Development of osmotin transgenics in *Hevea brasiliensis* Muell. Arg. using explants of zygotic origin

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Abstract

Hevea brasiliensis (para rubber tree) is a perennial outbreeding species and is the major source of commercial natural rubber (cis-1, 4-polyisoprene). In order to impart tolerance to the abiotic stresses such as drought and cold in *Hevea*, *Agrobacterium* mediated transformation was attempted with the gene coding for osmotin protein. *Agrobacterium* strain *GV 2260* harboring the plasmid osm/BinAR with osmotin gene under the control of CaMV35S promoter was employed for genetic transformation. Neomycin phosphotransferase gene (*npt*II) imparting kanamycin resistance was used as the selectable marker. The target explant used in this study was embryogenic callus induced from immature zygotic embryo following *half ovulo* embryo culture technique. A transformation frequency of about 44.8 per cent was achieved. Putative transgenic lines were proliferated and the gene integration was confirmed by PCR analysis. The sequence of the cloned PCR product showed 100 per cent similarity with the inserted gene sequence. Successful embryo induction (43.5%), maturation (46.0%) and plant regeneration (23.6%) were achieved. The regenerated plants were acclimatized in growth chamber and maintained in the containment facility in big polybags. Transgene integration and expression have been confirmed by PCR, Southern blot and RT-PCR.

Key words: *Agrobacterium;* GM; Genetic transformation; Osmotin; Rubber; Transgenic

Introduction

Hevea brasiliensis Muell. Arg. (para rubber tree), popularly known as the 'rubber tree', is a perennial outbreeding species and is the major source of commercial natural rubber (cis-1, 4-polyisoprene). Natural Rubber (NR), produced in specialized cells called laticifers, is one of the most important biological molecules used for the manufacture of about 40,000 products indispensible for the economic and commercial development of the world. Global projected gap between the demand and the supply of natural rubber by 2020 is one million tonnes and this necessitates production enhancement. The ever increasing demand for natural rubber can be met either by increasing the area under cultivation or by increasing the productivity and to this end, several NR producing countries are intensifying R&D in marginal areas which are unfit for cultivation. Unfavorable weather is the major challenge for successful rubber cultivation in such areas. For instance, prolonged drought and heat episodes affect plant growth and yield, causing annual losses estimated at billions of dollars. Hence the importance of genetic

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improvement in a perennial tree crop such as rubber, which faces the vagaries of the environment, needs no further emphasis. *H. brasiliensis* has a gestation period of 6 to 7 years and an economic life span of about 25 years. The identification and fixation of a particular gene through conventional methods of breeding requires several generations of crosses followed by long years of field evaluation. Recombinant DNA technology has become a powerful tool for crop improvement since it provides a means of genetic manipulation that can bypass sexual barriers and to some extent circumvent the limitations of the long breeding cycle of perennial trees. Among the different methods for gene transfer, *Agrobacterium* mediated gene transfer is the most widely used and powerful technique for the production of transgenic plants in the recent past. Since the major harvested products such as latex, wood and rubber seed oil are not used as food material, the biosafety concerns are less for the genetically modified rubber plants. Therefore, genetic engineering can definitely play an important role in the future of rubber industry.

Osmotin is a stress responsive multifunctional protein belonging to PR-5 protein family, providing osmotolerance to plants (Amjad and Malik, 2008). Gene expression studies revealed that its transcription can be activated by several factors like sodium chloride, desiccation, ethylene, wounding, abscisic acid, tobacco mosaic virus, fungi and UV light (Liu et al., 1994). Transgenic plants integrated with osmotin gene have been produced in different crop species (Subramanyam et al., 2011; Subramanian et al., 2012) for biotic/abiotic stress tolerance. Previously, we have reported the transformation of another clone of *Hevea brasiliensis* RRII 105 using the embryogenic calli derived from the anthers (Rekha et al., 2013). During this process, the major difficulty with the clonal explants was in hardening. Even though the plants survived the initial phase of hardening, they could not thrive beyond 3 months. This paper successfully demonstrates that the use of embryogenic calli derived from the zygote can solve this problem, obtaining high transformation frequency as well as better plant regeneration. Further, the use of juvenile explant was instrumental in overcoming the problem in hardening. The *Agrobacterium* mediated transfer of the gene that codes for osmotin protein, which was isolated from tobacco and is instrumental in imparting abiotic stress tolerance in *Hevea brasiliensis,* is detailed.

