENR as compared to pure SBR in both silica filled and mixed filler systems of carbon black/silica.

Replacement of carbon black by other filler systems can also be efficient in reducing rolling resistance. This can be accomplished by use of polymeric fillers. Compounds prepared by replacing about 30-40 parts of carbon black by about ten parts of polymeric filler, exhibit very good mechanical properties along with low hysterisis. Typical tread formulations with the polymeric filler have comparable cure characteristics along with better technological and dynamic properties in relation to conventional formulations. The density of the compound is also lower than the carbon black filled one. The added advantage of low hysterisis as seen from the lower heat build-up and higher resilience are realised in such mixes. The polymer filler containing compound also exhibits very good flex resistance.

Use of chemically modified form of rubber, partial replacement of carbon black with silica and complete replacement of carbon black with polymeric filler can contribute to low GHG causing emissions in the automobile industry.

In the present scenario of implementing regulatory legislations GHE emissions, the tyres are expected to be more fuel - efficient , high performance and environmentally friendly tyres.

Replacing synthetic rubbers with NR along with judicious choice of compounding ingredients mainly fillers contribute to reducing the GHE in Rubber industry. Production of synthetic rubbers is energy intensive and cause green house gas emissions NR is produced from a renewable means that removes carbon dioxide from atmosphere.

The tyre industry demands properties like low heat built up along with excellent abrasion resistance, good road grip and low rolling resistance. However products like gaskets and seals exposed to high temperature and fuels/solvents need resistance to these conditions. Better dynamic properties are shown by natural rubber while synthetic rubber shows improved resistance to fuel/solvents and high temperature . Globally about 22 million tons of rubbers produced per year and about 50 % of this is consumed in the tyre sector. The major synthetic rubbers consumed by the tyre industry are SBR, BR and halobutyl, the latter being used mostly as inner lining of tubeless tyres. The important rubbers used in non tyre applications where high temperature /fuel/solvent resistance is required are nitrile rubber, fluorocarbon rubbers and silicone rubbers while for gas impermeability applications butyl rubber is used. By suitable chemical modification the fuel resistance and gas impermeabilibity characteristics of natural rubber can be considerably improved. One of the promising rubbers of this group is epoxidised natural rubber (ENR) which has considerable good oil resistance along with gas impermeability. Due to the presence of epoxy group ENR helps in better dispersion of silca and can by used for preparing rubber compounds filled with silica without the aid of silane coupling agents.

In the present scenario of implementing regulatory legislations GHE emissions, the tyres are expected to be more fuel - efficient , high performance and environmentally friendly tyres. Reduced rolling resistance gives improvement in fuel efficiency leading to lesser emissions. Carbon black is predominantly used in tires, but the use of silica has become a primary alternative to improve rolling resistance. When silica is used to improve rolling resistance, it is traditionally used with a silane coupling agent, which helps in bonding the silica to the polymer.

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Rubber Tree (*Hevea brasiliensis* Muell. Arg)

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Summary

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is an important industrial crop
ber production. At present, more than 9.5 million hectares in about 40 count
to rubber tree cultivation with a production about 6.5 million tons of dry rubbe
world supply of natural rubber is barely keeping up with a global demand for
of natural rubber in 2020. Tapping panel dryness (TPD) is a complex physiolo
widely found in rubber tree plantations, which causes severe yield and crop l
rubber producing countries. Currently, there is no effective prevention or tre
serious malady. As it is a perennial tree crop, the integration of specific desire
conventional breeding is both time-consuming and labour-intensive. Genetic
with conventional breeding is certainly a more promising tool for incorpora
mically important genes that could improve existing Hevea genotype. This c
an *Agrobacterium*-mediated transformation protocol for rubber tree using in
derived calli as initial explants. We have applied this protocol to generate g
neered plants from a high yielding Indian clone RRII 105 of *Hevea brasilie*
were co-cultured with *Agrobacterium tumefaciens* harboring a plasmid vecto
Hb superoxide dismutase (SOD) gene and the reporter gene used was β-glucu
gene (*uidA*). The selectable marker gene used was neomycin phosphotransfe
kanamycin was used as selection agent. We found that a suitable transformat
Hevea consists of a 3-d co-cultivation with *Agrobacterium* in the presence of
ringone, 15 m*M* betaine HCl, and 11.55 m*M* proline followed by selection o
taining 300 mg/L kanamycin. Transformed calli surviving on medium conta
kanamycin showed a strong GUS-positive reaction. Upon subsequent subcu
media, we obtained somatic embryogenesis and germinated plantlets, which w
GUS positive. The integration of *uidA*, *npt*II, and HbSOD transgenes into *Hev*
confirmed by polymerase chain reaction (PCR) as well as Southern blot analy

