Rubber Science, **29**(2): 140-152, 2016 RESEARCH ARTICLE

EXPRESSION OF STRESS TOLERANCE IN TRANSGENIC CALLUS INTEGRATED WITH OSMOTIN GENE IN *HEVEA BRASILIENSIS*

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Stressful environment is a limiting factor for rubber productivity in the traditional rubber growing areas and limits the expansion of rubber cultivation to newer areas in several rubber producing countries, including India. Conventional methods of *Hevea* breeding take several years to produce plants with desirable traits. Genetic transformation offers a viable approach for producing plants with desired traits within a short period of time. Since osmotin gene is reported to impart tolerance to abiotic and biotic stress, the present study on *Agrobacterium* mediated gene transfer was carried out with the gene encoding osmotin protein. Osmotin gene construct with CaMV 35S promoter and kanamycin as selectable marker was used for the purpose. Transformation frequency of 48 per cent was obtained from anther derived embryogenic calli. Molecular analysis with PCR and RT-PCR could confirm the integration of gene and its expression. The calli were subjected to PEG as well as salt stress for fixed intervals. Higher proline accumulation in transgenic cell lines compared to control was observed under PEG stress. Transgenic cell lines could tolerate higher concentration of salt (150 mM), whereas the control calli showed growth retardation even in lower salt concentration (50 mM). The regenerated plants from these transgenic lines are expected to perform well under stressful environments.

Keywords: Genetic transformation, *Hevea brasiliensis*, Osmotin, Stress tolerance, Proline estimation

INTRODUCTION

Hevea brasiliensis Muell. Arg (Para rubber tree) 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 a nation. Unpredictable climatic conditions such as prolonged drought and heat period affect plant growth and yield, causing annual loss estimated at billions of dollars. Stressful environment is a limiting factor for rubber productivity in the traditional rubber growing areas and limits the expansion of

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cultivation to newer areas in several rubber producing countries including India. Hence, importance of genetic improvement in a perennial tree crop like rubber, needs no further emphasis. The identification and fixation of a particular gene through conventional methods of breeding requires several generations of crosses and field trials over many years. Genetic transformation is a viable option in this context (Venkatachalam *et al.*, 2007).

Among the several stress-related proteins, osmotin is one of the unique proteins isolated from tobacco cell cultures which are induced in response to both biotic and abiotic stresses (Singh *et al.*, 1985; 1987; LaRosa *et al.*, 1992; Raghothama *et al.*, 1997). Osmotin and osmotin-like proteins or the genes encoding these proteins have been extensively studied in several laboratories (Singh *et al.*, 1987; LaRosa *et al.*, 1989; Meeks-Wagner *et al.*, 1989; Grosset *et al.*, 1990a; 1990b; Neale *et al.*, 1990; Roberts and Selitrennikoff, 1990 and Woloshuk *et al.*, 1991). Stress conditions such as salinity, desiccation, ethylene, wounding, abscisic acid, tobacco mosaic virus, fungi, and UV light were found to be inducers of this protein (Singh *et al.*, 1987, LaRosa *et al.*, 1992). The large spectrum of cues, both abiotic and biotic, gave good indications that osmotin gene could always be activated under field conditions. Osmotin is positively associated with programmed cell death, inhibits cold-induced calcium signaling and affects cytoskeleton in response to cold stimuli (Angeli and Altamura, 2007). Transgenic plants integrated with osmotin gene have been produced in different crops like tobacco (Barthakur *et al.*, 2001), potato (Liu *et al.*, 1994; Li *et al.*, 1999), tea (Bhattacharya *et al.*, 2006 a & b), olive (Angeli and Altamura, 2007), strawberry (Amjad and Malik, 2008), tomato (Goel *et al.*, 2010) and soybean (Subramanyam *et al.*, 2012) which imparted tolerance to biotic as well as abiotic stresses.

The major objectives of the present study consists of the introduction of tobacco osmotin gene in to *Hevea* tissues via *Agrobacterium* mediated gene transfer, molecular confirmation of transgene integration, development of transgenic cell lines and their evaluation for intrinsic stress tolerance traits.

MATERIALS AND METHODS

Immature inflorescences were collected from rubber trees (clone RRII 105) growing in the experimental fields of Rubber Research Institute of India, Kottayam, Kerala. Young male flower buds were separated and sterilized as per the standard procedure and immature anther at diploid stage (before microsporogenesis) was dissected out aseptically and inoculated on callus induction medium (KumariJayasree *et al.*, 1999). The primary calli that emerged from the anthers were cultured for embryogenic callus induction as reported by Jayashree *et al.*(2003). Embryogenic calli that emerged were used as the target tissue for transformation.

