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Expression of hepatitis B surface antigen in potato hairy roots

G.B. Sunil Kumar [a,1], T.R. Ganapathi [a,1], L. Srinivas [a], C.J. Revathi [b], V.A. Bapat [a,*]

[a] *Plant Cell Culture Technology Section, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India*
[b] *R&D, Shantha Biotechnics Limited, Hyderabad, 501 401, India*

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Abstract

Transgenic potato plants with two different expression cassettes for hepatitis B surface antigen (HBsAg) expression were developed. These plants were used to induce hairy roots. The transgenic nature of the potato plants was confirmed by PCR and PCR-Southern analysis. A maximum expression of 19.11, 23.94 and 97.1 ng/g F.W. was noted in potato plants, microtubers and hairy roots, respectively. HBsAg expression in potato plants and microtubers was analyzed by RT-PCR and ELISA. The transgenic nature of the hairy roots was confirmed by PCR using *rol*B specific primers. B5 medium was found to be more suitable for hairy roots culture. The doubling time of the hairy roots was found to be 2.32 days. Spontaneous regeneration of plants from hairy roots was noted in hairy root line derived from pEFEHER transformed potato plants. Plants regenerated from hairy roots exhibited similar levels of HBsAg expression to that of transgenic plants. This is the first report on the expression of HBsAg in potato hairy roots.

Keywords: Hepatitis B surface antigen; Potato hairy roots; *Agrobacterium rhizogenes*

1. Introduction

Hepatitis B is one of the alarming diseases in the developing world. It is estimated that there are about 2 billion people infected by hepatitis B virus and 15–17% of them become chronic carriers, with very high endemicity in developing countries [1]. Although yeast derived hepatitis B vaccine proved to offer excellent safety and efficacy profiles and availability since the last 2 decades, the poor people in the developing countries cannot afford to purchase the currently available vaccine. Against this background, plants offer as an alternative system for the production of cheaper vaccines [2].

Potato has been extensively studied as an attractive system for the production of plant-based vaccines since the first report on oral immunization with plant-based vaccines [3]. Potato based hepatitis B surface antigen (HBsAg) was found to be orally immunogenic in mice [4] and in humans [5]. Recent incidences of inadvertent contamination of food supply by plant derived pharmaceutical proteins have resulted in heavy penalties and stricter USDA guidelines to cease food products with traces of pharmaceutical products [6], this restricts the use of transgenic plants for the production of biopharmaceuticals. The effect of environment could influence the expression levels of the recombinant protein in the transgenic plants and the implementation of good manufacturing practice (GMP) requires their cultivation under controlled conditions. This could be best achieved by using plant cell cultures or hairy root as expression systems. Though the plant cell cultures were extensively studied for the pharmaceuticals production, their genetic instability makes them less suitable for long-term cultures and scale up [7]. In this context, genetic stability, fast growth and growth in hormone free media makes hairy roots as an attractive system for the production of recombinant proteins.

Hairy roots have been used in a wide range of research application for plant improvement, plant secondary metabolism studies, plant interaction with the environment, and for the production of monoclonal antibodies [8]. However, there is no report on the expression of HBsAg in hairy root cultures. Here in we report for the first time the expression of HBsAg in potato hairy roots.

* Corresponding author. Tel.: +91 22 25595049; fax: +91 22 25505151.
E-mail address: vabapat@magnum.barc.ernet.in (V.A. Bapat).
[1] These authors contributed equally to work.

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2. Materials and methods

2.1. Plant material

Shoot cultures of var. Kufri Bahar were established from sprouted buds excised from the tubers. The buds were surface sterilized with 0.1% mercuric chloride for 5 min. After repeated washing in sterile distilled water, the buds were cultured on MS [9] medium. The pH of the medium was adjusted to 5.8 and all the cultures were gelled with 0.2% phytagel (Sigma). The cultures were exposed to 16 h light from fluorescent tubes and maintained at 25 ± 2 °C at a relative humidity of 50–60%. The buds were developed into plantlets in 4 weeks. Nodes from these plantlets were cultured on MS medium supplemented with 0.29 μM GA_3 and 0.54 μM NAA + charcoal 0.1% for raising the fresh cultures. Inter-nodal segments from these plantlets were routinely used for *Agrobacterium* mediated transformation.