Key Words: Rubber tree; *Hevea brasiliensis;* genetic transformation;

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faciens; polymeruse chain reaction (PCR) amplification; Southern blot hybridization; roxide dismutase gene (SOD).

roduction

ea brasiliensis, the para rubber tree, is the most important source of nat- bber; other rubber bearing plants are of minor importance. About 90% of l rubber is produced in Southeast Asia. Natural rubber is considered a aw material by developed countries and is valued for its high-performance teristics. Synthetic rubber, derived from petroleum, is not as elastic or nt and does not have the heat transfer properties of natural rubber. igh synthetic rubber is often blended with natural rubber, various prod- ich as airplane tires require natural rubber *(1)*. In the rubber tree, latex is ced in highly specialized cells called laticifers. When the bark of the rub- e is tapped, the cytoplasmic contents of these laticifers are expelled in the f latex. Latex is a milky substance, which upon coagulation and further sing yields natural rubber.

rubber tree (*Hevea brasiliensis*, $2n = 36$) is a perennial tropical crop, belongs to the genus *Hevea* and the family Euphorbiaceae. The genus encompasses ten species, all originating from the Amazon region *(2)* and ongly outcrossing and monoecious. Most of the natural rubber exploited world originates from this single species. As in other crops, rubber pro- n is influenced by various plant physiological conditions and pathogenic es. However, latex production still faces serious economic losses over the as a result of tapping panel dryness (TPD), or brown bast a syndrome terized by an abnormal reduction of latex flow. During tapping the latex healthy rubber tree flows through 3–4 h by turgour pressure inside the issue. Once the TPD occurs, the tapping incision is partly or entirely d and the amount of latex production is significantly decreased or stops etely. The incidence of TPD occurs in 12 to 50% of rubber trees in almost rubber producing country.

netic improvement of *Hevea* is very slow and time consuming as in many perennial species. The major limitations are the very narrow genetic base, nchronous flowering, low fruit set, long gestation period, heterozygous , and absence of fully reliable early selection parameters. Genetic engi- g is a powerful method for crop improvement when specific genetic es need to be made in a short time period without loss of genetic integrity. igh the transfer of DNA into plant cells via *Agrobacterium* and other meth- now routine for many plant species, the coupling of transformation with lection of transformed cells and regeneration of transgenic plants is still lt in many economically important tree species including *Hevea*. TPD is dered to be a serious physiological disorder caused by oxidative stress in

rubber tree that are frequently tapped for natural rubber in th *Hevea* an increase in the level of the free radical scavenging b of a superoxide dismutase (SOD) gene could enhance stress cells against oxidative stress. The genetic manipulation of *He* in the recent past. Transgenic plants have been developed wit *Agrobacterium* as well as particle bombardment methods transformation efficiency was generally low. Montoro et a response of calcium on *Agrobacterium*-mediated gene transf calli but did not obtain transgenic plants. So far no such agr tant gene has been successfully transformed into *Hevea*. developed transgenic *Hevea* plants by incorporating the SOD

The transformation protocol presented in this chapter wa author's laboratory in combination with review of the literat mation. For this *Agrobacterium*-mediated transformation, tive version of the *Hevea brasiliensis*-SOD gene (HbS 2-mo-old anther derived calli as initial explant. The β-g (*uidA*) was used for screening and neomycin phosphotrans was used for selection of the transformed calli. The transf was 4%. The overall scheme employed for this study is outli in Fig. 1. In this chapter we describe the steps involved in tion protocol and isolation of DNA as templates.