Osmotin gene construct

Agrobacterium strain GV 2260 harbouring the plasmid osm/BinAR under the control of CaMV35S promoter containing kanamycin resistance as the selectable marker (Barthakur *et al.*, 2001) was employed for genetic transformation (Fig. 1).

Agrobacterium infection and selection of transgenic cell lines

For infection, *Agrobacterium* suspension was prepared and the calli were infected following the procedure reported earlier by Rekha *et al.* (2013). Following infection, the cultures were co-cultivated for three days.

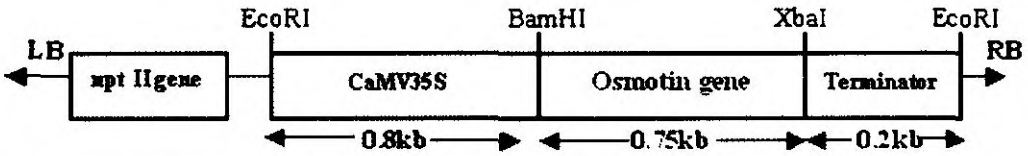


Fig.1.The osmotin gene construct with CaMV35S promoter and *npt* II gene as the selectable marker

The infected calli were blotted dry using a sterile filter paper and were transferred to selection medium containing 500 mg L^{-1} cefotaxime and 300 mg L^{-1} kanamycin (Jayashree *et al.*, 2003; Sobha *et al.*, 2003; Rekha *et al.*, 2006). The cultures were maintained at 25±2 °C in the dark. The cultures were observed weekly and those cultures with overgrowth of the bacteria were discarded. Sub-culturing to fresh selection media was done at three weeks interval.

After two sub-cultures, cefotaxime was omitted from the medium and selection for putative transgenic cell lines in the presence of kanamycin was continued. Putative transformed cell lines emerged from these cultures were selected and transferred to proliferation medium. For proliferation, MS medium supplemented with 1mgL^{-1} NAA and 10 gL^{-1} mannitol (Rekha *et al.*, 2013) was used.

Confirmation of the transgene integration by PCR analysis

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

Isolation of genomic DNA

Putative transgenic soft friable callus obtained after three or four weeks of culture in the proliferation medium were used for DNA isolation. Genomic DNA was extracted from the transformed as well as untransformed callus according to the modified CTAB method (Doyle and Doyle, 1990). The quality as well as the quantity of genomic DNA was checked on agarose gel in a UV trans-illuminator (Beckman USA). DNA quantification was also carried out using a Nano-Drop1000 spectrophotometer. Absorbance ratio (A 260/280) of 1.8 indicated good quality DNA without protein contamination. The DNA samples were stored at –20°C.

Plasmid DNA with the osmotin gene insert was isolated from *Agrobacterium* following the alkaline lysis method (Birnboim and Doly, 1979) and used as a positive control in molecular studies

Primer designing

Based on a previously published cDNA sequence of osmotin (Kumar and Spencer, 1992) gene specific oligonucleotide primers (forward and reverse) were designed with the help of 'Primer3' programme of 'Lasergene' software (DNASTAR, USA). The primer sequences and their Tm value are shown below:

Forward -	5'-ATG GGC AAC TTG AGA TCT TCT-3' Tm- 55.9
Reverse -	5'-CTA CTT AGC CAC TTC ATC- 3' Tm- 53.2

Polymerase chain reaction

The synthesized primers (M/s. Metabion, Deutschland) were dissolved in sterile double distilled water to get a concentration of 100 µM. The primer stock solutions were stored at –20°C.

PCR analysis was carried out with the osmotin gene specific primers to amplify the DNA fragment of 0.75 kb following the standard procedure. PCR amplification was performed with 50 ng of genomic DNA as templates. Plasmid DNA was used as the positive control, whereas DNA from the untransformed calli was used as the negative control. Amplifications were carried out in 20 µL reactions, containing the components mentioned in Table 1.