2.2. Plant transformation

Inter-nodal stem segments from in vitro grown plants were used for transformation and regeneration, essentially as described [10] with following modifications. The internode segments were pre cultured on MS basal medium for 48 h and then infected with *Agrobacterium tumefaciens* harboring two plant expression vectors pEFEHBS/pEFEHER with hepatitis B virus 's' gene encoding the surface antigen [11] for 30 min. The T-DNA portion of these vectors is depicted in Fig. 1. The explants were then blotted dry on sterile Whatman No. 1 filter paper and were re cultured on MS basal media for co-cultivation for 48 hours. Following co-cultivation, the explants were transferred to regeneration media (MS + Trans-Zeatin 13.68 μM + IAA 0.57 μM + GA_3 14.43 μM) containing cefotaxime (400 mg/l) and kanamycin (50 mg/l) for selection of transgenic shoots and inhibition of *Agrobacterium* growth. The regenerated kanamycin resistant shoots were sub cultured on MS medium supplemented with NAA (0.54 μM) and GA_3 (0.29 μM) and kanamycin (100 mg/l) for rooting.

2.3. Induction of microtubers

Microtubers were induced from the untransformed control and transgenic plantlets according to the protocol described [12]. The putative transgenic and control shoots were transferred to MS medium supplemented with BA (44.38 μM) and sucrose 8% (microtuber induction medium) and were kept in the dark for microtuber production.

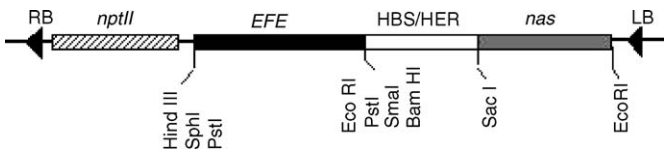

Fig. 1. T-DNA region of plant expression vectors pEFEHBS/pEFEHER. npt-II is a neomycin phosphotransferase; EFE is ethylene forming enzyme gene promoter of banana; nos is a nos terminator and RB and LB are right and left borders.

2.4. Induction of hairy roots

Hairy roots were induced from untransformed control and transgenic plants by co-cultivating inter-nodal segments with *Agrobacterium rhizogenes* strain ATCC 15834 for 30 min. Then the explants blotted dry and transferred to MS basal medium and maintained in dark. After 3 days, these were transferred to MS medium supplemented with Cefotaxime (400 mg/l). The roots initiated from the cut ends of the explants were excised and cultured on B5 medium [13]. The liquid cultures were initiated by taking a 4–5 cm long pieces of hairy roots. Six different lines were established for each construct and expression levels of HBsAg were assayed for each line.

2.5. Growth kinetics of hairy roots

Different media like MS, SH [14], WM [15] and B5 were used for optimizing the suitable medium for the growth of hairy roots. Four hundred milligrams of fresh weight (F.W.) of hairy roots were inoculated in 50 ml of liquid medium in a 250 ml Erlenmeyer flask and incubated in dark on a gyratory shaker. The growth of the hairy roots was measured by noting the mean fold increase in fresh weights at intervals of 2, 4, 8 and 10 days.

To optimize the amount of inoculum, different amounts (100–500 mg F.W.) of hairy roots were cultured in 50 ml B5 liquid medium in 250 ml Erlenmeyer flask and incubated on a gyratory shaker in dark for 10 days. The growth of the hairy roots was measured in terms of mean fold increase in fresh weight. All the experiments were carried out in triplicate and mean values were calculated.

The doubling time of the hairy roots was measured by taking fresh weights at 2 days intervals for 10 days. Three replicates were used at each time point. Doubling time was calculated by plotting a graph of $\log_2$ fresh weight (g) versus time (days) and calculating the inverse of slope for the linear part of the curve [16].