2. Materials

2.1. Plant Materials

Two-mo-old calli derived from immature anther or in clone RRII 105) (*see* **Fig. 2A**).

2.2. Agrobacterium tumefaciens *Strain and Gene Con*

We used the binary vector pDU96.2144 *(9)* which contai gene and *npt*II as selectable marker gene plus the HbSOD trol of constitutive promoter (CaMV 35S).

Binary vector is inserted into disarmed *Agrobacterium* EHA101 to create functional vector for transformation exp

2.3. Stock Solutions

1. 20X Modified Murashige and Skoog (MS) major salts stoc
   a. MS1: NH_4NO_3, 20.0 g/L; KNO_3, 32.0 g/L; $MgSO_4$ (a (anhy), 6.66 g/L; and KH_2PO_4, 3.4 g/L.
   b. MS2 and MS3: NH_4NO_3, 10.0 g/L; KNO_3, 16.0 g/L; M $CaCl_2$ (anhy), 3.32 g/L; and KH_2PO_4, 1.70 g/L.

Pre-culture of immature Anther derived calli (2-mo old) on proliferation medium prior to *Agro* infection

↓

Infect the calli with *Agrobacterium* containing gene of interest for 10 min

↓

Blotted dry the infected calli and transfer to co-cultivation medium (incubate in dark for 3 d)

↓

On completion of 3 d, transfer the infected calli to selection medium containing Kanamycin + cefatoxime

↓

Select putatively transformed callus lines

↓

Transfer to embryo induction medium and subculture at 2-wk interval

↓

fter GUS histochemical confirmation, subculture the well-developed bipolar embryos onto germination medium

↓

Leaves from germinated plantlets subjected to GUS staining before transfer to soil

↓

Molecular confirmation of transgene integration with *Hevea* genome by PCR and Southern blot analysis using leaf samples collected from established transgenic plants

Fig. 1. Flow chart of rubber transformation protocol.

MS minor stock solution: H_3BO_4, 620 mg/L; $CoCl_2 \cdot 6H_2O$, 2.5 mg/L; $_2 \cdot 5H_2O$, 2.5 mg/L; $MnSO_4 \cdot 4H_2O$, 1.69 g/L; $NaMoO_4 \cdot 4H_2O$, 25 mg/L; KI, g/L; and $ZnSO_4 \cdot 7H_2O$, 860 mg/L.

Iron Stock: Na_2EDTA, 3.72 g/L and $FeSO_4 \cdot 7H_2O$, 2.78 g/L.

B5 vitamin stock solution: Myo-inositol, 10 g/L; nicotinic acid, 100 mg/L; oxine HCl, 100 mg/L; and thiamine HCl, 1.0 g/L.

ns (Sigma, 1.0 mg/mL for each stock solution): 2,4-Dichlorophenoxy acetic acid D), indole-3-acetic acid (IAA), 1-naphthalene acetic acid (NAA). To prepare

