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Low light and low ammonium are key factors for guayule leaf tissue shoot organogenesis and transformation

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Abstract A new method has been developed for guayule tissue culture and transformation. Guayule leaf explants have a poor survival rate when placed on normal MS medium and under normal culture room light conditions. Low light and low ammonium treatment greatly improved shoot organogenesis and transformation from leaf tissues. Using this method, a 35S promoter driven BAR gene and an ubiquitin-3 promoter driven GUS gene (with intron) have been successfully introduced into guayule. These transgenic guayule plants were resistant to the herbicide ammonium-glufosinate and were positive to GUS staining. Molecular analysis showed the expected band and signal in all GUS positive transformants. The transformation efficiency with glufosinate selection ranged from 3 to 6%. Transformation with a pBIN19-based plasmid containing a NPTII gene and then selection with kanamycin also works well using this method. The ratio of kanamycin-resistant calli to total starting explants reached 50% in some experiments.

Keywords Guayule · *Parthenium argentatum* · *Agrobacterium* · Transformation · Low light intensity · Low ammonium · Glufosinate · BAR · GUS

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Introduction

Guayule (*Parthenium argentatum*), a shrub native to the Chihuahuan desert of Texas and Mexico, is a new crop candidate for the rubber industry. To date, it is the only hypoallergenic natural rubber source under commercial development in the world. Commercial guayule farming is established in California and Arizona although still on a small scale.

Transformation of guayule with genes of agronomic importance may lead to further improvements in latex yield, but efficient tissue culture and transformation systems are necessary. Tissue culture of guayule dates back to the early 1980s (Dastoor et al. 1981; Radin et al. 1982; Zavala et al. 1982; Smith 1983; Staba and Nygaad 1983; Dhar et al. 1989; Finnie et al. 1989; Trautmann and Visser 1990; Castillón and Cornish 2000). Although shoots, roots and hypocotyls were tested as explants, the only successful system was shoot node micropropagation. To this date, no report used leaf tissues as explants.

On the basis of the shoot node micropropagation system then available, Pan et al. (1996) developed an *Agrobacterium*-mediated transformation system. This method gently wounded each node at the axillary position, using the tip of a syringe needle, and introduced the *Agrobacteria*. This method has been successfully used to introduce several different genes into guayule, such as kanamycin resistance (Pan et al. 1996), and genes involved in rubber substrate biosynthesis, including farnesyl diphosphate synthase, and geranylgeranyl diphosphate synthase (Veatch et al. 2003). However, it is very time consuming because each node has to be wounded by hand. Also, the transformation efficiency was very low (0.71% or less).

Here we report a new leaf tissue method for guayule transformation that is more facile and more efficient than the shoot node method. A herbicide resistance gene (BAR gene) and an intron containing GUS reporter gene have been successfully introduced into guayule plants and expressed stably for more than 1 year.

Materials and methods

Maintenance of plant cultures

Shoot cultures of guayule lines G7-11, N6-5, and hybrid line AZ101Cl were established and maintained as described previously (Castillón and Cornish 2000). Half-strength MS medium (Murashige and Skoog 1962), containing 15 g/l sucrose and 8 g/l agar, pH 5.8, was used to maintain plant materials. Shoot tips ≥10 mm were excised and subcultured in Magenta boxes containing 80 ml fresh half-strength MS. Roots developed 1–2 weeks after the shoot tips were transferred to this medium. The cultures were maintained at 25°C under cool-white fluorescent light (~50 μmol/m^2/s, 12/12-h day/night photoperiod).

Leaf tissue organogenesis

One-month-old guayule plantlets growing in half-strength MS medium were used to prepare the leaf strips. First, the shoot tip was cut off and transferred to fresh half-strength MS medium to develop roots and grow into a new plant. Then the remaining leaves were cut into ~8 mm wide strips and cultured on either MSB2 medium (Pan et al. 1997) or (1/2NH_4)MSB2 medium (MS medium, NH_4NO_3 reduced to half and KNO_3 increased to 5 g/l, containing 2 mg/l BA, 30 g/l sucrose, 8 g/l agar, pH 5.8) under high light (~50 μmol/m^2/s), low light (~5 μmol/m^2/s), or dark conditions for shoot initiation. The explants were transferred to fresh medium every 2 weeks until new shoots emerged. The leaf strips with emergent shoots were then transferred to (1/2NH_4)MSB1 [same as (1/2NH_4)MSB2 medium except BA reduced to 1 mg/l] under high light (~50 μmol/m^2/s) for shoot elongation, and were transferred every 2 weeks to fresh medium. Once the shoots were 10 mm or longer, they were transferred onto 1/2MSI0.1 medium (half-strength MS medium plus 0.1 mg/l IBA) for rooting. Shoot tips from 1-month-old rooted plantlets were cut and inserted onto fresh half-strength MS medium for maintenance.

