BIOLOGIA PLANTARUM 51 (3): 467-471, 2007

Hepatitis B surface antigen expression in NT-1 cells of tobacco using different expression cassettes

G.B. SUNIL KUMAR*, T.R. GANAPATHI*, L. SRINIVAS*, C.J. REVATHI** and V.A. BAPAT*[1]

*Plant Cell Culture Technology Section, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, India**
*Shantha Biotechnics Limited, Medchal, Hyderabad-501401, India***

Abstract

Nicotiana tabacum 1 (NT-1) cells were transformed with four different expression cassettes of hepatitis B surface antigen (HBsAg). The transformed nature of the cells was confirmed by polymerase chain reaction (PCR). The expression levels were assayed by enzyme linked immunosorbent assay (ELISA). The expressivities varied among the different cassettes and the maximum expression of 16.6 ng g^{-1}(f.m.) of cells was noted in pEFEHER transformed cells. Salicylic acid (100 μM) treatment resulted in 1.8 fold increase of expression in pEFEHBS transformed cells. The effect of different concentrations of kanamycin and geneticin was studied on the growth of transformed cells and HBsAg expression. The cell growth was optimum at lower concentrations of the antibiotics, and the maximum expression was noted at 200 mg dm^{-3} of kanamycin.

Additional key words: ELISA, geneticin, jasmonic acid, kanamycin, *Nicotiana tabacum*, PCR, salicylic acid.

Introduction

There has been a growing interest in the use of plant cell cultures for the production of recombinant proteins, especially those of pharmaceutical interest. The cell culture system offers several advantages like production of the proteins under controlled conditions; no risk of contamination with agrochemicals and the expression is independent of the climate and soil quality (Hellwig *et al.* 2004, Min *et al.* 2006). The cells can be grown and multiplied continuously in hemizygous state, which could be advantageous as some transgene loci trigger silencing, when present in a homozygous condition (Wilde *et al.* 2000).

Hepatitis B is one of the major infectious diseases affecting several million people in the developing world. The late sequel of hepatitis B infection is hepatocellular carcinoma (Tiollais *et al.* 1981). The first vaccine for the disease consisted of HBsAg obtained from plasma of carriers, but concerns about safety and availability restricted its use. The recombinant vaccine produced in the yeast developed as an effective vaccine for mass immunization. However, the cost of the vaccine prohibits its use in the developing countries. Low cost vaccine is required to immunize the large segments of the population. Towards this goal, the production in plant cell cultures could be developed as an alternative strategy. HBsAg has been expressed in transgenic tobacco plants (Mason *et al.* 1992), cells (Sunil Kumar *et al.* 2003), potato (Richter *et al.* 2000), lettuce, lupin (Kapusta *et al.* 1999) and banana (Sunil Kumar *et al.* 2005a). In this communication, we report the optimization of HBsAg expression in NT-1 cells of tobacco using four different expression cassettes.

Received 11 May 2005, *accepted* 25 April 2006.
Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; EFE - ethylene forming enzyme gene promoter from banana; ELISA - enzyme linked immunosorbent assay; f.m. - fresh mass; HBsAg - hepatitis B surface antigen; HBS - hepatitis B surface antigen coding 's' gene; HER - hepatitis B surface antigen coding 's' gene with ER retention signal; NT-1 - *Nicotiana tabacum* 1; PCR - polymerase chain reaction; SHER - hepatitis B surface antigen coding 's' gene with an N-terminal pea vicilin secretory signal and a C-terminal ER retention signal.
[1] Corresponding author; fax: (+91) 022 25505151, e-mail: vabapat@magnum.barc.ernet.in

Materials and methods

Plant cell cultures and transformation: NT-1 cells were procured from Boyce Thompson Institute for Plant Research Inc., Ithaca, NY, USA. These cells were maintained on Murashige and Skoog (1962; MS) salts, 0.5 g dm^{-3} of 2-(N-morpholino) ethanesulfonic acid, 180 mg dm^{-3} KH_2PO_4, 100 mg dm^{-3} myoinositol, 1 mg dm^{-3} thiamine HCl, 2.21 mg dm^{-3} of 2,4-dichlorophenoxyacetic acid (2,4-D) and 30 g dm^{-3} sucrose. pH of the medium was adjusted to 5.7 and gelled with 2 g dm^{-3} *Phytagel* (*Sigma-Aldrich*, USA) (NT-1 medium). Cell cultures were initiated, maintained and *Agrobacterium* mediated transformation was carried out as described previously (Sunil Kumar *et al.* 2003). Four expression cassettes pEFEHBS, pEFEHER, pEFESHER and pSHER were used to transform NT-1 cells of tobacco. The construction of the expression vectors was described earlier (Sunil Kumar *et al.* 2005b).