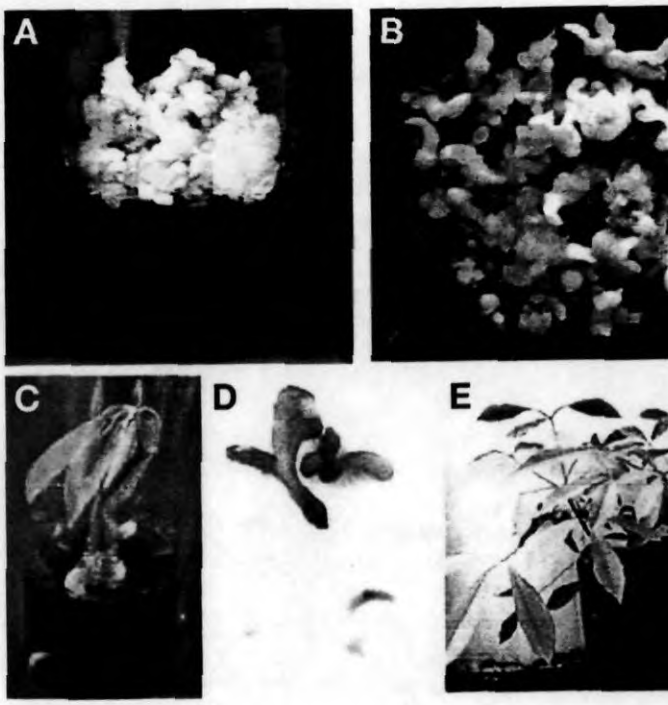

Fig. 2. Development of transgenic plants and molecular confirm of transgenes in rubber tree (*Hevea brasiliensis*). **(A)** Callus prolife stages of somatic embryos, **(C)** Germinated plantlet, **(D)** Histoc (transgenic embryos showing blue colour), **(E)** Plants established

individual solution, dissolve each powder in 50 µL of 1M KOH distilled water to 1 mL volume. Store at 4°C for up to 3 mo.

6. Cytokinins (Sigma, 1.0 mg/mL for each stock solution): 6 (BA), kinetin (KIN). To prepare individual solution, dissolve t of 1*M* HCl then make up to 1 mL volume with sterile distille indefinitely.
7. Abscisic acid (ABA) (1.0 mg/mL, Sigma): Dissolve the p *methanol make final volume with sterile water and store at 4*
8. Gibberellic acid (GA_3) (1.0 mg/mL, Sigma): Dissolve the (100%) and store at 4°C for up to 3 mo.
9. 100X B5 vitamins (Sigma): To make a 50 mL stock soluti myoinositol, 50 mg thiamine HCl, 50 mg nicotinic acid, and 5 in sterile distilled water and store at 4°C for up to 3 mo.
10. 20 mg/mL gentamycin stock solution (Sigma): Dissolve the vial with 1 mL sterile distilled water. Store at –20°C for up t

0 mg/mL kanamycin stock solution: Weigh 1 g of kanamycin monosulfate igma), place in a beaker, and add 8 mL of sterile distilled water. Allow namycin to dissolve completely. Make the final volume to 10 mL with sterile ater then filter sterilize using 42 μ*M* pore size membrane and divide into 1-mL iquots. Store at –20°C for up to 6 mo.

0 mg/mL Cefotaxime (Sigma) stock solution: Dissolve the chemical in sterile dis-led water then filter sterilize and aliquot into 1 mL. Store at –20°C for up to 3 mo.

0 m*M* Acetosyringone (Sigma) stock solution: Dissolve the chemical in sterile stilled water, filter sterilize, and aliquot into 1-mL Eppendorf tubes. Store ÷20°C for up to 3 mo.

0 m*M* Betaine HCl (Sigma) stock solution: Dissolve the chemical in sterile dis-led water and then filter sterilize and aliquot into 1-mL Eppendorf tubes. Store –20°C for up to 3 mo.

m*M* Proline (Sigma) stock solution: Dissolve the chemical in sterile distilled ater and then filter sterilize and aliquot into 1-mL Eppendorf tubes. Store at 0°C for up to 3 mo.

0 mg/mL Spermidine (Sigma) stock solution: Dissolve the chemical in sterile stilled water and then filter sterilize and aliquot into 1-mL Eppedondorf tubes. ore at –20°C for up to 6 mo.

edia

ELB medium: 10 g/L Bacto-tryptone (Sigma), 5 g/L yeast extract (Sigma), and g/L Bacto Agar (Sigma), pH 7.2. Autoclave, cool to 50°C, and add 50 mg/L namycin and 20 mg/L gentamycin. Mix well and pour into sterile 90-mm Petri ates.

llus induction and proliferation medium (MS-1): To make 1 L, add 50 mL of 20X odified MS major salt stock solution (7), 10 mL each of 100X MS minor stock solu-n, 100X iron stock solution, and 100X B5 vitamin stock solution, 5% sucrose (w/v), 0 mg/L 2,4-D, 0.5 mg/L KIN, 1.0 mg/L NAA, adjust to pH 5.7 with 0.1*M* KOH and d 0.25% (w/v) phytagel as solidifying agent then autoclave at 121°C for 20 min.