Materials and Methods

Target tissue

Embryogenic calli derived from zygotic embryo was used as the target tissue. The calli were raised through *half ovulo* embryo culture technique. Immature fruits (8-10 weeks old) were collected from field grown trees of the clone RRII 105, washed thoroughly in distilled water and dried. Fruits were then dipped in 80 per cent alcohol for 15 minutes and allowed to dry on a sterile filter paper in a petri plate. The developing seeds were isolated aseptically from the fruits and inoculated in the nutrient media. Since embryos are not visible at early stages and are difficult to dissect, the immature seeds were cut into two halves and placed in the medium with the micropylar end touching the medium. Nitsch (1960) basal medium with GA_3

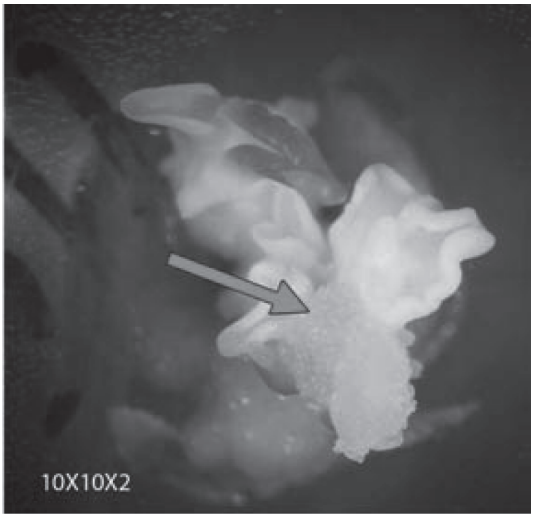


Figure 1. Emergence of embryogenic calli from the zygotic embryo

(3mg l^{-1}), kinetin (3 mg l^{-1}) and Zeatin (0.4 mg l^{-1}) was used for inducing the calli. Embryogenic calli emerged within 30 days after inoculation (Fig.1) and further proliferation was obtained by replacing the growth regulators with NAA (0.2 mg l^{-1}). This proliferated embryogenic calli was used as target tissue for transformation.

Osmotin gene construct

Agrobacterium strain GV 2260 harbouring the plasmid osm/BinAR under the control of CaMV35 S promoter containing kanamycin resistance as the selectable marker (Barthakur et al., 2001) was employed for genetic transformation (Fig.2). This

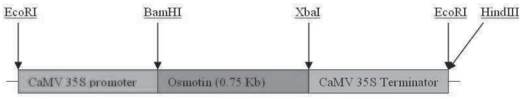


Figure 2. The construct used for transferring the osmotin gene in this study

gene construct was obtained from Dr. K.C. Bansal, Director, National Bureau of Plant Genetic Resources, New Delhi, India, under the Material Transfer Agreement.

Transformation protocol

50 µl *Agrobacterium* glycerol stock was added to 5 ml MGL medium with pH 7.0 (Jones et al., 2005) containing 50 mg l^{-1} kanamycin, 75 mg l^{-1} rifampicin and 100 mgl^{-1} carbenecillin. This was kept in an incubator shaker overnight at 23°C and 250 rpm. After 24 hours of growth, the bacterial culture was subjected to a pH shock by diluting 2.5 ml of the culture with 7.5 ml of TY medium (pH 5.5) (Rekha et al., 2013) containing the respective antibiotics and 40 mg l^{-1} acetosyringone. The cultures were again incubated overnight at 23°C and 250 rpm. The next day, 1.5 ml of the bacterial culture was diluted to 20 ml with TY medium (pH 5.5), containing 40 mg l^{-1} acetosyringone. The optical density (OD) was measured against TY blank at 600 nm and adjusted to the optimal level (0.1-0.2) and used for transformation. The explants were kept in a sterile filter paper placed in a 35 mm petri plate and soaked with *Agrobacterium* suspension using a micropipette. After 5 minutes, the calli were blotted dry and transferred to co- culture media and were incubated at 23°C for 3 days. The co culture media was supplemented with 40 mg l^{-1} acetosyringone, 115 mg l^{-1} proline and 113mg l^{-1} glycine betain hydrochloride.

Selection of transgenic callus lines

After 3 days of co culture, the infected calli were blotted dry using a sterile filter paper and were transferred to selection medium containing 500 mg l^{-1} cefotaxime for preventing the bacterial growth and 300 mg l^{-1} kanamycin for selection. After 30 days, cefotaxime was omitted from the medium and selection for putative transgenic cell lines in the presence of kanamycin was continued. The cultures were observed weekly and those cultures with overgrowth of the bacteria were discarded and the rest of the cultures were carried forward. Putative transformed cell lines emerging from these cultures were selected and transferred to proliferation medium. For proliferation, a combination of NAA and 2, 4, D was used as reported earlier (Rekha et al., 2013).