For transplanting, the shoot tip was cut at ~8 mm below the first shoot node from the maintained plantlet and inserted into a sterile cellulose plug (CUStarts, Caisson Laboratories, Inc.). Nine plugs, each with one shoot tip, were cultured in a sterile Magenta box containing 20 ml liquid half-strength MSI0.1 without sucrose and agar for 2 weeks. The plugs with the rooted plants were removed from the Magenta boxes, transplanted into the soil, and acclimated according to Castillón and Cornish (2000).

Leaf tissue transformation

Plasmid pND4 (Fig. 1) was constructed based on pPZP200 (Hajdukiewicz et al. 1994). It contained a double 35S promoter (Datla et al. 1991) driven BAR gene (Christensen and Quail 1996) and a potato ubiquitin-3 promoter (Garbaribo and Belknap 1994) driven intron containing GUS gene

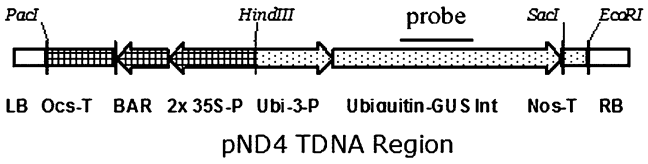

Fig. 1 Map of pND4-TDNA region. LB: left board; Ocs-T: octopine synthase gene terminator; BAR: herbicide glufosinate-resistant gene; 2×35-P: double 35S promoter; Ubi3-P: potato ubiquitin-3 promoter; UQ1-GUSInt: the first ubiquitin coding sequence fused to the GUS gene with an intron; Nos-T: nopaline synthase gene terminator; RB: right board

(Vancanneyt el al. 1990). Suspensions of *Agrobacterium* EHA101 harboring the pND4 binary vector, were prepared by inoculating 5 ml LB medium plus 20 mg/l rifampcin and 200 mg/l spectinomycin with 50 μl long-term glycerol stock in a 50 ml Falcon tube, and shaking overnight at 28°C. The suspension then was centrifuged for 15 min at 1,600×*g* at room temperature. The supernatant was discarded and the pellet was re-suspended in 20 ml of Inoculation Solution (1/10MS salts plus BA 2 mg/l, NAA 0.5 mg/l, glucose 10 g, acetosyringone 200 μM, pluronic F68 0.05%, pH 5.4).

Leaf strips were soaked in the *Agrobacterium* suspension at room temperature for 10 min. Soaked strips were blotted with filter paper and placed on 1/10MSBN medium (same as Inoculation Solution but with 9 g/l agar) for 3 days of co-cultivation under low light conditions (~5 μmol/m^2/s). After co-cultivation, they were transferred to (1/2NH_4)MSB2T medium [(1/2NH_4)MSB2 plus timentin (Cheng et al. 1998) 400 mg/l] for recovery for 1 week under low light, and then were transferred to (1/2NH_4)MSB2TG1 medium [(1/2NH_4)MSB2 plus timentin 400 mg/l, glufosinate 1 mg/l] for selection under low light, and transferred to fresh medium every 2 weeks. Timentin is an antibiotic used to eliminate *Agrobacterium*. After green shoots emerged, the explants were transferred to (1/2NH_4)MSB1TG0.6 medium [same as (1/2NH_4)MSB1 but with 200 mg/l timentin, and 0.6 mg/l glufosinate] for elongation and further selection. The explants were grown under high light thereafter. Green shoots 10 mm and longer were subcultured to 1/2MSI0.1TG0.5 medium (same as 1/2MSI0.1 but with 200 mg/l timentin and 0.5 mg/l glufosinate) for rooting for 2–4 weeks. Shoot tips of the rooted plantlets were subcultured in half-strength MS medium for maintenance or inserted into a sterile cellulose plug for rooting and then transplanted into the soil as described in the section on organogenesis.

An independent experiment was carried out to test this method using *Agrobacterium* ABI harboring pBIN19-based binary vectors containing a Nos promoter driven NPTII gene and a 35S promoter driving a number of other genes, to transform the guayule lines of G7-11, AZ101CL, and N6-5. The *Agrobacterium* culture medium was changed to include 75 mg/l kanamycin, 100 mg/l spectinomycin, and 25 mg/l chloramphenicol. The explants were recovered for 1 week on (1/2NH_4^+)MSB2 medium with timentin but without selection agent. Shoot selection media contained 30 mg/l kanamycin (instead of glufosinate) and the root selection medium contained 30 mg/l kanamycin.

GUS assay

Histochemical staining to detect GUS activity was conducted as described (Jefferson and Wilson 1991), except that the concentration of both NaH_2PO_4 and Na_2HPO_4 was increased to 200 mM to avoid false positive results.