matic embryo induction and maturation medium (MS-2): To make 1L, add mL of modified MS major salt stock solution, 10 mL each of 100X MS minor ck solution, 100X iron stock solution, and 100X B5 vitamin stock solution, 5% crose (w/v), 0.1 mg/L 2,4-D, 0.3 mg/L NAA, 0.3 mg/L BA, 2.0 mg/L GA_3, 0.1 g/L ABA, 200 mg/L casein hydrolysate, 150 mg/L malt extract, 100 mg/L banana wder, 10% coconut water (w/v), 2 mg/L spermidine, adjust to pH 5.7 with 0.1 *M* OH and add 0.4% phytagel as solidifying agent then autoclave at 121°C for 20 min.

mbryo germination and plantlet regeneration medium (MS-3): To prepare 1 L, add mL of modified MS major salt stock solution, 10 mL each of 100X MS minor stock ution, 100X iron stock solution, and 100X B5 vitamin stock solution, 0.3 mg/L A_3, 0.5 mg/L KIN, 0.1 mg/L IAA; 0.5 mg/L BA, adjust to pH 5.7 with 0.1*M* KOH d add 0.2% (w/v) phytagel as solidifying agent then autoclave at 121°C for 20 min.

-cultivation medium: To prepare 1 L, take the same MS-1 media components as scribed above and autoclave, cool to 50°C then add 20 μ*M* acetosyringone, 15 μ*M* aine-HCl, 11.55 μ*M* proline.

6. Selection medium: To prepare 1 L, take the same MS-1 described above and autoclave, cool to 50°C then add 300 500 mg/L cefotaxime, mix thoroughly and dispense 25 m petri dishes.

2.5. Other Reagents, Solutions and Supplies

1. Surface disinfection solution I: 0.5% (w/v) sodium hyp Tween-20.
2. Surface disinfection solution II: 0.1% (w/v) mercuric chlor
3. Sterile Whatman filter paper No. 3 (SD fine, India).
4. Sterile distilled water.
5. Sterile metal spatulas with spoon on one end (Fisher, USA
6. Soil mix: Soil rite (Himedia, India), sand in 1:1 ratio and thene bags (10 × 20 cm W × H).
7. X-Gluc solution (Sigma): 0.1 μ*M* phosphate buffer, pH diamine tetraacetic acid (EDTA), 0.5 m*M* potassium ferric sium ferrocyanide, 0.1% (v/v) Triton X-100, and 2 m*M* chloro-3-indolyl -β-D glucuronide).
8. Cetyltrimethyl ammonium bromide (CTAB) extraction b (hexadecyltrimethyl ammonium bromide), 1.4 *M* NaCl, 2 100 m*M* Tris-HCl, pH 8.0, 1% (w/v) polyvinyl polypyrrol (v/v) β-mercaptoethanol. Store at room temperature.
9. Phenol/chloroform/isoamyl alcohol (25:24:1): Mix 25 parts 100 m*M* Tris-Cl, pH 8.0) with 24 parts chloroform and 1 Add 8-hydroxy quinoline to 0.1% (w/v) Store in aliquots a
10. Chloroform/isoamyl alcohol (24:1).
11. RNase A (DNase free, 10 mg/mL): Dissolve RNase A in and 15 m*M* NaCl; boil for 10 min and allow to cool to ro aliquots at –20°C to prevent microbial growth.
12. Proteinase K: 10 mg/mL in H_2O. Store at –20°C.
13. 100% isopropyl alcohol, ice-cold.
14. 70% (v/v) ethanol, ice-cold.
15. TE buffer; 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0
16. Organic solvent-resistant Oak-ridge centrifuge tube (Tarso

3. Methods

3.1. Callus Initiation and Pre-culture

The following tissue culture protocol is modifications of

1. Collect flower buds (0.5–1.0 cm size) and surface disinfec disinfection solution I for 5 min followed by thorough w tilled water 5 times.
2. Surface sterilizes the flower buds with 100 mL of surface for 3 min followed by 5 rinses in sterile distilled water.