2.6. PCR analysis

Total genomic DNA was extracted from the four putatively transgenic plants for each construct and control untransformed plants using modified CTAB method [17]. A 50 μl of PCR mix contained the primers (100 ng each), Taq DNA polymerase (1.0 unit), 200 μM of each dNTP, 1× PCR buffer and 100 ng of genomic DNA as template. The PCR conditions were 94 °C initial melting for 5 min followed by 35 cycles of amplification with each cycle consisting of following steps: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min with a final extension for 10 min. The primer sequences used to amplify a 681 bp fragment HBsAg 's' gene are as follows:

1. 5′-TACTGGATCCACCATGGAGAACATCAACA-3′ and
2. 5′-TCTAGAGCTCTTAAATGTATACCCAGAAGA-CAAAAGAA-3′.

The transgenic nature of the hairy roots was analyzed using *rolB* specific primers to amplify 780 bp fragment. The genomic

DNA was extracted from the hairy roots as described above. The PCR reactions were carried out in 50 μl volume with 100 ng genomic DNA, 10 pmol of each primer, 200 μM dNTPs, 1 unit Taq DNA polymerase, 1× PCR buffer and 2 mM $MgCl_2$. PCR conditions were 94 °C initial melting for 5 min followed by 40 cycles of 94 °C for 1 min, 52.5 °C for 1.5 min and 72 °C for 2 min and final extension for 10 min at 72 °C. The primer sequences used to amplify a 780 bp fragment of the *rolB* gene are as follows:

1. 5′-ATGGATCCCAAATTGCTATTCCCCCACGA-3′ and
2. 5′-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3′.

2.7. Southern analysis of PCR products

The PCR products of the transgenic plants after gel separation were blotted onto nylon membranes (Hybond N+; Amersham-Pharmacia). The 681 bp *Bam*HI fragment from pBSHER containing the HBsAg 's' gene was radioactively labeled with α-[^{32}P]dCTP using the Random primer Kit from BRIT (India) according to the manufacturer's instructions, and used for the hybridization. The blotting and subsequent hybridization were carried out as described [18].

2.8. Reverse transcription (RT)-PCR of transgenic potato plants/microtubers

Total RNA was isolated from the microtubers of four transgenic plants/microtubers for each construct as well as from the untransformed control using Rneasy Plant Mini Kit (Qiagen, USA) and RT-PCR was carried out according to manufacturer's instructions. Ten microliters of this cDNA was used as template for PCR and the PCR conditions and the primers used are same as it is mentioned in PCR analysis. PCR with total RNA from transgenic plants did not give any amplification indicating that RNA preparation is free from genomic DNA.

2.9. ELISA analysis

Total protein was extracted from the leaves, microtubers and hairy roots of untransformed control as well as transgenic plants as described previously [19]. The extracts were clarified and analyzed for the levels of HBsAg expression by ELISA (Shan kit HBsAg ELISA, Shantha Biotechnics Ltd., India). The positive control (human serum derived HBsAg) as a standard and negative control (protein extracted from untransformed control) was used.

3. Results and discussion

3.1. Development of transgenic potato plants and induction of microtubers

Four weeks after co-cultivation shoots were developed from inter-nodal segments. One to three shoots developed per explant in 40% of the cultures. These shoots were excised and transferred to fresh medium of the same composition and the shoots elongated in 3 weeks. These elongated shoots were transferred to rooting medium with kanamycin selection. About 20% of the shoots developed roots and grew vigorously on this medium. Six rooted plants for each construct were randomly selected and used for molecular analysis. The transgenic plants produced microtubers from the terminal and axillary regions of the shoots in microtuber induction medium in 4 weeks. An average of eight microtubers were obtained from each shoot. These microtubers were used for the expression analysis. The yield of microtubers was similar to that of untransformed control.