The reaction mix was overlaid with a drop of mineral oil and amplification was carried out in a Perkin-Elmer 480 DNA thermal cycler. The PCR conditions were as follows:

Step I	Initial denaturation	4 min.94°C
Step II	Denaturation	30 sec. 94°C
	Annealing	1 min. 58°C
	Extension	2 min. 72°C
Step III	Repeat step II	35 times
Step IV	Final elongation	10 min.72°C

The PCR products were analyzed in 1.5 per cent agarose gel electrophoresis. Gel images were captured using 'EDAS 290' (Electrophoresis Documentation and Analysis System - Kodak, USA). Molecular weight of the amplified products was determined using Kodak I D Image Analysis software.

Cloning and sequencing of PCR amplicon

Cloning was performed using pGEM-T Easy vector (M/s.Promega).

Ligation reaction set up: The following components were added and incubated at room temperature for one hour.

5X ligation buffer	:	5 µL
PCR product	:	2 µL
pGEM-T Easy vector	:	1 µL
DNA ligase enzyme	:	1 µL
Water	:	1 µL

Cloning

The competent cells were thawed on ice and 2 µL of the ligated product was transferred into 50 µL competent *E.coli* cells and kept in ice for twenty minutes. The components were subjected to heat shock at 42°C for 1 min. and returned to ice for about 2-3 min. SOC medium (250 µL) was added and grown at 37°C for an hour and then plated on LB agar plates containing ampicillin (100 µg mL^{-1}) and 40 µL X-gal (2%). After incubation overnight, transformed white colonies grown were selected and analysed for osmotin PCR amplification.

Reverse transcription PCR (RT-PCR) analysis

In order to study the expression of osmotin gene in *Hevea*, total RNA was isolated from the transgenic as well as non-transgenic tissues according to the procedure of Venkatachalam *et al.* (1999) and cDNA synthesis was carried out following standard procedure (Sambrook *et al.*,1989). PCR was performed using osmotin specific primers with the cDNA as the template.

Evaluation of drought tolerance

Proliferated calli from transgenic as well as non-transgenic cell lines were used

for the experiment. Both the calli were sub-cultured to fresh proliferation medium. After ten days, free proline content was estimated in transgenic and non-transgenic calli according to Bates *et al.* (1973).

The plant tissue (0.5 g) was homogenized in 5 mL of 3 per cent sulphosalycylic acid. The homogenate was filtered and 2 mL of extract was taken in a test tube to which 2 mL glacial acetic acid and 2 mL ninhydrin reagent were added. The reaction mixture was incubated at 100 °C in a water bath for one hour till a brick red colour developed. After cooling, 4 mL of toluene was added and transferred to a separating funnel. The chromospheres containing toluene is separated and the absorbance was measured at 520 nm in a spectrophotometer against toluene blank. By referring to a standard curve made from known concentrations of proline, free proline content in the samples were estimated and expressed on fresh-weight basis as follows:

$$\text{Free proline content } (\mu M\ g^{-1}\ \text{tissue}) = \frac{(\mu g\ \text{proline mL-1} \times \text{mL toluene}}{115.5} \times \frac{5}{\text{wt. of sample (g)}}$$

where, 115.5 is the molecular weight of proline.

Proline estimation after PEG stress

Both the transgenic and control calli was subjected to PEG stress by culturing the callus in proliferation media supplemented with 6 per cent PEG for a period of three weeks. After three weeks of culture in this medium, the proline content was again estimated in both the cell lines as mentioned above.

Evaluation of salt tolerance

The transgenic cell lines along with the non-transgenic control were sub-cultured to callus proliferation media. After three weeks, the calli were subjected to salt stress by culturing the callus in medium containing different concentrations of sodium chloride (50-200 mM) for a period of twenty days. About fifty callus clumps were kept in each treatment and the treatments were replicated five times. The survival rate of the callus was observed.

All the experiments were carried out according to the Biosafety Guidelines, Department of Biotechnology, Govt. of India for recombinant DNA work.

RESULTS AND DISCUSSION

Selection of putative transgenic cell lines

In the selection medium, all the callus clumps turned black within two weeks of culture (Fig. 2b). Fresh creamy yellow cell clusters started emerging from the clumps after forty to sixty days of culture in the selection medium (Fig. 2c). In the present experiment, the selectable marker used was kanamycin (300 mgL^{-1}), which had proven effective in previous gene transfer experiments of *Hevea* with other gene constructs (Jayashree *et al.*, 2003; Rekha *et al.*, 2006; Kala *et al.*, 2006). To eliminate false positives, fresh cell clusters were recovered from dying explant tissue and transferred to fresh selection medium. After two to three weeks of culture with kanamycin, putative transformed cell lines, showing resistance to kanamycin proliferated (Fig. 2d) with a transformation frequency of 48 per cent. These lines were separated individually and transferred to proliferation medium fortified with kanamycin. Since each line represents a single transformation event, each transgenic