PCR and Southern blot analysis

One gram leaf tissue was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Total DNA was extracted from the powder using Plant DNAzol (Invitrogen, Cat# 10978-021) according to the manufacturer's protocol. PCR was carried out in 50 µl of mixture containing 5 units of Taq DNA polymerase (New England Biolabs, Cat# M0267L) and 5 µl of 10× ThermoPol Reaction Buffer (supplied with the enzyme), 4 µl of dNTP (2.5 mM each), 200 ng genomic DNA or 20 pg plasmid DNA, and 100 ng of GUS specific forward (5′-caacgaactgaactggcaga-3′) and reverse (5′-tttttgtcacgcgctatcag-3′) primers. After heating the samples to 94°C for 2 min, the reaction proceeded with 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s. A final elongation step was carried out at 72°C for 10 min. PCR products were separated by electrophoresis on 0.9% (w/v) agarose gels.

For the Southern blot analysis, the non-radioactive DIG products (Roche, Cat# 1636090, 1093274, 1759051, 1603558, 1585762) were used following the protocol described on the Roche website with some modifications. Ten micrograms DNA from each sample was digested with *Hin*dIII (6 units/µg DNA) for 24 h at 37°C. The digested DNA was then electrophoresed in a 0.9% agarose gel in TAE buffer at 35 V for 14 h. The gel was depurinated, denatured and neutralized. DNA was transferred to a positively charged nylon membrane and crosslinked. The membrane was prehybridized in DIG Easy Hyb. at 50°C for 1 h and then hybridized with the DIG-labeled GUS-specific probe overnight at the same temperature. High stringency washing was carried out at 68°C for 2×20 min in a buffer containing 0.5× SSC and 0.1% SDS. The hybridization signal was detected by a chemiluminescent assay using anti-DIG alkaline phosphatase-conjugated Fab fragment and its substrate CDP-star with the Kodak Image 2000 for 100 min exposure.

Results

Shoot organogenesis

Effect of low light intensity

When leaf strips were cultured on MSB2 medium (Pan et al. 1996) they turned brown and withered within 2 weeks. Modifications of the medium, such as changing the BA concentration from 1 to 8 mg/l, adding 20–160 mg/l ascorbic acid or 1–8 mg/l $AgNO_3$, or replacing sucrose with glucose, did not significantly change explant health. The same results were found using leaf strips from 2-week-old and 1-month-old plantlets.

However, low light intensity considerably improved the survival of, and shoot organogenesis from, the guayule leaf strips. Leaf strips that grew under low light were greener and healthier than those grown under high light (Fig. 2).

Different light intensities were tested to determine the optimal intensity for shoot organogenesis from guayule leaf strips. The rate of organogenesis significantly improved when the light intensity dropped to 12 µmol/m^2/s (Fig. 3); both the number of explants producing shoots and the total shoots produced were doubled. Lowering of the light intensity appeared to enhance shoot production, down to 1.5 µmol/m^2/s, although the differences were not statistically significant. Dark grown leaf strips had less brown sectors than those grown under low light, but they produced fewer shoots.

Duration of low light treatment also was tested. Petri dishes were cultured under the high light condition (50 µmol/m^2/s) but covered with paper. Light intensity under the paper was ~5 µmol/m^2/s. The paper was removed at 1, 2, 3 or 4 weeks. Control plates were not covered with paper (Fig. 4). Shoot production increased with longer low light treatment, reaching a maximum at 4 weeks. Explants grown under low light more than 4 weeks appeared yellow compared with those grown under low light for 3 or 4 weeks.

Effect of low ammonium

Preliminary experiments showed that under high light conditions, B5 medium (containing lower ammonium than MS, Gamberg et al. 1968) and half-strength MS medium plus 2 mg/l BA produced fewer brown sectors on the leaf strips than MSB2 medium. However, the greener tissues did not produce more shoots as would be expected. This may be because of inadequate nutrient status in the B5 and half-strength MS media. A medium named (1/2NH_4)MSB2 was then made by reducing NH_4NO_3 in half and increasing KNO_3 to 5 g for MS medium. For G7-11, (1/2NH_4)MSB2 doubled the shoot production (Fig. 5). Guayule leaf strips cultured on (1/2NH_4)MSB2 medium under low light produced green shoots within 4 weeks (Fig. 6).

Transformation

Selection with glufosinate

Untransformed guayule leaf strips were very sensitive to glufosinate. Shoot formation was prevented by 0.5 mg/l glufosinate. pND4 transformed leaf strips were selected on 1 mg/l glufosinate after the 3-day co-cultivation and the 7-day recovery period (Fig. 7a). Green shoots appeared 4–6 weeks after culturing on this medium (Fig. 7b). However, shoot elongation on 1 mg/l glufosinate medium was very slow so once shoots appeared, the concentration of