Confirmation of the transgene integration by PCR analysis

Osmotin gene integration was tested in randomly selected cell lines developed from independent transformation events, by PCR analysis using osmotin gene specific primers as per the standard procedure.

Embryo induction

The proliferated embryogenic callus was subcultured on to different media combinations for achieving embryo induction. The basal medium used was the one already reported for embryo

induction from transgenic cell lines of rubber (Rekha et al., 2013). Based on the preliminary observations, experiments were designed with varying concentrations and combinations of growth regulators *viz*. NAA (0-5 mg l^{-1}), Kin (0.1- 0.5 mg l^{-1}), along with 50 g l^{-1} sucrose and B5 vitamins. Media constituents (as mentioned above) other than the growth regulators were kept the same and the culture medium was solidified with 0.3 per cent phytagel. Each treatment contained 25 calli groups and the experiments were repeated 5 times. Observations on embryo induction were recorded after two months and percentage embryo induction was worked out.

Embryo maturation

Embryos were transferred to the maturation medium one month after embryo induction. For maturation, the same basal medium which was used for embryo induction was employed. Since ABA is an established growth regulator for achieving embryo maturation in several crops, different levels of ABA (0.2-1.0 mg l^{-1}) were supplied with varying levels of phytagel (0.2- 0.6%) in the hormone free basal medium for embryo maturation.

Germination and plant regeneration

Mature somatic embryos with well developed cotyledons were transferred to the germination medium. Poorly developed and abnormal embryos were discarded. MS medium, supplemented with different proportions of the growth regulators BA and GA (0.5-5 mg l^{-1}) were tried for germination. Embryos transferred to these media combinations were incubated in dark condition for a period of 2 weeks and then transferred to light for shoot development. Experiments were repeated 5 times with 20 mature embryos per set. The germinated embryos were transferred to MS medium for plant regeneration. For plant regeneration, hormone free medium with 20 per cent coconut water was used. The pH of all media was adjusted to 5.7 and autoclaved at 121ºC for 10 minutes. Cultures were raised in culture tubes containing 20 ml medium and incubated at 25±2ºC under 16 h photoperiod. Subcultures were done every 3 weeks.

Acclimatization

Well developed plants with 2-3 mature leaves were transferred for hardening. Plantlets were carefully removed from the culture tubes and washed gently in running tap water to remove adhering medium. Dead tissues near the cotyledons, if any, were also removed. Plantlets were dipped in 0.2 per cent fungicide solution for 3 minutes in order to reduce fungal infection and were blotted to remove the adhering water particles. The seedlings were then planted in earthenware pots containing sterile sand, soil and soil-rite mixture and kept in controlled conditions in growth chamber at 25°C with 90 per cent relative humidity. Humidity was decreased by 2 units at 2 days interval. Simultaneously, temperature was gradually increased from 25°C to 30°C. These plants were supplied with dilute Hoagland mixture once weekly and watering was done at two days' interval. After about three weeks, the surviving plants were transferred to poly bags filled with garden mixture (1:1:1) and kept in the growth chamber itself. After the emergence and maturation of a new flush, the hardened plants were taken out from the growth chamber and maintained in the containment facility.

Molecular assays

PCR analysis

Agrobacterium strain GV 2260 was grown in MG/L liquid medium. Plasmids were isolated from overnight grown liquid cultures of *Agrobacterium* using standard protocol. From the hardened transgenic plants and control plants, genomic DNA was extracted according to CTAB method (Doyle and Doyle, 1990). PCR analysis was carried out with the osmotin-specific primers designed to amplify the DNA fragment of 0.75 kb using standard procedure (Sambrook et al., 1989). The forward and

reverse primers specific for the osmotin coding region were 5'-ATGGGCAACTTGAGATCTTCT-3' and 5'-CTACTTAGCCACTTCATC- 3' respectively. Plasmid DNA was used as a positive control whereas DNA from the untransformed plant was used as the negative control. PCR analysis was repeated with npt II specific primers in order to detect the presence of marker gene in transgenic plants. The forward and reverse primers are 5'-GAG GCT ATT CGG CTA TGA CT 3' and 5'-AAT CTC GTG ATG GCA GGT TG 3' respectively.

PCR reactions were carried out using 50 ng template, 100 mM each of dATP, dGTP, dTTP, dCTP, 250 nM of each primer, 0.5 µl *Taq* DNA polymerase and 1.5 mM $MgCl_2$ in a final volume of 20 µl. The reaction mixture was incubated in a thermal cycler under the following conditions. The initial denaturation step was carried out at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, and with a final extension step of 72°C for 7 min. The amplified PCR products were visualized on a 1.2 per cent agarose gel stained with ethidium bromide using 0.5 X TBE as the running buffer.