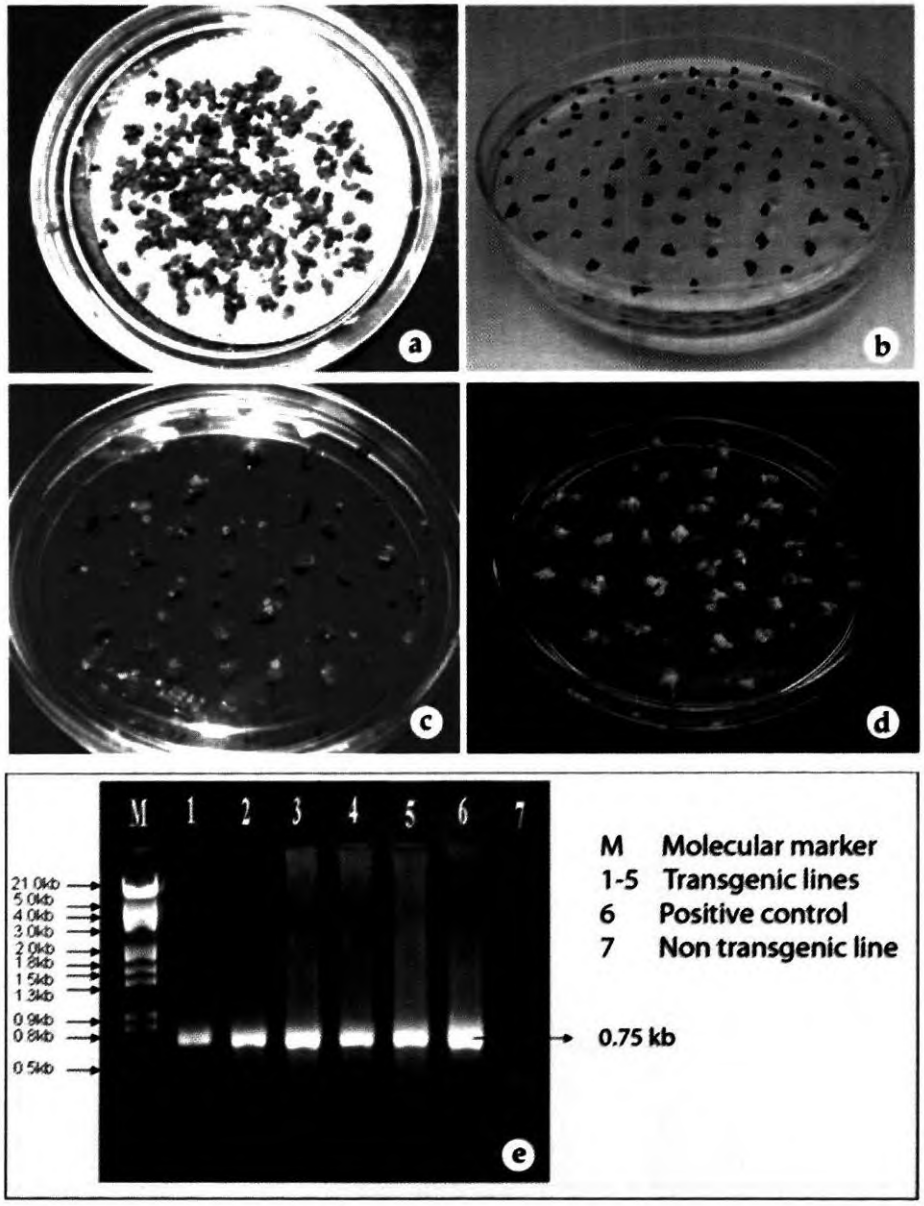
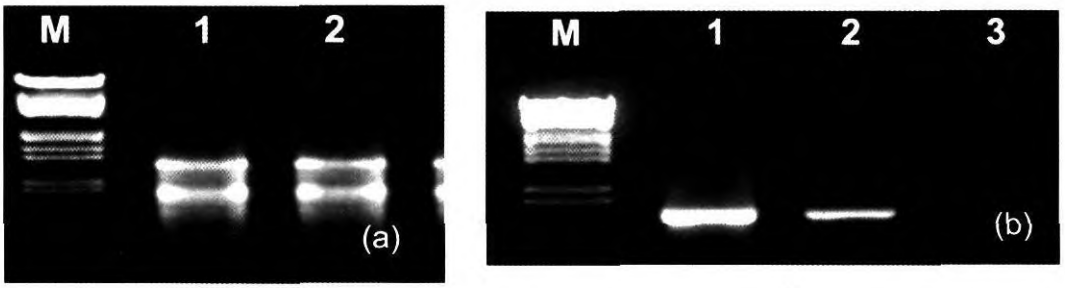


