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BRIEF COMMUNICATION

Expression of hepatitis B small surface antigen in *Santalum album* embryogenic cell suspension cultures

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Abstract

Embryogenic cell suspension cultures of *Santalum album* were transformed with *Agrobacterium tumefaciens* harboring pD35SHER plant expression vector having hepatitis B small surface antigen (HBsAg) with a C-terminal ER retention signal. The transformed colonies were selected on culture medium supplemented with kanamycin and subsequently the transgenic nature of these colonies was confirmed by PCR analysis. The expression of HBsAg was confirmed by RT-PCR analysis and Western blot analysis and the expression was quantified using monoclonal antibody-based ELISA. Cell suspension cultures were initiated from the colony with expression of 11.09 μg(HBsAg) g^{-1}(f.m.). To further increase the expression of HBsAg, transgenic *S. album* suspensions were cultured on media with various medium additives and cells growing in medium with 30 mM trehalose showed the expression of 19.95 μg(HBsAg) g^{-1}(f.m.).

Additional key words: *Agrobacterium tumefaciens*, C-terminal ER retention signal, genetic transformation, medium additives.

Plants and plant cell cultures have emerged as viable alternatives to microbial and mammalian hosts for expression of high-value recombinant proteins (Čeřovská *et al*. 2008, Lhernould *et al*. 2008). Plant cell cultures grown in bioreactors using simple and inexpensive medium are especially useful as they can undertake correct folding and assembly of multimeric proteins like mammalian systems and carry zero risk of contamination from human pathogens (Hellwig *et al.* 2004). Cell cultures of tobacco, soybean, tomato and rice have served as expression hosts for production of different recombinant proteins (Sijmons *et al.* 1990, Huang *et al*. 2001, Smith *et al.* 2002, Kwon *et al.* 2003). Use of specific medium additives which enhance protein production and stability in transformed cell cultures along with exploration of novel plant species like *Santalum album* for efficient heterologous expression of recombinant proteins promises to overcome some of the drawbacks associated with this technology. Native as well as genetically transformed *S. album* cell suspension cultures have been reported to be efficient protein producers in the past (Pal *et al.* 2003, Shekhawat *et al.* 2008). Medium additives when used at appropriate concentrations can act as enhancers of protein synthesis, protein stabilizing agents or inhibitors of intracellular protein degradation leading to enhanced recovery of the foreign protein (Hellwig *et al.* 2004). In this communication, we describe optimized expression of hepatitis B small surface antigen, a pharmaceutically important recombinant protein, in *S. album* cell suspension cultures through the use of a variety of medium additives.

Double-enhanced CaMV 35S promoter was released from pCAMBIA 1301 (*CAMBIA*, Canberra, Australia) by using *Hin*dIII and *Xho*I and cloned in to the corresponding sites of pGBSSHER (Shekhawat *et al.* 2007) by replacing the potato-derived granule-bound starch synthase gene promoter. This construct (Fig. 1), henceforth called pD35SHER, was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation and used to transform *S. album* embryogenic cell suspension

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Abbreviations: ELISA - enzyme linked immunosorbent assay; HBsAg - hepatitis B surface antigen; HER - hepatitis B small surface antigen coding 's' gene tagged with a C-terminal ER retention signal; RT-PCR - reverse transcriptase-polymerase chain reaction.

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cultures as described previously (Shekhawat *et al.* 2008). Five putatively transformed colonies growing on semi-solid selection medium (containing 50 mg dm^{-3} kanamycin) were initiated as cell suspension cultures in liquid selection medium.

Genomic DNA isolated from untransformed and transformed cells using *GenElute* plant genomic DNA miniprep kit (*Sigma*, St. Louis, USA) was used in PCR analysis. The primer pair used to amplify hepatitis B small surface antigen gene tagged with a C-terminal ER-retention signal (HER) sequence was: forward primer 5' ATGGAGAACACCACATCAGGA 3' and reverse primer 5' TTACAGCTCGTCCTTCTCGGACAT 3'. A 50 mm^3 volume of PCR mix contained 10 pmol of each primer, 200 μM of each dNTP, 1.5 units of Taq DNA polymerase, 1× PCR buffer and 25 ng of plant genomic DNA as template. PCR conditions used were 94 °C for 5 min followed by 35 amplification cycles each consisting of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 10 min. A 699-bp amplification band specific for HER was noted in transformed cells whereas it was absent in untransformed control cells, confirming the transformed nature of selected colonies (Fig. 2*A*).