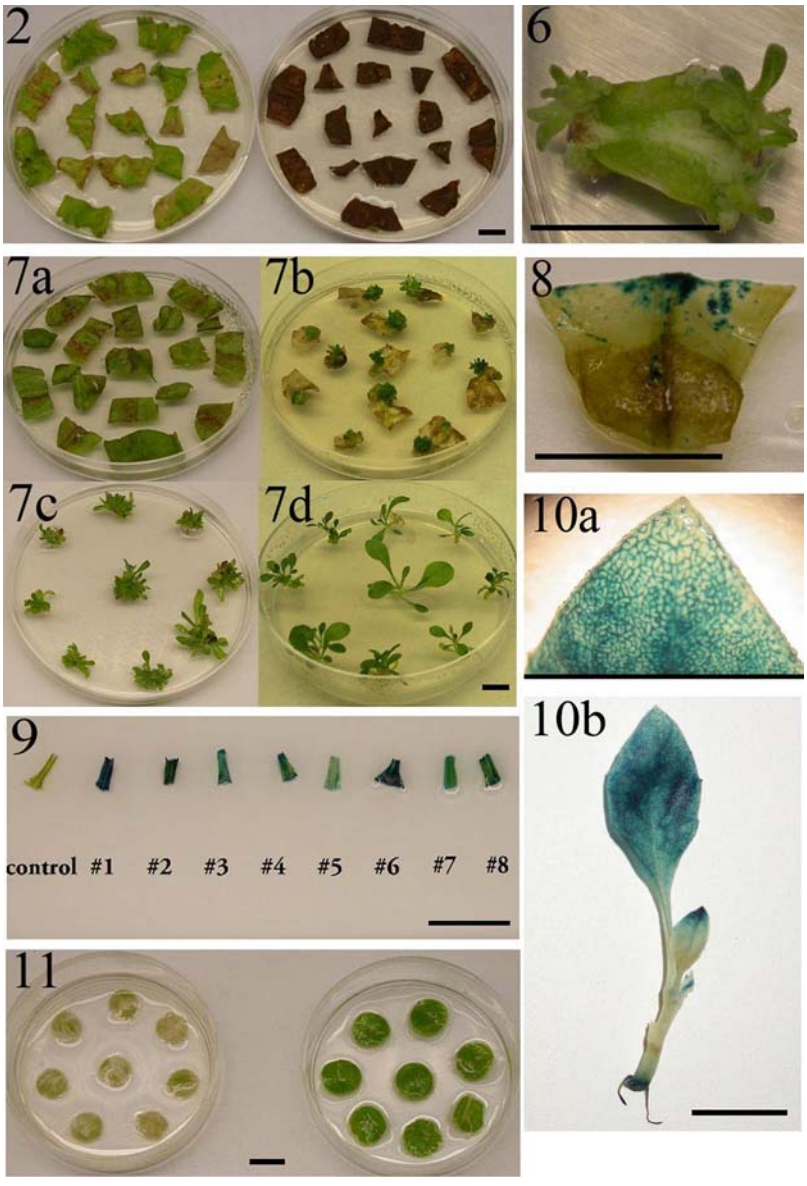

Fig. 2 Low light intensity is a key factor for shoot organogenesis from guayule leaf tissue. Leaf strips were prepared from 1-month-old G7-11 plantlets grown on half-strength MS medium in Magenta boxes. They were cultured on MSB2 medium for 3 weeks in high and low light intensities. Bar: 1 cm. *Left*: Low light (~5 μmol/m^2/s); *Right*: High light (~50 μmol/m^2/s). **Fig. 6** Shoots regenerated from leaf strips cultured on (1/2NH_4^+)MSB2 medium for 4 weeks under low light. Bar: 1 cm. **Fig. 7** Guayule leaf strip transformation: (**a**) leaf strips were inoculated with *Agrobacterium tumefaciens* and co-cultured on 1/10MSBN medium for 3 days; (**b**) shoot initiation on (1/2NH_4)MSB2TG1 medium for 6 weeks (three subcultures); (**c**) shoot elongation on (1/2NH_4)MSB1TG0.6 medium for 8 weeks (four subcultures); (**d**) rooting on half-strength MSI0.1TG0.5 medium for 4 weeks. Bar: 1 cm. **Fig. 8** Transient GUS expression. A leaf strip showing blue spots after GUS staining conducted 3 days after co-culture. Bar: 1 cm. **Fig. 9** GUS test after rooting. Leaves were cut from the plantlets grown in the Magenta box and they were cut into strips for GUS staining. Control: non-transformed guayule G7-11; #1–#8: transformed G7-11 plantlets. Bar: 1 cm. **Fig. 10** Stable GUS expression of #1 plantlet. **a** View through a dissecting microscope. **b** A small guayule plantlet with roots showing GUS activity. Bar: 1 cm. **Fig. 11** Resistance to glufosinate. Non-transformed G7-11 (left) and transformed G7-11 #4 (*right*) leaf discs were soaked in (1/2NH_4)MSB1 liquid medium containing 1 mg/l glufosinate ammonium. Photo was taken 7 days after soaking. Bar: 1 cm

glufosinate was reduced to 0.6 mg/l. It took 8 more weeks for some of the shoots to reach ≥10 mm (Fig. 7c). These shoots were transferred to rooting medium containing 0.5 mg/l glufosinate. After 2–4 weeks, rooted plantlets (Fig. 7d) were transferred to half-strength MS medium in the Magenta boxes for maintenance. The shoot tips from 1-month-old plantlets were inserted into a sterile cellulose plug for rooting and then transplanted to the soil.