Fig. 2. Development of transgenic cell lines
a. Co-cultivation of the infected callus in liquid medium
b. Drying of explants in the selection medium after 2 weeks
c. Emergence of transgenic cell lines from the dried explants
d. Proliferation of transgenic cell lines
e. PCR amplification of the transgene from the randomly selected putative transgenic lines



a b

Fig. 3. Expression analysis of the transgene by RT-PCR
(a) RNA (b) Amplification of osmotin gene insert from cDNA
M – *Eco*RI +*Hind* III double digest Lambda DNA marker (21Kb)
Lane 1 – positive control (plasmid)
Lane 2 – Amplification of c DNA isolated from transgenic cell line

cell line showing kanamycin resistance was handled individually. Later, the proliferated putative transgenic lines were tested for the presence of transgene by PCR analysis for further confirmation of gene integration.

Molecular confirmation of transgene integration

PCR analysis

Since the gene construct is not having any reporter gene, the only way for the preliminary confirmation of transformation is by the amplification of PCR products. DNA isolated from the proliferated transgenic cell lines were subjected to PCR analysis for the confirmation of gene integration with non-transgenic calli (negative control) and plasmid (positive control). Results showed that all the cell lines tested were PCR positive. The 0.75 kb osmotin gene insert could be positively amplified from all the transgenic cell lines, whereas no amplification was obtained from the non-transgenic negative control (Fig. 2e) indicating the presence of transgene in the transformed cell lines of *H.brasiliensis* genome. The PCR positive lines were used for further experiments. For further confirmation of gene integration, the osmotin amplicon was cloned and sequenced. The sequence showed 100 per cent similarity with the inserted gene confirming the transgene integration.

RT-PCR

Molecular confirmation of the transgene expression was done by RT-PCR. Positive amplification of osmotin gene insert from cDNA synthesized from transgenic callus was obtained in the RT-PCR analysis. The amplification was absent in control calli. Since the inserted osmotin gene is from tobacco and not a native gene in *Hevea*, amplification of 0.75 kb osmotin gene insert in the RT-PCR reaction confirmed the expression of transgene in *Hevea* (Fig. 3a & b).

Evaluation of drought tolerance

The estimation of free proline content under unstressed condition revealed that transgenic cell lines showed a slightly higher level of proline content compared to control. Under water stress induced by PEG (6%), a hike in proline content was noticed for both transgenic and non-transgenic cell lines.