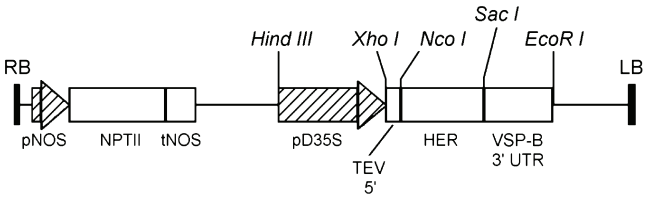

Fig. 1. T-DNA region of pD35SHER. RB - right border, LB - left border, pNOS - nopaline synthase promoter, NPT II - neomycin phosphotransferase type-II coding sequence, tNOS - nopaline synthase terminator, pD35S - double enhanced CaMV 35S promoter, TEV 5' - 5' untranslated region from tobacco etch virus, HER - HBsAg 's' gene coding sequence with C-terminal ER retention signal, VSP-B 3' UTR - 3'untranslated region from soybean vegetative storage protein-β.

Total cellular RNA was isolated from untransformed control and transformed tissues using RNeasy plant mini kit (*Qiagen*, Maryland, USA) for performing RT-PCR analysis. 1 μg of this RNA was used for cDNA synthesis using *cMaster* RT-PCR system (*Eppendorf*, New York, USA). A part of this cDNA (0.5 μg) was then used as template for PCR done as above. A 699-bp amplification band was observed in the transformed cell cultures and was absent in untransformed control cells confirming the presence of HER mRNA in transformed cell suspension cultures (Fig. 2*B*).

Transformed *S. album* suspension cultures growing in selection medium were treated with following eight medium additives at an initial inoculum density of 1 g(f.m.) in 10 cm^3 liquid medium: DMSO (at 0.4, 0.75, 2 and 3 %; v/v), *Tween-20* (at 0.001, 0.002, 0.005, 0.0075 and 0.01 %; v/v), GA_3 (at 0.58, 1.45, 2.9, 14.5 μM), $CaCl_2$ (at 0, 1.5, 3, 6, and 9 mM), chloroquine (at 62.5, 125, 312.5 and 500 μM), NH_4Cl (at 2, 5, 10, 25 and 50 mM) trehalose (at 10, 30, 60 and 75mM) and mannitol (at 27.44, 54.88, 109.76, 164.64 and 219.52 mM). The cultures were maintained on a gyratory shaker at 100 rpm under a 16-h photoperiod, irradiance of 45 μmol(photon) m^{-2} s^{-1} and a temperature of 25 ± 2°C. Total soluble proteins extracted from 100 mg tissues using 1 cm^3 chilled lysis buffer (containing 50 mM sodium phosphate, pH 7.3, 100 mM NaCl, 10 mM EDTA, 25 mM sodium ascorbate and 0.1 % *Triton X-100*) were kept on ice for 30 min and then clarified first at 17 000 *g* for 30 min and then at 20 000 *g* for 30 min. HBsAg expression was estimated using *Eliscan* HBsAg ELISA kit (*RFCL*, Haridwar, India). Total soluble proteins from untransformed *S. album* cell suspension culture were used as a negative control. All experiments were carried out in triplicates and standard errors were calculated. All the data were subjected to analysis of variance and the Tukey test for mean significance, using the *SPSS* software package (*version 12*). Maximum expression of 11.09 μg(HBsAg) g^{-1}(f.m.) were noted in transformed untreated cells. Further, the use of 30 mM trehalose improved the expression of HBsAg in *S. album* cell suspension cultures by about almost 2-fold to 19.95 μg(HBsAg) g^{-1}(f.m.) (Table 1).

Total soluble proteins from selected *S. album* suspension cultures (50 mm^3) were suspended in sample buffer (50 mmol dm^{-3} Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS, 1 % 2-mercaptoethanol, 0.0025 mM bromophenol blue and 100 mM dithiothreitol) and used for Western analysis as described (Ganapathi *et al.* 2007). A 24-kDa HBsAg specific band was noticed in transformed cells while being absent in untransformed control cultures. The intensity of HBsAg specific band was higher in the transformed cultures grown with trehalose as compared to transformed untreated cells (Fig. 2*C*).

We have demonstrated the usefulness of transgenic *S. album* cell suspension cultures for stable expression of a recombinant protein like β-glucuronidase at very high contents intracellularly as well as in secreted form in the spent culture medium (Shekhawat *et al.* 2008). Here, we have tested the *S. album* cell suspension cultures, to efficiently express hepatitis B small surface antigen. A C-terminal ER retention signal (SEKDEL) has been incorporated in the HBsAg coding sequence here as previous studies have shown that ER retention permits

concentration of HBsAg subunits concomitant with better subunit interaction and disulphide cross linking leading to an improved expression level in heterologous expression systems (Richter *et al.* 2000). Further, we have used monoclonal antibody-based ELISA for estimating HBsAg levels in transformed cell cultures as monoclonal antibodies recognize epitopes in highly conformational immuno-dominant 'a' region of HBsAg giving an accurate estimate of functionally active antigen produced in transgenic expression systems. Further, the use of efficient regulatory sequences like double enhanced CaMV 35S promoter and TEV 5' leader sequence along with soybean vegetative storage protein 3'UTR in the expression construct facilitated excellent yield of HBsAg in transformed *S. album* cell suspension cultures.