The transformation efficiency (Table 1) ranged from 3 to 6%, a significant improvement over the shoot node method (0.7%).

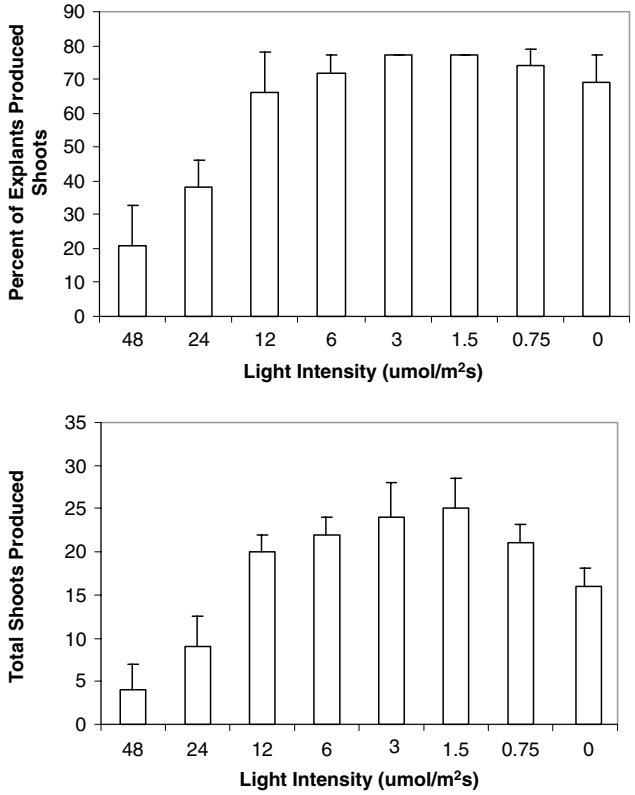

Fig. 3 Effect of different light intensity. G7-11 leaf strips cultured on MSB2 medium in different light intensities. Data collected 4 weeks after culture started. **A** Number of explants produced shoots under different light intensity. **B** Total shoots produced under different light intensity

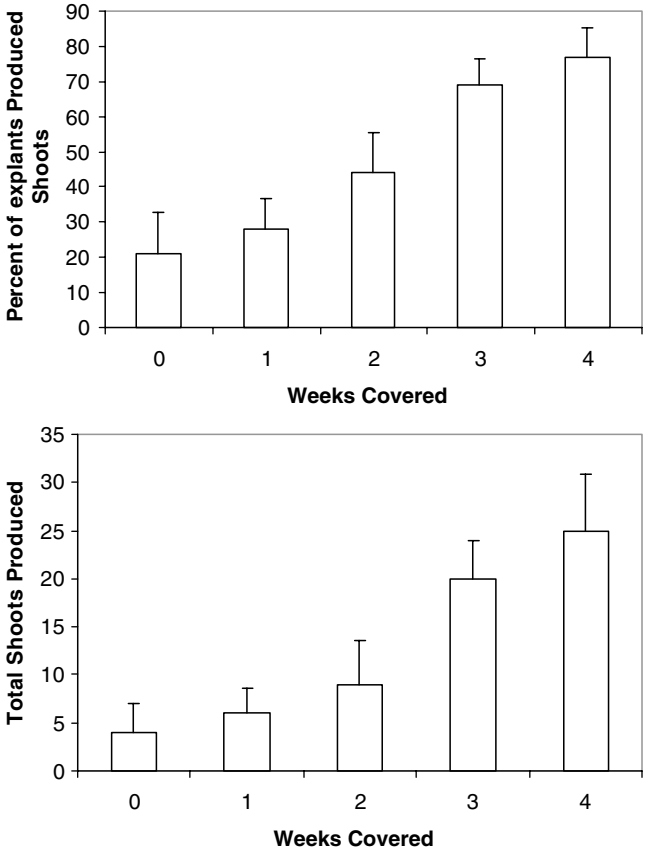

Fig. 4 Effect of duration of low light treatment. G7-11 leaf strips cultured on MSB2 medium under high light intensities but covered with paper. The light intensity under paper was ~5 μmol/m^2/s. Data collected 4 weeks after culture started

GUS assay

Transient GUS expression from the pND4 transformed leaf strips could be detected after 3 days of co-cultivation (Fig. 8). Endogenous GUS activity was not detected in leaf tissues from non-transformed control plants (Fig. 9). Eight plantlets from different leaf strips and transformed by pND4_EHA101 all showed GUS activity. Stable GUS expression could be detected 1 year after transformation (Fig. 10a and b).

Herbicide resistance

When leaf disks from regenerated plantlets were soaked in 1 mg/l glufosinate, the leaf discs from the non-transformed control plants turned yellow in 1 week, while the leaf discs from transformed plantlets remained green (Fig. 11).