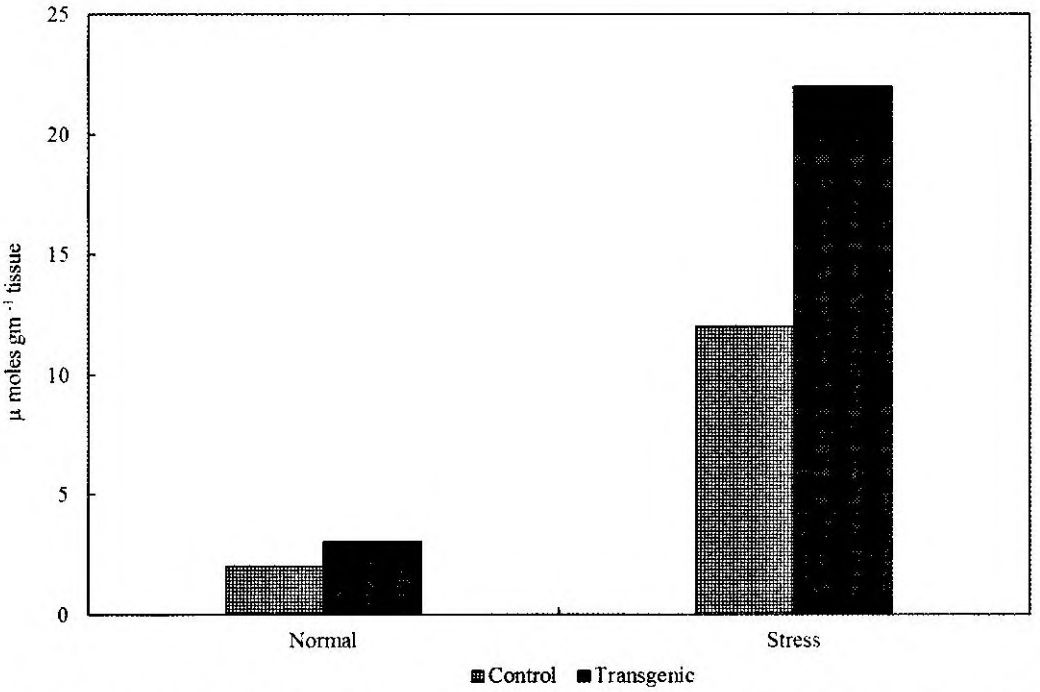


Fig. 4. Estimation of proline from transgenic (T) and control calli (NON-T) under stressed as well as unstressed conditions

However the percent increase in proline content was much higher for transgenic cell lines compared to non-transgenic calli (Fig. 4). An almost two-fold increase in the free proline content was observed for the transgenic cell line compared to control.

Among the different compatible solutes, proline is an important quaternary amino acid derivative that accumulates during salt stress, drought, and low temperature. Proline accumulation under various abiotic stresses (heat, cold, drought, moisture and salinity) in crop plants is considered as a tolerance mechanism. Osmotin induced proline accumulation under stress has been reported in transgenic plants of different crops like strawberry, wheat, cotton, tomato, chilli pepper, mulberry and soybean (Goel *et al.*, 2010; Subramanyam *et al.*, 2012; Das *et al.*, 2011). Results of the present study are in conformity with these reports. Since the newly inserted osmotin gene is under the control of constitutive promoter, some amount of osmotin is produced under unstressed conditions. This induces proline pathway and result in higher accumulation of proline. Under stressed conditions, more amount of osmotin was synthesized and a corresponding increase in proline was noticed. Under stress, the rise in proline content in control calli may be due to the inherent tolerant mechanism of these plants. In transgenic plants, the presence of the osmotin gene resulted in two fold increase in proline content. Positive correlation between proline accumulation and plant stress tolerance has been reported earlier by

Table 1. **Components of PCR reaction**

Component	Volume (mL)	Final concentration
Template DNA	1.00	50 ng
Reaction buffer *	2.00	1 X
dNTP mix	2.00	100 mM of each dNTPs
Forward primer	1.00	10 pmol
Reverse primer	1.00	10 pmol
Taq DNA polymerase **	0.15	0.75 U
Sterile distilled water	12.85	
Total volume	20 mL	

* Tris-HCl (10 mM) pH 9; KCl (50 mM); $MgCl_2$ (15 mM)
** AmpliTaq from Thermo Fisher Scientific, USA

different workers (Barthakur *et al.*, 2001; Gao *et al.*, 2009). It is suggested that proline act as an osmolyte as well as a source of nitrogen during recovery from stress. Recent reports indicate enhanced stress tolerance when proline is supplied exogenously at low concentrations (Hayat and Hayat, 2012). The free proline plays important roles in osmotic adjustment, protecting cellular macromolecules and scavenging hydroxyl radicals in salinity-stressed plants (Chen *et al.*, 2011). The proline accumulation induced by osmotin in the transgenic *Hevea* callus is a positive indication of stress tolerance. There are reports that constitutive over-expression of osmotin gene modulates the transcript abundance of other stress related genes (Patade *et al.*, 2013).

Salt tolerance of transgenic cell lines

Effect of salt on the growth of transgenic and control calli is presented in Table 1. At 50 mMsalt concentration, transgenic calli proliferated profusely without any growth retardation, whereas, non-transgenic calli showed growth retardation with only 23 per cent survival. With increase in salt concentration, growth of the calli in both transgenic and control retarded. However, the rate of retardation was minimum in transgenic calli. Transgenic calli tolerated up to 150 mM salt with a survival rate of 72 per cent, while growth retardation was observed in non-