3.2. PCR and PCR-Southern analysis of transgenic plants

Four putatively transformed plants for each construct were analyzed for their transgenic nature by PCR and PCR-Southern analysis. Transgenic plants showed the amplification of 681 bp fragment by PCR of genomic DNA with HBsAg 's' gene specific primers, while it was absent in untransformed control plants (Fig. 2a and b). The amplified PCR products were hybridized to the radioactively labeled 681 bp HBsAg 's' gene, while no hybridization signal was detected in untransformed control confirming the transgenic nature of the plants (Fig. 3a and b).

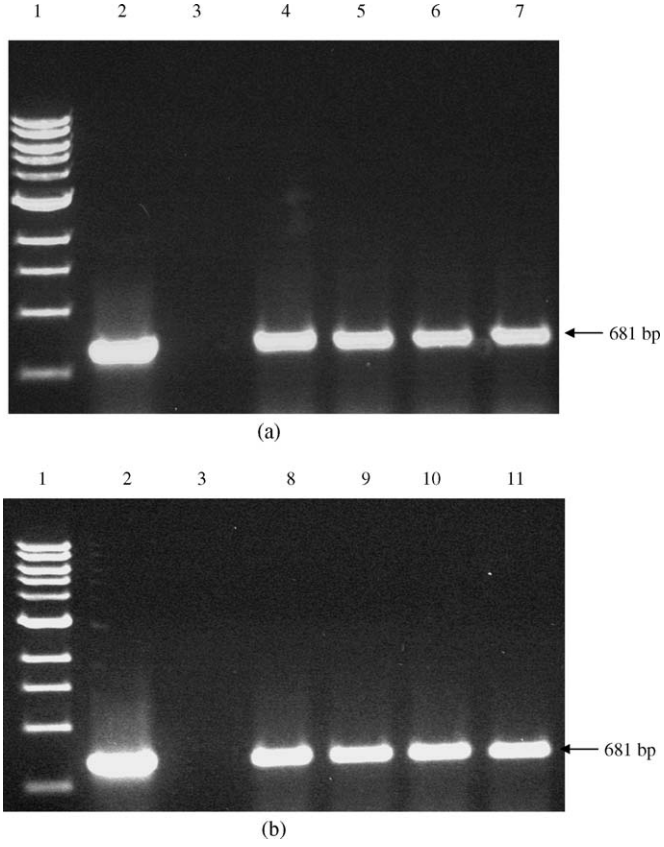

Fig. 2. PCR analysis of potato plants. Lane 1: 1 kb ladder; lane 2: positive control (pEFEHBS); lane 3: negative control (genomic DNA extracted from untransformed control plants); (a) lanes 4–7: pEFEHBS transformed plants and (b) lanes 8–11: pEFEHER transformed plants showing the amplification of 681 bp fragment of HBsAg 's' gene.