Molecular analysis

PCR analysis of guayule plantlets revealed a 0.8 kb band (part of the GUS gene) in all glufosinate-resistant plantlets, as predicted, but not in the non-transformed control (Fig. 12). Southern blot analysis also showed a positive GUS signal in all glufosinate-resistant plantlets, the copy number ranging from 1 to 5, but no signal was detected in the non-transformed control (Fig. 13).

Table 1 Transformation efficiency

Vectors	Total explants	Resistant plantlets[a]	GUS+ plantlets[a]	PCR+ plantlets[a]	Transformation efficiency (%)
pND4	200	10	8	8	4
pND4	200	17	12	12	6
HMGR_AN pND4	200	9	na (HMGR_AN)	6	3

Note. na, not applicable; the GUS gene in pND4 was replaced by HMGR gene from *Aspergillus nidulans*

[a] These plantlets were regenerated from different leaf strips. They represent independent transformation events

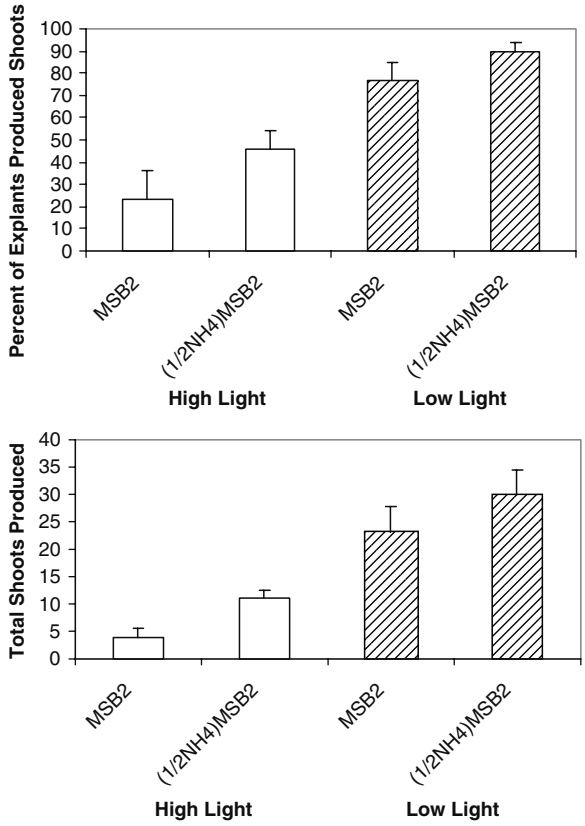

Fig. 5 Effect of low ammonium on shoot organogenesis from guayule leaf tissue. G7-11 leaf strips cultured on MSB2 and $(1/2NH_4^+)$MSB2 medium under high and low light intensities. Data were collected 4 weeks after culture started

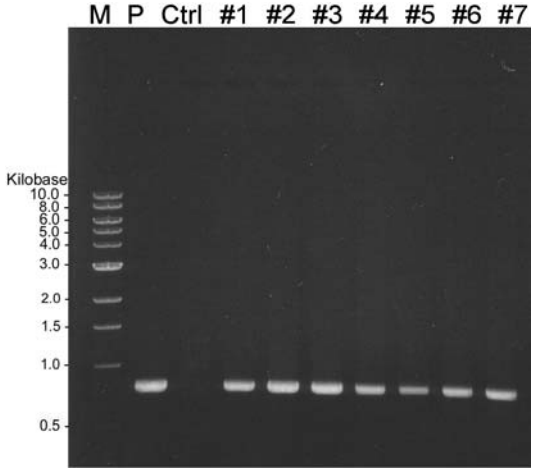

Fig. 12 PCR analysis. P: plasmid pND4; Ctrl: non-transformed control; #1-#7: transformed G7-11; M: DNA marker

Selection with kanamycin

Three lines of guayule, G7-11, AZ101CL, and N6-5, were transformed with *Agrobacteria* harboring pBIN19-based binary vectors. Kanamycin-resistant calli grew out from the leaf strips under low light and low ammonium conditions (Fig. 14), and produced shoots and roots. Preliminary data showed the ratio of green calli produced to total explants transformed ranged from 0 to 50%.

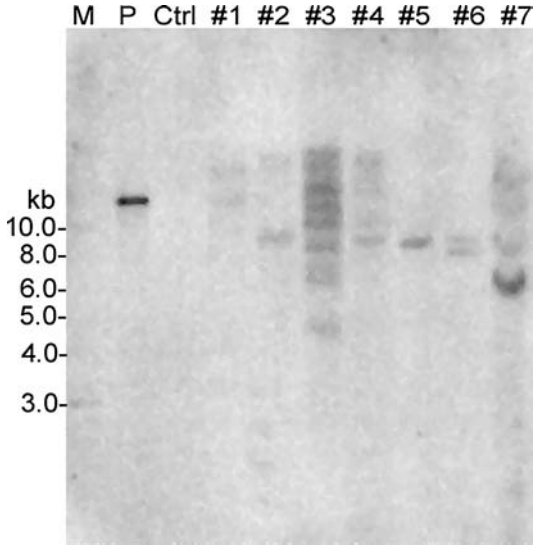

Fig. 13 Southern blot analysis with DIG labeled probe. P: plasmid pND4; Ctrl: non-transformed control; #1–#7: transformed G7-11; DNA samples were digested by *Hin*dIII. M: DNA marker