PCR and ELISA analysis: Transgenic nature of the cells was determined by PCR analysis. Genomic DNA was extracted from the transformed and control untransformed cells using CTAB method (Stewart and Via 1993). PCR amplification was carried out essentially as described (Sunil Kumar *et al.* 2003).

Total soluble proteins were extracted from transformed and untransformed cells as described earlier (Mason *et al.* 1992). The expression levels were assayed in triplicate by enzyme linked immunosorbent assay (ELISA) using Shan kit HBsAg elisa (*Shantha Biotechnics Ltd*., Hyderabad India). Human serum derived HBsAg was used as a positive control and protein extracted from untransformed cells as a negative control.

Effect of antibiotics on the growth of cells: The growth of transformed and untransformed cells was determined under 0 - 500 mg dm^{-3} kanamycin or 0 - 50 mg dm^{-3} geneticin and each concentration was taken in five replicates. The filter-sterilized antibiotics were added to autoclaved semi-solid NT-1 medium and poured into test tubes. These were inoculated with 200 mg fresh mass (f.m.) of the cells. The growth after three weeks was measured by taking f.m. The effect of kanamycin on the expression of HBsAg in pEFEHBS transformed cells was assayed by ELISA analysis.

Salicylic or jasmonic acid treatment of the transformed cells: Salicylic or jasmonic acid (10 mM) stocks were prepared in dimethyl sulfoxide (DMSO). Different concentrations of salicylic acid or jasmonic acid (0 - 500 µM) were added to 50 cm^3 autoclaved liquid NT-1 medium and 1 g (f.m.) of pEFEHBS transformed NT-1 cells were inoculated and the cultures were maintained in dark on gyratory shaker. After 10 d, the cells were harvested, total protein was extracted and amount of HBsAg was estimated by ELISA.

Results

NT-1 cells of tobacco were co-cultivated with *Agrobacterium* harboring either of the pEFEHBS, pEFEHER, pSHER, or pEFESHER expression cassettes. The T-DNA portion of the four plant expression cassettes is depicted in Fig. 1*A-D*. The transformed cells developed into distinct colonies in three weeks on selection medium. These were isolated and transferred individually to fresh selection medium. After four to six passages in liquid NT-1 medium, fine cell suspensions of transformed cells were obtained.

The effect of kanamycin and geneticin on the growth of control untransformed cells and pEFEHBS, pEFEHER transformed cells was carried out. The growth of control-untransformed cells was inhibited at 50 mg dm^{-3} of kanamycin and 5 mg dm^{-3} of geneticin, while the transformed cells grew luxuriantly at these concentrations (Fig. 2*A,B*).

The genomic DNA was isolated from transformed and untransformed cells and PCR was carried out. A diagnostic 681 bp fragment amplification was noted in all the transformed cell lines and in positive control, while it was absent in control-untransformed cells (Fig. 3).

ELISA analysis showed the varied levels of expression among the different lines of transformed

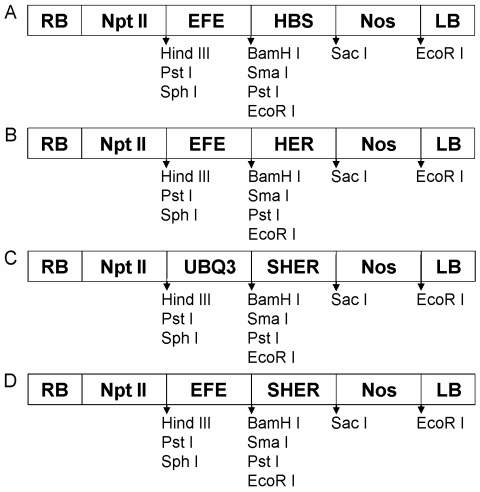

Fig. 1. T-DNA region of pEFEHBS (*A*), pEFEHER (*B*), pSHER (*C*), and pEFESHER (*D*). RB and LB are right and left borders, Npt-II is neomycin phosphotransferase, UBQ3 is ubiquitin promoter from *Arabidopsis*, EFE is ethylene forming enzyme promoter of banana, HBS is HBsAg's' gene, HER is HBsAg's' gene with ER retention signal, SHER is HBsAg's' gene with N-terminal pea vicilin secretory signal and C-terminal ER retention signal and Nos is nos terminator.