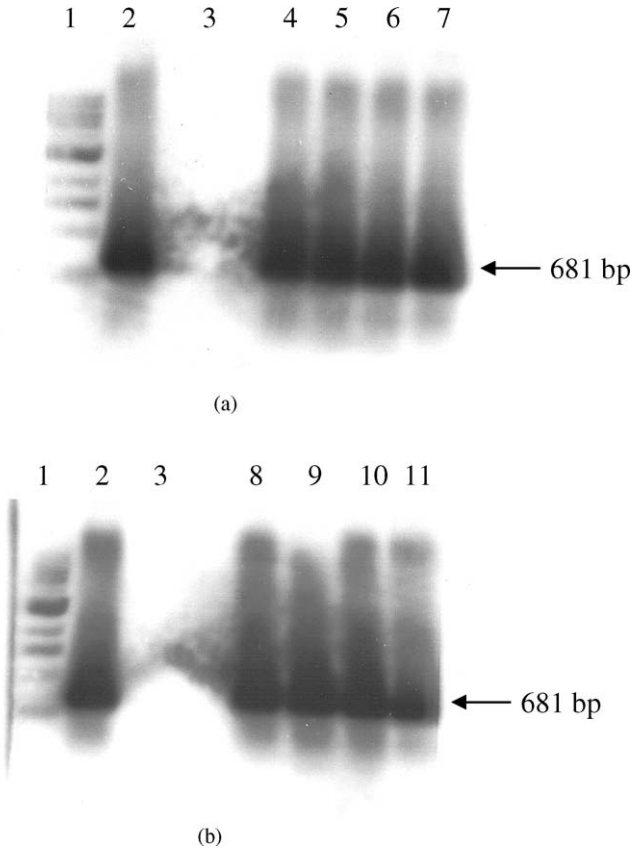

Fig. 3. Southern analysis of PCR products. Lane 1: 1 kb ladder; lane 2: positive control (pEFEHBS); lane 3: negative control (genomic DNA extracted from untransformed control plants); (a) lanes 4–7: pEFEHBS transformed plants and (b) lanes 8–11: pEFEHER transformed plants showing the hybridization of 681 bp fragment of HBsAg 's' gene.

3.3. Expression analysis

The expression of HBsAg in transgenic plants and microtubers was confirmed by RT-PCR analysis (Fig. 4a and b). Expression levels in different transgenic plant tissues were estimated by ELISA. A maximum expression of 19.11, 23.94 and 97.1 ng/g F.W. was noted in transgenic plants, microtubers and hairy roots with pEFEHER expression cassette (Table 1). Expression levels in the hairy root derived plant were almost similar to that of transgenic plant.

Table 1
Expression levels of HBsAg in different transgenic potato tissues

Transgenic potato samples	Construct	Expression levels (ng/g F.W.)
Plant	pEFEHBS	14.31 ± 0.015
Plant	pEFEHER	19.11 ± 0.051
Microtuber	pEFEHBS	18.93 ± 0.3
Microtuber	pEFEHER	23.94 ± 0.8
Hairy roots	pEFEHBS	92.50 ± 0.4
Hairy roots	pEFEHER	97.10 ± 0.6
Hairy root derived plants	pEFEHER	13.40 ± 0.4