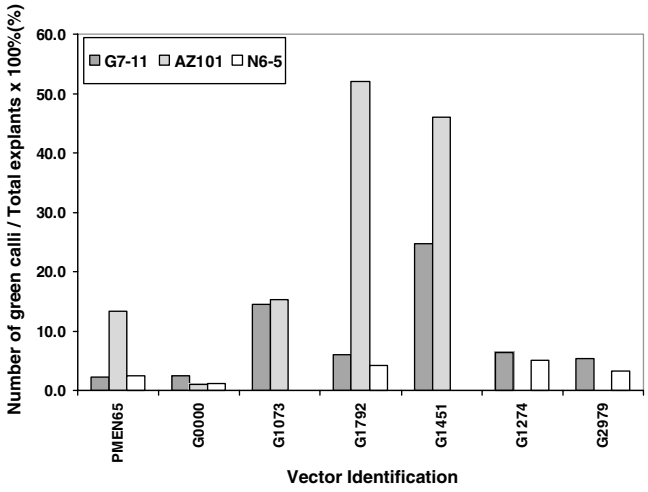

Fig. 14 Selection with kanamycin. *Agrobacteria* ABI harboring each of the following vectors were used to transform three genotypes of guayule. PMEN65: empty vector; G0000: no vector; G1073, G1792, G1451, G1274 and G2979: vectors containing kanamycin-resistant gene and different target genes

Discussion

This is the first report of shoot organogenesis from, and transformation of, guayule leaf tissue. By using low light intensity, guayule leaf strips produced healthy green shoots within 4 weeks on $(1/2NH_4)$MSB2 medium. Light intensities below 12 $\mu mol/m^2/s$ increased the regeneration efficiency by 3–4-fold compared to the normal culture light intensity of 40–50 $\mu mol/m^2/s$. The optimal light intensity for guayule leaf tissue regeneration is 1.5 $\mu mol/m^2/s$.

Reduced ammonium and supplemental nitrate further increased the regeneration efficiency. Also, low light and low ammonium conditions greatly helped *Agrobacterium*-mediated guayule leaf tissue transformation. We have successfully obtained glufosinate-resistant and GUS positive guayule plants from guayule leaf tissue. Transformation efficiency ranged from 3 to 6%, much higher than the shoot node method. Our method also avoids the time consuming wounding process previously used.

Dark or low light conditions are commonly used for somatic embryogenesis. Somatic embryogenesis of wheat (Wang and Wei, 2003), barley (Ganeshan et al. 2003), and oat (Nuutila et al. 2002), using leaf base segments as explants, were all induced in the dark. Somatic embryo induction in poplar (Michler and Bauer 1991) and Podophyllum (Arumugam and Bhojwani 1990) requires absolute darkness. Complete darkness was needed for embryogenesis of Norway spruce when ammonium was present in the medium (Verhagen and Wann 1989). Darkness was effective for induction of carrot somatic embryos, as was green or red light, while blue light or white light at higher intensities was inhibitory (Micheler and Lineberger 1987). For soybean, low light intensities (10 μmol/m^2/s), or light provided by Grolux fluorescent tubes, which provide more light in the red spectrum, resulted in higher frequencies of embryogenesis (Lazzeri et al. 1987). Another report showed that the immature cotyledons of soybean cultivars Jack and Fayette, produced more somatic embryos under lower light intensity (5–10 μmol/m^2/s) than higher light intensity (50–60 μmol/m^2/s), see http://www.cropsoil.uga.edu/homesoybean/light.htm.

Since the female gamete is included in the embryo sac embedded in the ovule (Dodeman et al. 1997), zygotic embryogenic cells are light protected. Somatic embryogenesis may be similar to zygotic embryogenesis in this point and therefore dark or low light is necessary for induction of somatic embryogenesis.