Eight medium additives chosen based on their unique properties were used in this study for improving the expression of HBsAg in *S. album* cell suspension cultures. In general, these additives did not adversely affect the growth of cell suspension cultures except in some treatments where cell growth was inhibited due to high additive concentrations. Dimethyl sulfoxide (DMSO) acts as a protein-stabilizer both for intracellularly and extracellularly accumulated proteins in transgenic tobacco suspension cultures (Whal *et al.* 1995). Herein, DMSO at a concentration of 0.4 % (v/v) improved the expression of HBsAg in *S. album* suspension culture cells by 1.36 times. Gibberellic acid (GA_3), postulated to increase the rate of protein synthesis

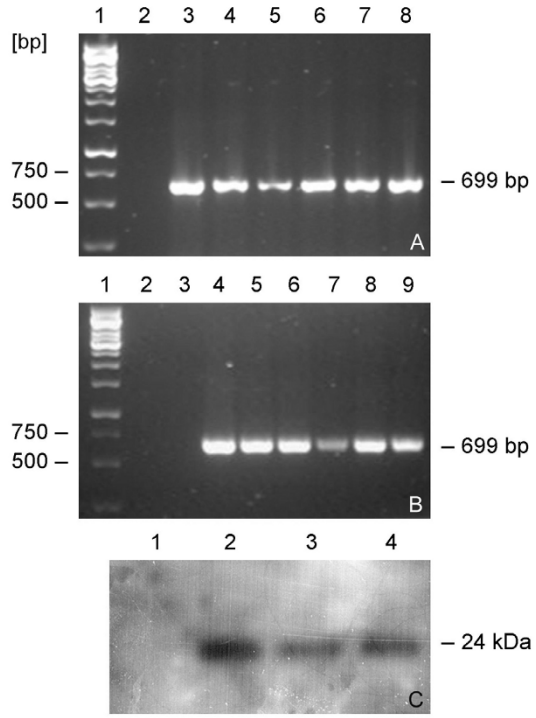

Fig. 2. *A* - PCR analysis of genomic DNA isolated from pD35SHER transformed *S. album* cell suspension cultures. *Lane 1* - 1 kb marker, *lane 2* - untransformed control, *lane 3* - positive control (pD35SHER), *lanes 4 to 8* - transformed cell cultures. *B* - RT-PCR analysis for confirming the expression of HBsAg in the pD35SHER transformed *S. album* cell suspension cultures. *Lane 1* - 1 kb marker, *lane 2* - total RNA control, *lane 3* - untransformed control, *lane 4* - positive control (pD35SHER), *lanes* 5 to 9 - transformed cultures. *C* - Western blot analysis of HBsAg expression in the pD35SHER transformed *S. album* cell suspension cultures. *Lane 1* - untransformed control, *lane 2* - yeast-derived HBsAg, *lane 3* - untreated pD35SHER-transformed cultures, *lane 4* - pD35SHER-transformed cultures treated with 30 mM trehalose.

Table 1. Amount of HBsAg in untransformed and pD35SHER-transformed *S. album* cell cultures grown with or without medium additives after 10 d of growth. Means ± SE, $n = 3$. Different letters denote significant differences at $P \leq 0.05$ between particular compound treatments.