sect out the immature anthers and place approx 10 anthers/tube on MS-1 ium (*see* **Note 1**). Incubate cultures at 26°C in the dark and subculture at k intervals into fresh medium of the same formulation.
culture the 2-mo-old callus (*see* **Note 2**) on fresh MS-1 medium prior to infec- with *Agrobacterium* (*see* **Notes 3** and **4**).
e the culture in controlled environment at 26 ± 2°C for 2 d. Photoperiod of 8-h light/dark cycle with cool-white florescent light (60 µmol/m^2/s).

robacterium *Culture Preparation*

ak *Agrobacterium* using loop from a glycerol stock onto AELB medium supple- ted with 20 mg/L gentamycin and 50 mg/L kanamycin. Incubate at 28°C for 2 d.
nsfer a single well-grown *Agrobacterium* colony from plate into a 25 mL id AELB medium with appropriate antibiotics.
p 25 mL cultures onto an incubator shaker (28°C) for 24 h with agitation rpm).
w cells overnight until an A_{600} of 0.5; adjust the bacterial cell density to 5×10^8 s/mL and use for transformation.

robacterium *Infection and Co-cultivation*

nsfer the precultured calli into the *Agrobacterium* suspension and immerse for nin.
lect the infected calli using sterile spatula and blot dry on sterile Whatman 3 filter paper to remove excess of bacterial suspension.
nsfer the calli on coculture medium (10 callus pieces/plate) and incubate them er 26 ± 2°C in the dark for a period of 3 d (*see* **Note 5**).

ection of Transformed Callus

er 3 d of coculture, subculture the calli into selection medium. Seal plates with film and maintain at 26 ± 2°C in the dark. Subculture every 2 wk for a period mo (*see* **Note 6**).
it cefotaxime from the selection medium and screen for putatively transformed us lines in the presence of kanamycin (*see* **Notes 7–10**).
ect 8-wk-old kanamycin resistant callus lines for embryo induction.

matic Embryogenesis and Plant Regeneration

nsfer the putatively transformed calli growing on selection medium and culture MS-2 medium for embryo formation.
culture the kanamycin resistant embryogenic callus lines to fresh MS-2 lium at 2-wk intervals. Transfer embryos into fresh MS-2 medium for matura- and incubate for 4 wk with 16-h photoperiod.
ect mature bipolar embryos (*see* **Fig. 2B**) and subject them to histochemical S assay (*see* **Fig. 2D**) (*see* **Note 11**).
e mature bipolar embryos containing cotyledons on fresh MS-3 medium for mination and keep at 16-h photoperiod.

5. Mature embryos germinate into plantlets in the MS-3 regener 2 wk of culture (*see* **Fig. 2C**) (*see* **Note 12**).
6. Transfer the plantlets (>5-cm long) formed in the medium into MS liquid medium for 2 wk. Establish these plants into small autoclaved soil mix, cover with a plastic bag and keep under c Then make a small hole in the plastic bag for aeration (*see* N
7. Remove the cover at the end of 2 wk and transfer plants to l to glass house for hardening (*see* **Notes 15 and 16**). Initially k shade to avoid direct sunlight.
8. After 2 wk, shift the acclimatized rubber transgenic plants to normal field condition with sunlight (*see* **Fig. 2E**).
9. Budgraft the well-established transgenic plants to normal roo house for multiplication.
10. Collect the leaves of the acclimatized transgenic plants for hi and molecular analysis such as GUS histochemical assay, PC hybridization.

3.6. Isolation of Plant Genomic DNA, PCR, and Southe

The following is the modifications of Dellaporta et al. *(1*

1. Label the 50-mL centrifuge tubes, collect 2 g of leaves from t well as untransformed control plant and place them in the lab **17 and 18**).
2. Rinse the leaves with cold sterile water three times in beaker, b No. 1 paper.
3. Place the leaves in a mortar, add liquid nitrogen, and grind to a pestle (*see* **Note 19**).
4. Transfer the frozen powder to 50-mL Oak ridge centrifug organic solvent-resistant tube.
5. Immediately add 20-mL warm CTAB extraction buffer to the der and gently mix to wet thoroughly. Incubate for 30 min at mixing (*see* **Note 20**).
6. Extract the homogenate with an equal volume of 25:24:1 pheno alcohol. Mix well by gentle inversion. Spin for 10 min at 10,00
7. Transfer about 18 mL of aqueous fraction (upper phase) by fresh centrifuge tube. Be careful not to take the interphase your DNA quality (*see* **Note 21**).
8. Add 10 µL each of 10 mg/mL proteinase K and 10 mg/m homogenate mix well by inversion several times. Incubate th for 20 min, with occasional agitation.
9. Add an equal volume of 24:1 chloroform/Isoamyl alcohol. inversion. Spin for 10 min at 10,000*g*, at 4°C. Collect top aq
10. Precipitate the DNA by adding exactly 0.6 volume of isopro mix well. *If precipitate is visible, proceed to step 11. If not,* p at –20°C.