Southern hybridization analysis

For the confirmation of osmotin gene integration Southern hybridization was performed as per the standard procedure (Southern, 1975). Sufficient quantity of genomic DNA with good quality was isolated from transgenic as well as non-transgenic leaves. Around 30 ìg of genomic DNA from the transgenic plants was digested with *Eco*R1 in separate reactions. The digestion was performed overnight at 37°C. The fragments were size fractionated on 1 per cent agarose gel. Blotting was carried out following the standard procedure developed by Southern (1975). DNA digests were loaded in 1 per cent agarose gel and electrophoresis was carried out at 40V for 5h. The osmotin gene from the construct was used as the probe for Southern hybridization. It was amplified from the cloned binary vector (osm/Bin AR) through PCR using gene specific primers, purified and radio labeled using 'Multiprime DNA labeling system' from M/S Amersham (UK) following manufacturer's instructions. Hybridization of the labelled probe to the nylon membrane was performed according to Sambrook and Russell (2001).

RT-PCR analysis

In order to study the expression of osmotin gene in *Hevea,* total RNA was isolated from the transgenic and non-transgenic leaves (both callus and leaves), cDNA synthesis was carried out using manufacturer's instructions (M/S Promega ImpromII reverse transcription kit), PCR was performed using osmotin specific primers with the cDNA as the template and PCR amplification was carried out as described earlier, using osmotin gene specific primers with non-transgenic cDNA as the negative control, plasmid as the positive control and transgenic cDNA as the test sample.

Results and Discussion

Agrobacterium mediated genetic transformation experiments resulted in successful integration of osmotin gene into *H. brasiliensis* callus. Transgenic cell lines were developed, proliferated, embryos induced, matured and germinated to develop transgenic plants integrated with osmotin gene. The transgene integration and expression were confirmed by molecular analysis. Plants were hardened and grown in containment facility.

Selection of putative transgenic cell lines

After co-culture for three days, the infected calli were transferred to selection medium with appropriate antibiotics (kanamycin 300 mg l^{-1} and cefotaxime 500 mg l^{-1}). Initially, in the selection medium, most of the calli clumps gradually turned brown. Later, fresh creamy yellow cell clusters started emerging from the clumps after 40 to 60 days of culture in the selection medium. With a view to

eliminate false positives, these cell clusters were recovered from dying explant tissue and transferred to fresh selection medium. After 2 to 3 weeks of culture in the fresh medium with kanamycin, putative transformed cell lines, showing resistance to kanamycin proliferated, whereas the false positives dried out (Fig. 3a). A high frequency transformation (46.3%) was obtained and numerous cell lines were emerged. Explant selection is a critical parameter while performing transformation and regeneration experiments. Embryogenic calli derived from different sources such as anther (Montaro et al., 2003; Blanc et al., 2006) leaf (Kala et al., 2006) and ovule (Rekha et al., 2006) have been used as potential target tissues for genetic transformation in *Hevea* with different gene constructs and high frequency transformation have been reported. The availability of totipotent cells as targets for transformation is today the limiting factor in genetic transformation of recalcitrant woody species (Birch, 1997). Juvenility of the explant, friability and texture of the calli are the factors influencing transformation frequency. Generally it is observed that the texture of the callus and friability vary with initial explants and this will be reflected in the transformation and regeneration frequencies. In the present study, embryogenic calli emerged from the immature zygotic embryo is the target tissue. Hence more regeneration and better acclimatization potential are expected. Embryos and embryogenic calli derived from embryos are used in many crops for somatic embryogenesis (Gupta and Grob, 1995; Klimaszewska and Cyr, 2002; Von Arnold et al., 2002). In citrus, juvenile tissues showed higher sensitivity to *Agrobacterium* than the mature ones (Cervera et al., 2004). The high transformation efficiency observed in the present study may be attributed to the juvenility, proliferation potential and fine texture of the callus derived from zygotic embryos. The proliferated lines were subjected to PCR analysis and the PCR positive lines were used for further experiments.