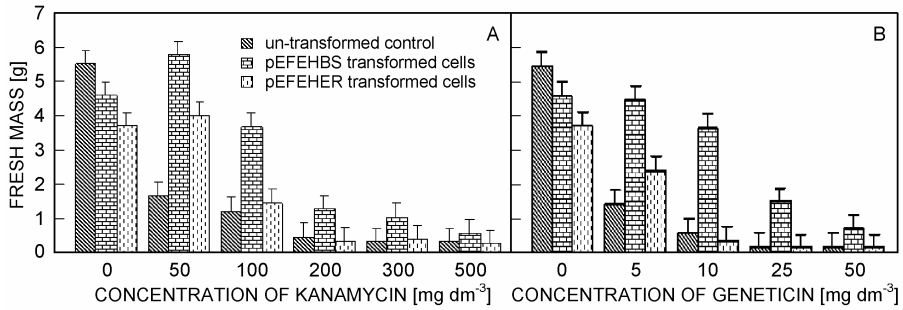

Fig. 2. Effect of different concentrations of kanamycin (*A*) or geneticin (*B*) on the growth of untransformed and pEFEHBS or pEFEHER transformed NT-1 cells of tobacco. Kanamycin (0 - 500 mg dm^{-3}) or geneticin (0 - 50 mg dm^{-3}) was added to semisolid NT-1 medium and inoculated with 200 mg(f.m.) of the cells. Each concentration was taken in five replicates. The growth after three weeks was measured by taking the fresh mass of the cells.

cells for each expression cassettes used. The maximum expression of 5.36 ng g^{-1}(f.m.) was obtained in pEFEHBS transformed cells, 16.6 ng g^{-1}(f.m.) in pEFEHER transformed cells, 7.45 ng g^{-1}(f.m.) in pSHER transformed cells and 4.49 ng g^{-1}(f.m.) was noted in pEFESHER transformed cells (Table 1).

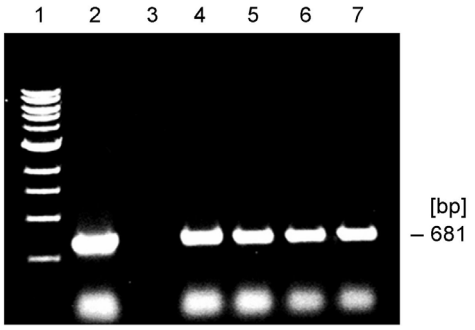

Fig. 3. PCR analysis of transformed NT-1 cells of tobacco. *Lane 1* - 1 kb ladder, *2* - positive control (pEFEHBS plasmid), *3* - negative control (genomic DNA from untransformed cells), *4* - pEFEHBS, *5* - pEFEHER, *6* - pEFESHER, *7* - pSHER transformed cells. The band of interest (681 bp) is pointed *on the right*.

Table 1. Expression levels [ng g^{-1}(f.m.)] of HBsAg in six independent transformed cell lines of tobacco for each expression cassette. Values indicate means ± SE of three replicates.

Line	pEFEHBS	pEFEHER	pSHER	pEFESHER
1	2.55 ± 0.30	16.6 ± 0.50	2.10 ± 0.40	4.49 ± 0.60
2	5.36 ± 0.10	4.09 ± 0.20	1.98 ± 0.10	3.01 ± 0.05
3	0.70 ± 0.01	2.33 ± 0.08	6.45 ± 0.60	1.69 ± 0.06
4	4.98 ± 0.30	7.15 ± 0.50	3.05 ± 0.06	2.76 ± 0.03
5	5.02 ± 0.50	5.85 ± 0.07	7.45 ± 0.30	2.57 ± 0.05
6	3.06 ± 0.10	1.73 ± 0.05	2.48 ± 0.05	2.75 ± 0.07

The effect of different concentrations of kanamycin was studied on the expression of HBsAg in pEFEHBS transformed cells. The expression levels were low up to 100 mg dm^{-3} of kanamycin and maximum expression was obtained at 200 mg dm^{-3}. Then the expression levels were gradually decreased at higher concentrations (Fig. 4).

The pEFEHBS transformed cells, when treated with salicylic or jasmonic acid accumulated higher levels of HBsAg. 100 μM salicylic acid treatment resulted in 1.8 fold increase in HBsAg expression, whereas 500 μM jasmonic acid treatment gave 1.3 fold increase (Fig. 5*A*,*B*).