pin for 15 min at 14,000*g* at 4°C. Discard the supernatant without disturbing the ellet.
Vash the DNA pellet with ice-cold 70% (v/v) ethanol, spin for 2 min at 10,000*g*. iscard the supernatant and air dry for 10 min (*see* **Note 22**).
esuspend the DNA pellet in a minimal volume of TE buffer (100–500 µL/gram f starting tissue material).
ake 2 µL DNA and analyze the undigested DNA on 1% agarose gel to determine e integrity and quality (*see* **Note 23**).
mplification of transgenes from genomic DNA by PCR using gene specific rimers (*7*).
onfirm the integration and presence of the HbSOD transgene in the putatively ansgenic plants through Southern blot analysis (*7*).

tes

nmature anthers at diploid stage are very essential for 100% callus initiation.
/e found that the 2-mo-old callus is ideal for genetic transformation in *Hevea*.
re-culture of calli in the proliferation medium is necessary to increase the trans-rmation frequency.
ound with sterile dissection needle enhances the transformation efficiency.
3-d co-cultivation period is optimal for transformation experiments and cocul-vation beyond 3 d results in bacterial over-growth that will destroy the callus.
is found that subculture is to be performed at 2-wk intervals into fresh medium the same formulations to avoid drying of tissues.
/e observed that 300 mg/L kanamycin is the optimal concentration to select ansformed cell lines in *Hevea*.
the kanamycin concentration in the medium was increased beyond 350 mg/L, a ecrease in transformation frequency was noticed.
our experiment, profuse growth of nonstransgenic calli was observed from to 200 mg/L kanamycin, which indicated that kanamycin concentrations up to 0 mg/L were ineffective for selecting transformed cell lines.
e used 20 m*M* acetosyringone in the co-cultivation medium to enhance the trans-rmation frequency.
ght-wk-old kanamycin-resistant mature bipolar embryos were subjected to GUS say before transfer to embryo germination medium. It is very important to con-m the transgene integration events before the development of plants.
edia supplemented with ABA, polyamines, and organic supplements and with timal agar concentrations favored embryogenesis and the regeneration of trans-nic plants.
ne leaves from germinated plantlets were subjected to GUS expression assays. d all were found to be GUS-positive.
itially, we kept germinated plantlets in liquid half-strength hormone free MS edium for 2 wk; this step is essential before transfer to soil rite.
e used plastic bags to cover the plants. It is important to cover each plant immediately after transfer to soil rite to prevent wilting.

16. If the plantlets established in soil rite are kept in direct sunli under the plastic cover and dry the plant.
17. GUS positive plantlets were used for molecular confirmati *uidA*, *npt*II, and HbSOD transgenes by PCR using specific p
18. The integrity of the nucleic acids will be improved by maintai cold. We have observed that modified CTAB method give D
19. In order to get better results, the following precautions shou the preparation of reagents for DNA isolation and during Southern blot hybridization procedures wear gloves at all time molecular biology grade reagents. Use sterile, disposable plas
20. The success of this DNA isolation procedure hinges on the rupt cellular integrity while maintaining DNA in an intact, h form. Thus, mixing of the tissue sample and phenol shou gentle inversion, which minimizes shearing forces on the D
21. It is important that aqueous phase must be collected without interphase to get good quality DNA.
22. The DNA pellet may be air dried for 10 min.
23. To ensure the DNA isolated is of high quality, it is advisab are examined by running in a 1% (w/v) agarose gel before performed.

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IV

TROPIC PLANTS