Embryo induction

These lines were subcultured to different formulations of embryo induction medium. Embryos could be developed from the proliferated calli after subculture into embryo induction medium. From the different experiments carried out for embryo induction, it was clearly observed that growth regulators play a pivotal role in

Table 1. Effect of NAA and Kinetin on embryo induction from transgenic cell lines derived from zygote derived embryogenic calli

	Embryo induction percentage					
NAA→	1.0	2.0	3.0	4.0	5.0	10
Kini↓						
0.1	14	25.5	25.0	15	15.75	12.25
	(21.97)	(30.18)	(30.34)	(22.79)	(23.39)	(20.44)
0.2	17.5	30	25.25	27.25	17.75	15
	(24.74)	(33.13)	(30.18)	(27.46)	(24.93)	(22.79)
0.3	20.75	43.5	38	32.75	27.75	19.5
	(27.11)	(41.29)	(38.01)	(34.93)	(31.80)	(26.21)
0.5	21	31.5	37	33.25	27	20.75
	(27.29)	(34.16)	(37.48)	(35.23)	(31.32)	(27.11)
1.0	17.25	31.75	27.75	25.75	23	7.75
	(24.55)	(34.31)	(31.80)	(3 0.5)	(28.67)	(16.17)
				CD (0.05)	(Kin x NAA)	= 1.23

The data were subjected to arc sine transformation and transformed means are given in parentheses

embryogenesis from the osmotin transgenic cell lines. The optimum level of the growth regulators, NAA and kinetin for embryo induction were derived from a factorial experiment and the results are presented in Table 1.

NAA and kinetin influenced induction of somatic embryos from transgenic cell lines integrated with osmotin gene. The highest percentage of embryo induction was obtained in a combination of 2 mg l^{-1} NAA and 0.3 mg l^{-1} kinetin. Further increase in concentration of NAA resulted in excess proliferation of the callus and reduction of embryo induction frequency. Higher concentrations of kinetin also showed significant reduction in embryo induction from transgenic cell lines. The time taken for embryo induction was about 80-100 days in this medium. Initially the embryos were translucent, then turned milky white and gradually turned to ivory colour (Fig. 3b).

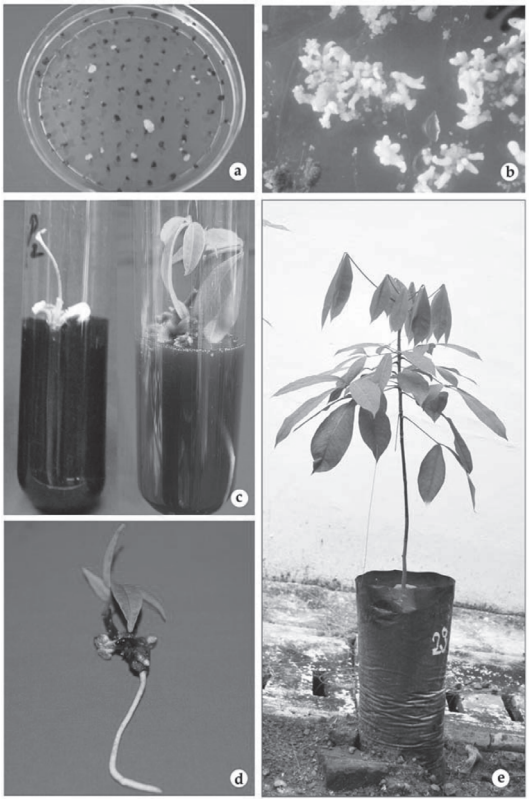


Figure 3. Different stages of transgenic plant development (a) Emergence of transgenic cell lines (b) Embryo induction (c) Germination (d) Plant regeneration (e) Acclimatized transgenic plant in the polybags

Induction of the embryogenic capacity is influenced by several factors, including basal medium components, plant growth regulators and culture conditions. Somatic embryogenesis has been studied extensively in *Hevea* by many workers (Etienne et al., 1993; Carron et al., 1995; Montaro et al., 2003; Jayasree et al., 1999; Sushamakumari et al., 2000). The culture conditions and basal media have already been standardized. However, the insertion of a foreign gene can alter the genetic makeup of the cells and change the regeneration capacity. Hence optimization of conditions for establishing somatic embryogenesis is highly essential for the transgenic cell lines. In the present study, a combination of NAA and kinetin worked well for the induction of somatic embryos from the transgenic cell lines of *Hevea* integrated with osmotin gene and an embryo induction frequency of 43 per cent was obtained. Jayasree et al., (1999) reported a profound influence of NAA on somatic embryogenesis in *Hevea.* NAA at 0.2 mg l^{-1} promoted embryo induction whereas higher concentrations beyond 0.3 mg l^{-1} resulted in low embryo induction frequency. However in the present study, the maximum percentage of embryo induction was observed at a higher concentration of NAA (2 mg l^{-1}). NAA beyond 3 mg l^{-1} resulted in reduction in the embryo induction frequency. Lelu et al. (1999) reported that auxins and cytokinins are necessary for inducing and sustaining embryogenesis except in a few cases. In *Centella asiatica,* it was reported that NAA and kinetin were superior in the induction of somatic embryogenesis, over 2, 4-D and kinetin (Martin, 2004).