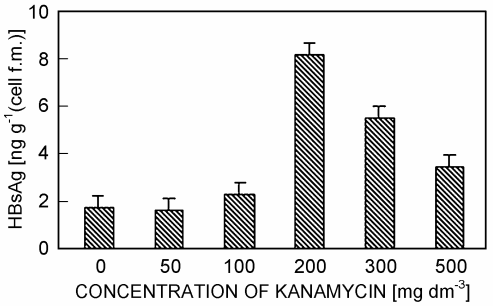

Fig. 4. Effect of different concentrations of kanamycin on HBsAg expression in pEFEHBS transformed NT-1 cells of tobacco. Kanamycin was added to the semisolid NT-1 medium and inoculated with 200 mg(f.m.) of the cells. Each concentration was taken in five replicates. The effect of kanamycin on the HBsAg expression was assayed by ELISA analysis.

Discussion

Plant cell cultures have been used over twenty years to produce variety of secondary metabolites and more recently to produce recombinant proteins. Although there are limitations of slow growth and low levels of expression, plant cell based system can provide an economical alternative to other systems, as their only

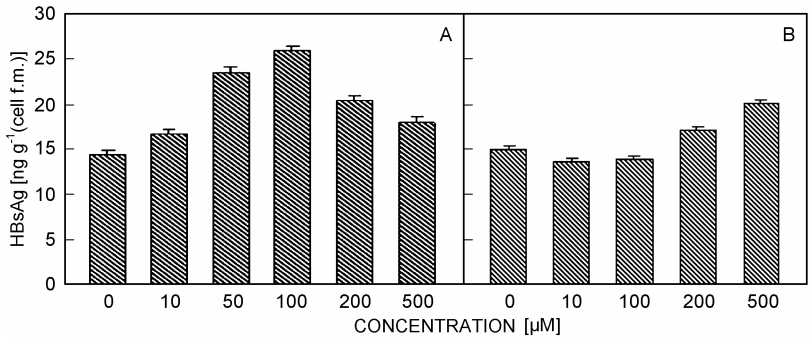

Fig. 5. Effect of salicylic acid (*A*) and jasmonic acid (*B*) on the expression of HBsAg in pEFEHBS transformed NT-1 cells of tobacco. Salicylic acid or jasmonic acid were added to NT-1 medium and 1 g(f.m.) of the pEFEHBS transformed NT-1 cells were inoculated and the cultures were maintained in dark on gyratory shaker. After 10 d, cells were harvested, total protein was extracted and amount of HBsAg was estimated by ELISA.

media requirements are nutrients, salts, vitamins and growth hormones (Bodeutsch *et al.* 2001). The well-characterized plant cell lines include Bright Yellow 2 (BY-2) and *Nicotiana tabacum* 1 (NT-1) of tobacco (Hellwig *et al.* 2004).

In our studies, four different expression cassettes were used to optimize HBsAg expression in NT-1 cells of tobacco. Among the different expression cassettes used, the cassette with *EFE* promoter (pEFEHER) showed the higher expression levels of HBsAg. The ethylene produced under *in vitro* conditions may be responsible for higher expression. It is known that ethylene is produced by plant tissues during tissue cultures (Biddington 1992). ACC oxidase expression increases with increase in ethylene accumulation or wounding of the tissue (Gomez *et al.* 1997). In the present study the HBsAg expression is under the control of *EFE* gene promoter (pEFEHBS, pEFEHER and pEFESHER), therefore the higher levels of HBsAg expression were obtained in the cells transformed with these expression cassettes.

The incorporation of C-terminal ER retention signal might have also contributed to the higher expression of HBsAg. Mason and Arntzen (1995) reported that recombinant B subunit of heat labile enterotoxin (LT-B) produced in tobacco and potato showed enhanced accumulation when a C terminal microsomal retention signal was added. The expression of HBsAg in pEFEHBS transformed cells was further enhanced by salicylic acid treatment. The salicylic acid response element (TGACGT) (Kim *et al.* 1993) in the *EFE* promoter might have contributed to higher expression of HBsAg in the treated cells. The copy number of salicylic acid response element in the promoter region influences the degree of induction by salicylic acid. The expression of β-glucuronidase was enhanced by adding four copies of the response element (Qin *et al.* 1994). Similarly, increasing the copies of salicylic acid response element and treatment of the transformed cells with salicylic acid could further enhance the expression of HBsAg from the *EFE* promoter.