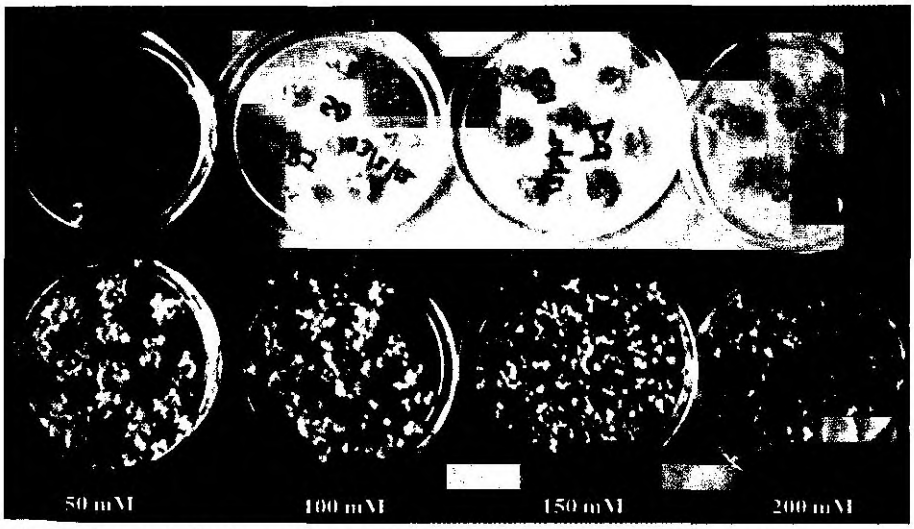


Fig. 5. Survival of callus under salt stress control (top), transgenic (bottom)

Table 2. **Effect of salt on the survival of transgenic and non-transgenic calli (survival %)**

	50 mM	100 mM	150 mM	200 mM
Transgenic calli	100 (100)	88.8 (70.56)	72.8 (58.63)	46.4 (42.96)
Non-transgenic calli	23.8 (4.98)	13 (3.74)	3.2 (2.04)	0.8 (1.31)
			CD	0.28

Arcsine transformed values are given in parenthesis.

transgenic calli even at lower concentrations (50 mM) of NaCl (Fig. 5). About 70 per cent of the transgenic calli could survive at high NaCl concentrations (150 mM) whereas non-transgenic calli could not tolerate even 100 mMNaCl (Table 2, Fig. 5). On sub-culturing in a medium without salt, the transgenic calli from all the treatments, except 200 mM, retained the growth and proliferated. The calli treated with 200 mMNaCl showed poor proliferation. It was hypothesized that osmotin might be involved in the modulation of plant responses to salinity stress either by acting as transcriptional regulator for the genes encoding key enzymes responsible for salinity tolerance or as signaling molecule acting through intracellular receptors (Abdin *et al.*, 2011). Previous reports also proved that the over expression of osmotin in tobacco (Barthakur *et al.*, 2001), tomato (Sarad *et al.*, 2004; Goel *et al.*, 2010), strawberry (Husaini and Abdin, 2008) and chilli pepper (Subramanyam *et al.*, 2011) improved the salinity tolerance. One of the main consequences of salinity stress is the loss of intracellular water which causes rise in osmotic pressure. Plants accumulate compatible solutes in the cytoplasm to increase their hyper osmotic tolerance (Turkan and Demiral, 2009). In the present investigation, under the salt stress conditions, the transgenic calli expressing tobacco osmotin gene might have accumulated more proline than their non-transgenic counter parts. It indicates that the expression of osmotin gene might activate the key enzymes of proline biosynthetic pathway, which in turn enhance the tolerance to salinity. The correlation between salinity tolerance and proline accumulation is reported in many crops like tobacco, wheat (Gao *et al.*,2009), *Lycium barbarum* (Chen *et al.*, 2009), Chrysanthemum (Chen *et al.*, 2011) and in mulberry (Checker *et al.*, 2011). The salt tolerance exhibited by the transgenic calli corroborates with the above findings and is another positive indication for stress tolerance.

CONCLUSION

Hevea callus was transformed with the gene coding for osmotin through *Agrobacterium* mediated gene transfer. The gene integration and expression was confirmed through PCR and RT-PCR, respectively. The *in vitro* stress tolerance studies conducted with transgenic cell lines of *Hevea* revealed positive indications of drought tolerance as indicated by the higher accumulation of proline under water stress and active proliferation of the callus under salinity stress. Plants have been regenerated from these cell lines. The gene integration and expression have been confirmed through molecular studies. These transgenic plants integrated with osmotin gene are expected to show stress tolerance and also to perform well under stressful environments.

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