Embryo maturation

The globular embryos developed into cotyledonary embryos in the embryo induction medium itself. These cotyledonary embryos were transferred to embryo maturation medium. The effect of ABA and phytagel on maturation of transgenic embryos is evident (Table 2). The maturation of embryos

Table 2 .Effect of phytagel and ABA on maturation of transgenic embryos integrated with osmotin gene (%)

	Embryo maturation percentage				
ABA (mg l^{-1}) →	0.2	0.4	0.6	0.8	1.0
Phytagel (%)↓					
0.2	14.75 (22.58)	17.75 (24.91)	21.75 (27.79)	27.25 (31.47)	30.75 33.68) *
0.3	13.75 (21.75)	22.75 (28.48)	28.25 (32.105)	32.00 (34.443)	35.0 (36.27)
0.4	20.75 (27.08)	27.75 (31.78)	31.75 (34.29)	34.0 (35.67)	35.75 (36.72)
0.5	28.0 (31.94)	35.0 (36.27)	41.25 (39.963)	46.0 (42.70)	38.0 (38.055)
0.6	29.5 (32.86)	24.0 (29.33)	24.5 (29.66)	22.5 (28.30)	20.25 (26.74)
			CD(0.05)	ABA X phytagel	=1.16

Data were subjected to arc sine transformation and transformed means are given in parentheses.

improved with higher concentration of phytagel as well as ABA. Maximum maturation was observed in a combination of 0.5 per cent phytagel along with 0.8 mgl^{-1} ABA. During the period, embryos enlarged in size and the cotyledons turned ivory coloured and opaque. Maturation of the embryos needed four to six weeks under dark incubation. Embryo maturation is a process in which the embryos accumulate enough reserve material for germination and is a key phase between embryo development and germination (Quatrano, 1987). According to Ammirato (1983), poor quality and incomplete maturation of the somatic embryos are considered to be the main factors limiting the conversion of embryos into plants, and the culture conditions must be changed in a sequential manner. Embryo maturation is affected by various physical factors, depending on the requirement of the species. In *Hevea*, a study of the zygotic model has revealed the need to induce a maturation phase prior to embryo germination, primarily for the completion of cauline meristems formation, accumulation of starch / protein and desiccation (Carron et al., 1995). In the present study, different concentrations of ABA along with varying levels of phytagel were tried for embryo maturation. Embryo maturation occurred at a higher level of phytagel (0.5%) along with 0.8 mgl^{-1} ABA. The importance of water relations in controlling embryo maturation has been supported by evidence from both zygotic and somatic embryo culture experiments (Xu et al., 1990). High concentrations of phytagel have been used for reducing the vitrification of coconut somatic embryos (Perera et al., 2011). The positive influence of ABA for cotyledon development, organization of meristem, procambial bundles, the epidermis and the latex bearing vessels, protein reserve accumulation (Etienne et al., 1993) and for preventing precocious germination in Hevea have been reported earlier. In contrast to the present study, Sushamakumari et al., (2000) reported that high sucrose level has favoured embryo maturation rather than ABA.

Embryo germination and plant regeneration

Among the growth regulators tried, a maximum germination of 23.6 per cent was obtained in a combination of 1.5 mg l^{-1} BA and 1.5 mg l^{-1} GA_3 in MS basal medium. Higher concentrations had a negative effect on the germination of embryos (Table 3) (Fig. 3c). In our experiments, maximum germination occurred in half strength MS medium supplemented with 1.5 mg l^{-1} BA and 1.5 mg l^{-1} GA_3.

Table 3. Effect of BA and GA_3 on germination of transgenic embryos

BA (mg l^{-1}) → GA_3 (mg l^{-1}) ↓	0.5	1	1.5	2	5
0.5	10.40 (3.22)	12.40 (3.64)	12.8 (3.57)	14.40 (3.78)	10.80 (3.28)
1	10.80 (3.27)	14.40 (3.79)	17.20 (4.14)	15.60 (3.94)	12.0.00 (3.46)
1.5	14.40 (3.78)	18.40 (4.23)	23.6 (4.86)	17.20 (4.15)	14.40 (3.46)
2	13.6 (3.78)	17.2 (3.68)	17.20 (4.14)	16.40 (4.05)	9.60 (3.08)
5	9.60 (3.08)	10.00 (3.15)	14.40 (3.78)	14.40 (3.78)	7.60 (2.74)
			CD (0.05)	interaction	0.28

The values were subjected to square root transformation and transformed values are given in parentheses