The concentration of antibiotics used in the culture medium influences the growth of transformed cells. Appropriate levels of the antibiotics are required to obtain optimum biomass and expression of recombinant proteins. In our studies, it was observed that 50 mg dm^{-3} of kanamycin concentration, maximum amount of biomass was obtained and at 200 mg dm^{-3} of kanamycin maximum expression of HBsAg was obtained. Further studies in this direction are required to optimize the concentration of kanamycin to obtain maximum growth and expression levels for the successful scale up of these cells.

Though plant cell cultures offer several advantages for the production of recombinant proteins, challenges still remain in several areas before the system becomes acceptable and commercially feasible. Importantly, stronger promoters need to be identified, reasons for protein instability and strategies to enhance protein recovery have to be worked out (Hellwig *et al.* 2004).

References

Biddington, N.L.: The influence of ethylene in plant tissue culture. - Plant Growth Regul. **11**: 173-187, 1992.

Bodeutsch, T., James, E.A., Lee, J.M.: The effect of immobilization on recombinant protein production in plant cell culture. - Plant Cell Rep. **20**: 562-566, 2001.

Gomez, R.L., Campbell, A., Dong, J.G., Yang, S.F., Lim, M.A.G.: Ethylene biosynthesis in banana fruit: isolation of a genomic clone to ACC oxidase and expression studies. - Plant Sci. **123**: 123-131, 1997.

Hellwig, S., Drossard, J., Twyman, R.M., Fischer, R.: Plant cell culture for the production of recombinant proteins. - Nat. Biotechnol. **22**: 1415-1422, 2004.

Kapusta, J., Modelska, A., Figlerowicz, M., Pniewski, T., Letellier, M., Lisowa, O., Yusibov, V., Koprowski, H.,

Plucienniczak, A., Legocki, A.B.: A plant derived edible vaccine against Hepatitis B virus. - FASEB J. **13**: 1796-1799, 1999.

Kim, S.R., Kim, Y., An, G.: Identification of methyl jasmonate and salicylic acid response elements from the nopaline synthase (*nos*) promoter. - Plant Physiol. **103**: 97-103, 1993.

Mason, H.S., Arntzen, C.J.: Transgenic plants as vaccine production systems. - TIBTECH. **13**: 388-392, 1995.

Mason, H.S., Lam, D.M.K., Arntzen, C.J.: Expression of hepatitis B surface antigen in transgenic plants.- Proc. Natl. Acad. Sci. U.S.A. **89** : 11745-11749, 1992.

Min, S.R., Woo, J.W., Jeong, W.J., Han, S.K., Lee, Y.B., Liu, J.R.: Production of human lactoferrin in transgenic cell suspension cultures of sweet potato. - Biol. Plant. **50**: 131-134, 2006.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Qin, X.F., Holulgue, L., Horvath, D.M., Chue, N.H.: Immediate early transcription by salicylic acid via the cauliflower mosaic virus as-1 element. - Plant Cell **6**: 863-874, 1994.

Richter, L.J., Thanavala, Y., Arntzen, C.J., Mason, H.S.: Production of hepatitis B surface antigen in transgenic plants for oral immunization. - Nat. Biotechnol. **18**: 1167-1171, 2000.

Stewart, C.N., Jr., Via, L.E.: A rapid CTAB isolation technique for RAPD fingerprint and other PCR applications. - BioTechniques **14**: 748-749, 1993.

Sunil Kumar, G.B., Ganapathi, T.R., Revathi, C.J., Prasad, K.S.N., Bapat, V.A.: Expression of hepatitis B surface antigen in tobacco cell suspension cultures. - Protein Exp. Purification **32**: 10-17, 2003.

Sunil Kumar, G.B., Ganapathi, T.R., Revathi, C.J., Srinivas, L., Bapat, V.A.: Expression of hepatitis b surface antigen in transgenic banana plants. - Planta **222**: 484-493, 2005a.

Sunil Kumar, G.B., Ganapathi, T.R., Srinivas, L., Revathi, C.J., Bapat, V.A.: Secretion of hepatitis B surface antigen in transformed tobacco cell suspension cultures. - Biotechnol. Lett. **27**: 927-932, 2005b.

Tiollais, P., Charnay, P., Vyas, F.: Biology of hepatitis B virus. - Science **213**: 406-411, 1981.

Wilde, C.D., Houdt, H.V., Buck, S.D., Angenon, G., Jaeger, G.D., Depicker, A.: Plants as bioreactors for protein production avoiding the problem of transgene silencing. - Plant mol. Biol. **43**: 347-359, 2000.