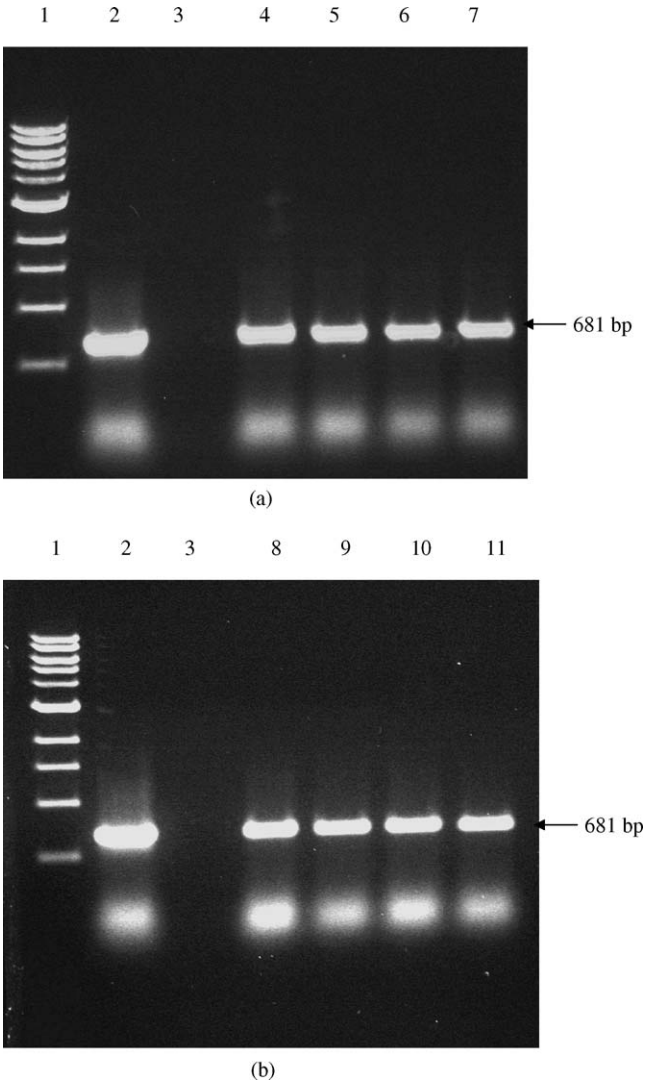

Fig. 4. RT-PCR analysis of HBsAg expression in (a) transgenic potato plants; (b) microtubers derived from transgenic plants. Lane 1: 1 kb ladder; lane 2: positive control (pEFEHBS); lane 3: negative control (genomic DNA extracted from untransformed control plants); lanes 4 and 5: pEFEHBS transformed plants; lanes 6 and 7: pEFEHER transformed plants; lanes 8 and 9: microtubers of pEFEHBS transformed plants; lanes 10 and 11: microtubers of pEFEHER transformed plants.

3.4. Induction of hairy roots

The inter-nodal stem segments from transgenic and untransformed control potato plants showed the initiation of hairy roots with in a week after co-cultivation with *Agrobacterium rhizogenes* (Fig. 5a–c). The roots differentiated were excised and grown on fresh medium (Fig. 5d and e). Six lines for each construct and control hairy root cultures were established in B5 medium. Among the six lines, the highest expressing line was selected and used for further analysis.

3.5. Growth kinetics and doubling time of hairy roots

Hairy root cultures were grown in different liquid media to determine the medium for optimal growth. Among the five

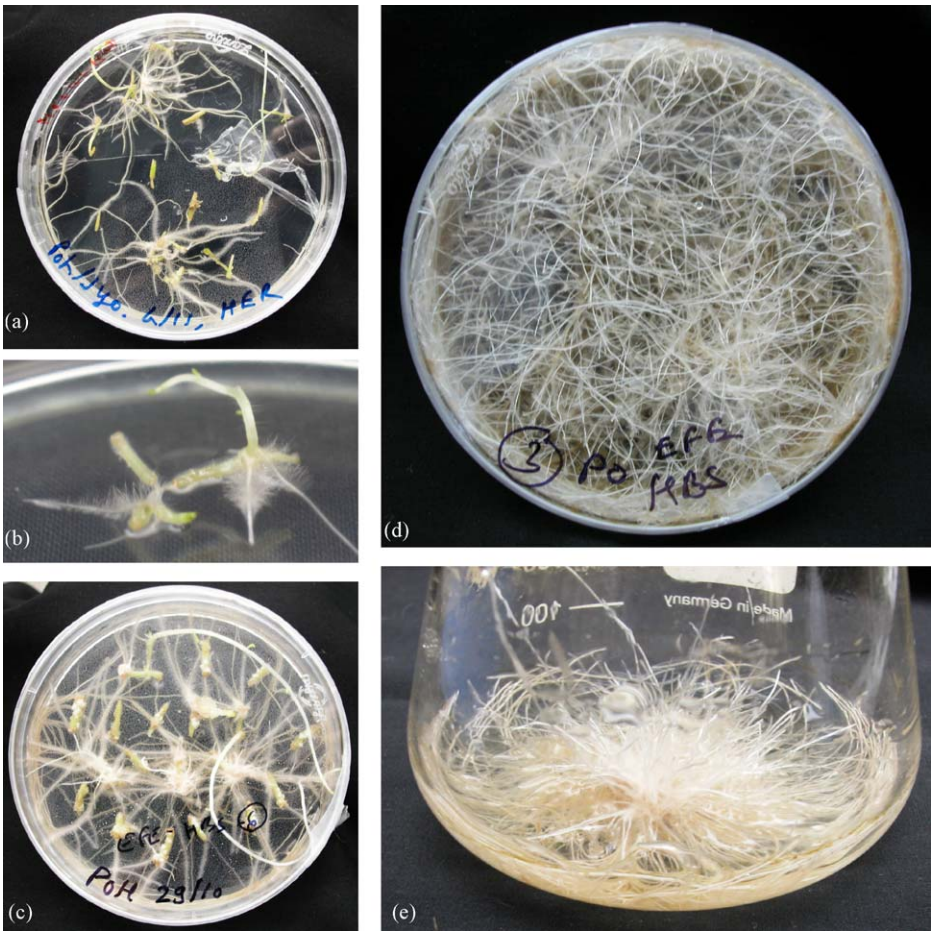

Fig. 5. (a) Induction of hairy roots from inter-nodal segments of transgenic potato plants; (b) close view of an explant showing the development of hairy roots; (c) growth of hairy roots on MS solid medium after 4 weeks of culture; (d) luxuriant growth of excised hairy roots on B5 solid medium after 4 weeks of culture; (e) growth of hairy root cultures in 25 ml B5 liquid medium.