Beneficial effect of GA_3 on germination has already been reported in *Hevea* (Carron et al., 1995; Jayasree and Thulaseedharan, 2001). A combination of BA and GA_3 induced 67 per cent germination in somatic embryos developed from root explants in *Hevea* (Sushamakumari et al., 2006). Similarly BA (0.3 mg l^{-1}) and GA_3 (0.3 mg l^{-1}) were used for the germination of rescued zygotic embryos in the immature stage in *Hevea* (Rekha et al., 2006). Incorporation of GA_3 in combination with BA and IBA in the germination medium favoured bipolar differentiation and improved germination and plant regeneration in transgenic embryos of *Hevea* integrated with MnSOD (Sobha et al., 2003). Even though the exact mechanism of the beneficial role of GA_3 on embryo germination is not clear, ultrastructural studies carried out by Choi et al., (1999) showed that somatic embryos developed *in vitro* could be dormant after maturation and a breakage of the dormancy is required.

During *in vitro* culture, many factors other than growth regulators were found to affect somatic embryo germination. In the present study, sucrose was reduced to 20 g l^{-1} for germination and plant regeneration. In *Hevea,* use of reduced levels of sucrose in the germination as well as in the plant regeneration medium has been reported earlier (Carron et al.1995; Kala et al., 2008). The germinated embryos, after root and shoot elongation, were transferred to plant regeneration medium.

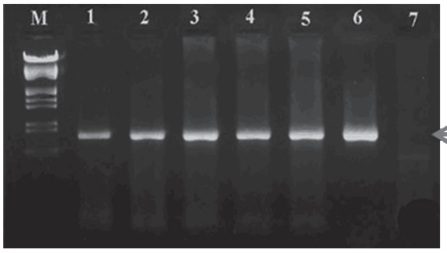
Improvement of transgenic embryo germination and conversion is a long term effort, as many factors contribute to germination and conversion capacity especially in a perennial crop like *Hevea*. The germinated embryos were transferred carefully into the plant regeneration medium so that the root tips were not damaged. During the plant regeneration phase, repeated subculturing of the regenerated plants resulted in great damage of the root and shoots leading to senescence of the plantlets. In order to overcome this, culture tubes were prepared with medium enough to last for about two months. About 23 per cent plant regeneration frequency could be obtained in hormone free medium with 20 per cent coconut water.

Acclimatization

Transgenic plants integrated with osmotin gene could be acclimatized successfully, when the plantlets were kept in environment controlled growth chamber with gradual reduction in humidity. The hardened plants were transferred to containment facility and maintained in larger polybags (Fig. 3e). Acclimatization of the plantlets is a major problem in woody plant species. A large number of *in vitro*

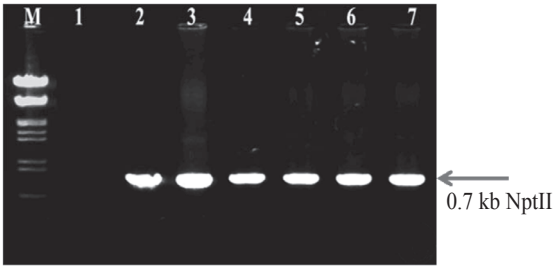
produced plants do not survive the transfer from *in vitro* to the *ex vitro* environment under greenhouse or field conditions. Compared to *in vitro* conditions, the greenhouse and field have substantially lower relative humidity, higher temperature and higher light level and septic environment that are stressful to micropropagated plants (Hazarika, 2003; Hazarika and Bora, 2010). According to Gutiérrez-Mora et al. (2012), during acclimatization process, the *in vitro* developed plants should develop cuticle, epicuticular waxes, and should establish an effective stomatal regulation of transpiration, leading to stabilization of water status. In *Hevea* also acclimatization is reported to be a bottleneck in developing transgenics. The hardening percentage reported was as low as 4 per cent for MnSOD transformants (Jayashree et al., 2003). In earlier experiments using the same gene construct with clonal material even though an initial success of 10 per cent was obtained, gradual retardation in growth was observed after 3 months and the final success was meager (Rekha, 2013). However in the present study, a better acclimatization was observed and about 50 per cent hardening was obtained. Explant juvenility is the major factor behind this. The more juvenile the tissue, more will be the response. Juvenile tissues, such as zygotic embryos, have better potential and competence to produce embryos and organs compared to more differentiated and mature tissues. Zygotic embryo has been used as explant for somatic embryogenesis and for developing transgenic plants in different crop species (Elhiti and Stasolla, 2011). The increased efficiency of embryogenic callus formation and regeneration of plantlets was observed in many crops by using polyembryos derived from the zygote as the explant. The secondary somatic embryos derived from the cotyledonary explants of zygotic

A) Amplification of osmotin gene (0.75 kb)