Shoot organogenesis from leaves, however, occurs from the palisade mesophyll cell(s) next to the epidermis of the explant and usually it first appears as a greener shoot primordium. This is photomorphogenic development and light is important in this process. For most plant species, high light intensity (above 20 μmol/m^2/s) was used. Examples are tobacco (Dhaliwal et al. 2003), *Lilium* (lily) (Bacchetta et al. 2003), *Arachis pintoi* (Leguminosae) (Rey et al. 2000), *Lycium barbarum* L. (Ratushnyak et al. 1990), *Melia azedarach* L. (Meliaceae family) (Vila et al. 2003) *Prunus domestica* L. (plum) (Nowak et al. 2004), *Saintpaulia ionantha*×*confusa* hybrids (African violet) (Lo et al. 1997), *Cajanus cajan* (L.) Millsp. (pigeonpea) (Dayal et al. 2003), *Gypsophila paniculata* L. (Gypsophila) (Zuker et al. 1997), *Plumbago* (Plumbaginaceae family) (Das and Rout 2002), *Solanum melongena* L. (eggplant) (Rotino and Gleddie 1990), *Pyrus communis* var *pyraster* L. (wild pear) (Caboni et al. 1999), *Rosa hybrida* (roses) (Ibrahim et al. 1998), *Pothomorphe umbellate* (pariparoba or caapeba) (Pereira et al. 2000), *Coleus forskohlii* Briq. (Lamiaceae) (Reddy et al. 2001), *Scrophularia buergeriana* Miq. (figwort) (Park et al. 2003), *Murraya koenigii* (curry leaf tree) (Babu et al. 2000), *Paphiopedilum philippinense* (orchids) (Chen et al. 2004), *Echinacea purpurea* (purple coneflower) (Koroch et al. 2002) *Datura meteloides* D. C. (Curtis et al. 1999), and many more have been reported.

Few reports mentioned using low light or dark for shoot organogenesis. Dolcet-Sanjuan et al. (1991) reported adventitious shoot regeneration in *Cydonia oblonga* L. (quince). Young leaves were kept in the dark for the first 3 weeks and then in 40 μmol/m^2/s for the following 3 weeks. Cambecèdes et al. (1991) regenerated leaf explants of *Lonicera nitida* Wils. cv. 'Maigrün' (Caprifoliaceae) using a 3-week-dark period following low light (5.6 μmol/m^2/s) treatment. Faure et al. (1998) reported in vitro regeneration of spearmint and peppermint by culturing leaf disks in the dark for 2 weeks and then under low light (6 μmol/m^2/s) for 6 weeks. Yadav and Padmaja (2003) reported that initial incubation for 5 days in the dark followed by transfer to 10/14 h light/dark cycle (12.1 μmol/m^2/s) for 45–50 days favored regeneration from leaf segments of pigeonpea (*Cajanus cajan* L.). However, none of these reports discuss the function of the dark treatment.

Our results revealed one of the rare cases where organogenesis benefited from low light. When leaves are cut into strips and infected by *Agrobacterium*, they suffer wounding. Orozco-Cardenas and Ryan (1999) reported that tomato plants generated hydrogen peroxide (H_2O_2) in response to wounding. Guayule, a plant rich in secondary metabolites, exhibits a more severe wounding response than some other plant species. The leaf strips turn brown, wither and die. The wounding response of guayule leaf strips is enhanced by high light and alleviated by low light. High light may accelerate the octadecanoid pathway, or increase the speed of H_2O_2 generation, or both. Low light may function in an opposite direction and allow the leaf strips to recover from wounding. It seems possible that shoot organogenesis from leaf tissue in other plant species rich in secondary metabolites also may be achieved under similar low light conditions.

Pellegrineschi (1997) reported shoot organogenesis from hypercotyls and cotyledons of *Vigna unguiculata* (L.) Walp. (cowpea) cultured under low light (10 μmol/m^2/s) for 20 days and then stronger light (40 μmol/m^2/s) for 20 days. The two-step low light to high light culture is very similar to our culture conditions. Also, similar to our results with guayule, shoot regeneration was higher in cultures of *Prunus avium* (sweet cherry) leaves incubated with a 16/8 h light/dark photoperiod (14 μmol/m^2/s) than those incubated in continuous darkness (Bhagwat and Lane 2004).

Our results also showed that guayule leaf strips grown on (1/2NH_4^+)MSB2 medium had a lower wounding response than those on MSB2 medium. One possible reason might be that the higher concentration of ammonia is toxic to guayule leaf strips and accelerates the wounding response. MS medium contains 1650 mg/l or 20 mM NH_4^+. This concentration is optimal for tobacco but may be too high for some other plants. Chio et al. (1998) reported that elimination of ammonium nitrate from MS medium promoted the root growth of Korean ginseng plantlets. Rugini and Mu-

ganu (1998) reported that media composition affected the shoot maintenance, but not induction of shoot regeneration from calli of *Malus×domestica* Borkh. cv. Golden Delicious (apple) secondary leaflets; the best composition was found to be high calcium (calcium nitrate 580 mg/l), low ammonium (ammonium nitrate 230 mg/l) and low hormone levels. Bassüner and Bauwe (1992) modified MS medium by halving the NH_4^+ level for in vitro *Flaveria pubescens* (Asteraceae) plant regeneration. These results indicate that lower NH_4^+ in MS does benefit some plant tissue cultures.

In conclusion, an efficient shoot organogenesis and gene transformation method has been established for guayule leaf tissue. Low light and low ammonium are key factors in making this method successful. Both herbicide resistance and kanamycin resistance are effective selective markers for transformed guayule cells. Transformation efficiencies ranged from 3 to 6%, a significant improvement over the previous best of 0.7%

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