Treatments	Concentration	HBsAg [μg g^{-1}(f.m.)]
Untransformed control		0.003 ± 0.00
untreated control		11.090 ± 0.39
GA_3	0.58 μM	14.468 ± 1.27*a*
	1.45 μM	16.477 ± 0.75*a*
	2.90 μM	16.407 ± 0.47*a*
	14.50 μM	8.886 ± 1.38*b*
Tween-20	0.0010 %	14.904 ± 1.11*a*
	0.0020 %	14.216 ± 0.58*a*
	0.0050 %	13.263 ± 0.61*a*
	0.0075 %	14.048 ± 1.26*a*
	0.0100 %	13.540 ± 1.87*a*
DMSO	0.40 %	15.119 ± 1.41*a*
	0.75 %	14.880 ± 1.37*a*
	2.00 %	5.283 ± 0.52*b*
	3.00 %	4.046 ± 0.33*b*
$CaCl_2$	0.00 mM	3.917 ± 0.60*a*
	1.50 mM	7.457 ± 1.15*b*
	6.00 mM	14.256 ± 0.51*c*
	9.00 mM	11.460 ± 0.47*c*
NH_4Cl	5.00 mM	12.470 ± 0.95*a*
	10.00 mM	17.051 ± 1.02*b*
	25.00 mM	15.588 ± 0.63*ab*
	50.00 mM	13.022 ± 1.12*ab*
Chloroquine	62.5 μM	9.828 ± 1.00*a*
	125.0 μM	9.760 ± 1.00*a*
	312.0 μM	12.509 ± 0.83*b*
	500.0 μM	11.100 ± 0.89*ab*
Trehalose	10 mM	18.134 ± 2.06*a*
	30 mM	19.944 ± 0.40*a*
	60 mM	19.335 ± 1.01*a*
	75 mM	18.060 ± 0.74*a*
Mannitol	27.44 mM	18.735 ± 0.52*a*
	54.88 mM	19.622 ± 0.76*a*
	109.76 mM	18.390 ± 0.23*a*
	164.64 mM	19.095 ± 1.45*a*
	219.52 mM	16.272 ± 0.30*b*

in plant cell cultures (Tsoi and Doran 2002), increased the accumulation of HBsAg in *S. album* suspension culture cells by 1.48 fold when used at a concentration of 1.45 μM. $CaCl_2$ is known to promote the uptake of nutrients like amino acids and sulphates (Harrington *et al.* 1981). In fact, doubling the concentration of $CaCl_2$ in basic MS medium improved HBsAg accumulation moderately by 1.28 times. *Tween-20*, a non-ionic surfactant, when used at low concentrations increases the permeability of plant cells as demonstrated by an increase in transient *Agrobacterium* infection in presence of *Tween-20* in lettuce leaves (Negrouk *et al.* 2005). By the same analogy using *Tween-20* at low concentrations can facilitate efficient uptake of nutrients from the culture medium leading to increased growth rates and improved accumulation of recombinant proteins in cell cultures. *Tween-20* at 0.001 % improved the accumulation of HBsAg in *S. album* suspension culture cells by 1.34 times. Trehalose, a naturally occurring disaccharide with two glucose units is one of the best protein stabilizers known (Kaushik and Bhat 2003). Trehalose has been used previously to stabilize HBsAg in vaccine formulations (Jaganathan *et al.* 2004, Maa *et al.* 2007). Trehalose was found to be the best medium additive used in this study. Addition of trehalose at a concentration of 30 mM improved the HBsAg accumulation by 1.8 times. NH_4Cl and chloroquine are known lysosomal enzyme inhibitors (Tanaka *et al.* 1986). At appropriate concentrations they can minimize intracellular degradation of recombinant foreign proteins by lysosomal proteases. Addition of NH_4Cl at a final concentration of 10 mM and chloroquine at 312 μM to the liquid culture medium resulted in a 1.54 and 1.13 fold enhancement in HBsAg expression, respectively. Application of osmotic stress using metabolically inert osmolytes like mannitol improves the yield of recombinant proteins in transgenic plant cell suspension cultures (Lee *et al.* 2002, Soderquist and Lee 2005). Mannitol proved to be one of the best medium additives in the present study with a 1.77 fold increase in the expression of HBsAg in *S. album* suspension cultures when culture medium was supplemented with 54.88 mM mannitol.

HBsAg has been expressed in other plant tissues including tobacco leaves and cell suspension cultures, lettuce leaves, banana fruit, tomato fruit, potato tubers and soybean cell suspension cultures (reviewed in Streatfield 2005, Sunil Kumar *et al.* 2007). Highest expression levels of ~25 μg(HBsAg) g^{-1}(f.m.) (determined using monoclonal ELISA) have been reported in soybean cell suspension cultures (Smith *et al.* 2002). However, genetic instabilty and loss of expression during repeated subcultures were the major constraints in their study. Recently, Ganapathi *et al.* (2007) have reported the restoration of HBsAg expression in soybean suspension cultures by upto 50 % of the original expression using leupeptin hemisulphate. We have demonstrated the inherent stability of recombinant protein expression in cell cultures derived from a tree species like *S. album* (Shekhawat *et al.* 2008). Herein, β-glucuronidase expression remained stable even after continuous subcultures at an interval of 12 - 14 d for more than 7 months. High HBsAg expression obtained in the present study coupled with our earlier results on stable expression of recombinant proteins in *S. album* cell suspension cultures establish the usefulness of *S. album* cell suspension cultures for efficient expression of recombinant proteins.

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