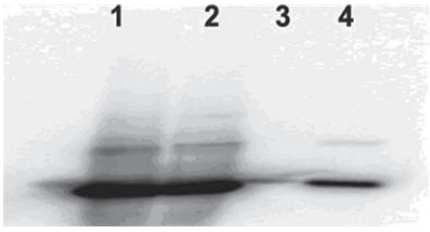
Lane 1-5 Trangenic cell lines
Lane 6 Plasmid
Lane 7 Non Trangenic control

B) Amplification of npt II(0.75 kb) gene



Lane 1 Non Trangenic control
Lane 2-6 Trangenic cell lines
Lane 7 Plasmid

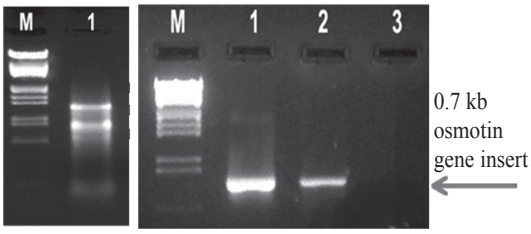
C) Southern Hybridisation



Positive signal obtained in the southern blot of DNA isolated from transgenics (*Eco*RI digest)

D) RT-PCR

RNA



Lane M Marker
Lane 1 Positive control
Lane 2 Trangenic sample
Lane 3 Non Trangenic sample

Figure 4. (A-D) Molecular analyses of transgenic plants

embryos were successfully utilized for the development of transgenic plants in walnut (Dandekar et al., 1989).

Molecular confirmation of gene integration and expression in transgenic plants

The recovered plants after transformation were analyzed at different levels to ensure that they were transgenics. When the plants were large enough to spare some leaves without compromising the vigour, they were analyzed for the presence of transgene and its expression.

PCR analysis

Polymerase chain reaction was carried out at two stages. The putative transgenic lines after kanamycin screening were separated individually and transferred to proliferation medium fortified with kanamycin. Since each line represents a single transformation event, each transgenic cell line showing kanamycin resistance was handled individually. Both *npt11* and osmotin gene specific primers were used for the experiment. Results had shown that all the lines tested were PCR positive. The 0.75 kb osmotin gene insert has been positively amplified from all the transgenic cell lines whereas no amplification was obtained from the non-transgenic cell (Fig. 4a). This indicated the presence of the transgene in the transformed cell lines of *H. brasiliensis* genome. The PCR positive lines were used for further experiments.

Further, 0.7kb *nptII* gene was also amplified from the transgenic plants (Fig. 4b). The osmotin amplicon was cloned and sequenced and the sequence showed 100 per cent similarity with the inserted gene, confirming the transgene integration.

Southern blot analysis

Southern hybridization had proven the successful integration of the transgene in to *Hevea* genome. Fig. 4c shows the blot with *Eco*RI digestion, and positive signals were obtained at different lanes indicating the random integration of gene insert. Three bands were obtained in the first two transgenic plants and 2 bands were obtained for the 4th transgenic plant. From the results obtained, we can infer that the transgene is stably integrated in *Hevea* genome.

RT-PCR-analysis

The 0.75 kb osmotin gene insert was amplified from the cDNA prepared from the RNA isolated from the leaves of transgenic plants, which in turn confirmed the transgene expression (Fig. 4d). The study of transgene expression is of vital importance whenever transgenic plants are produced. RT-PCR enables researchers to quickly identify plants that are expressing transgenes. The availability of kits has made RT-PCR a fast and reliable test for transgene expression analysis, circumventing the need for Northern analysis. It is identical to conventional PCR, except that the template DNA is created by reverse transcription of RNA. The reverse transcription PCR utilizes the ability of the enzyme reverse transcriptase to synthesize DNA from RNA template. The positive or negative PCR results represent the presence or absence of RNA transcription in the original RNA sample and therefore, are an indicator of specific gene expression. In the present study, positive PCR amplification was obtained from the DNA synthesized from RNA isolated from putative transformants, indicating the transgene expression. The amplification was absent in the negative control. *Agrobacterium* mediated genetic transformation was attempted in *Hevea* using zygote derived embryogenic calli as the explant. High frequency transformation was obtained. Transgenic plants were developed *via* somatic embryogenesis. Plants were successfully hardened and maintained in big polybags. The gene integration and expression was confirmed by PCR, Southern and RT-PCR. These plants are expected to perform well under stressful environments. Use of zygote derived explants is a new approach in *Hevea* transformation and this

ensures easy regeneration and hardening. Even though the clonal integrity is not maintained here, the plants are equivalent to half sibs incorporated with a novel gene. Development of transgenic root stocks is yet another possibility.

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