media tested B5 and SH media were found to be suitable for the optimal growth of potato hairy roots. Maximum mean fold increase in fresh weight of 12- and 8-fold were observed in B5 and SH media, respectively (Fig. 6). An optimum inoculum of 100 mg F.W. was required to obtain 21.28-fold increase of fresh weight in 10 days (Fig. 7). The growth of the hairy root lines in terms of fresh weight showed exponential pattern with doubling time of 2.32 days. The biomass accumulated at the end of 10 days was 12 times the weight of initial inoculum of hairy roots. Spontaneous regeneration of shoots was noted in hairy roots with pEFEHER expression cassette (Fig. 8).

3.6. Molecular analysis of hairy roots

The transgenic nature of the hairy roots was confirmed by PCR amplification of a portion of *rolB* gene. A 780-bp fragment

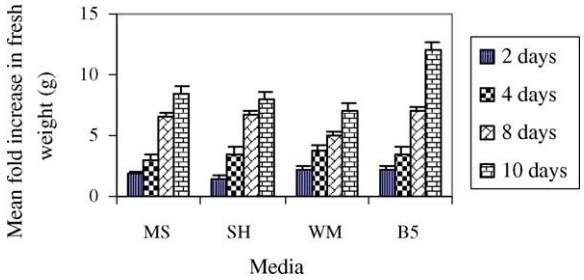

Fig. 6. Effect of medium composition on the growth of potato hairy roots. Different media like MS, SH, WM and B5 were used for optimizing the suitable medium for the growth of hairy roots. Four hundred milligram F.W. of hairy roots were inoculated in 50 ml of liquid medium. The growth of the hairy roots was measured by noting the mean fold increase in fresh weights at intervals of 2, 4, 8 and 10 days.

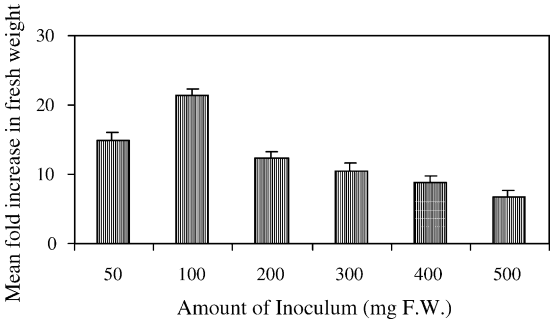

Fig. 7. Optimization of inoculum amount for the potato hairy root cultures. Different amounts (100–500 mg F.W.) of hairy roots were cultured in 50 ml B5 liquid medium and incubated on a gyratory shaker in dark for 10 days. The growth of the hairy roots was measured in terms of mean fold increase in F.W.

Fig. 8. (a) Spontaneous regeneration of a shoot from hairy root cultures grown in B5 liquid medium and (b) 4-week-old fully grown plantlets regenerated from hairy roots.

amplification was noted in all the hairy root lines, while it was absent in the control roots (Fig. 9). The expression levels of HBsAg in hairy roots were determined by ELISA analysis (Table 1).

Potato has been extensively used for molecular farming, especially for vaccines [20]. It has historic importance as the first plant to be used for oral delivery of plant-based vaccines. Its rapid growth rates, amenability to genetic transformation and ability to produce microtubers makes it an attractive choice for the production of vaccines. Laboratory animals like mice readily accepts potatoes as food, thus allowing immunogenicity studies of orally delivered plant-based vaccines.

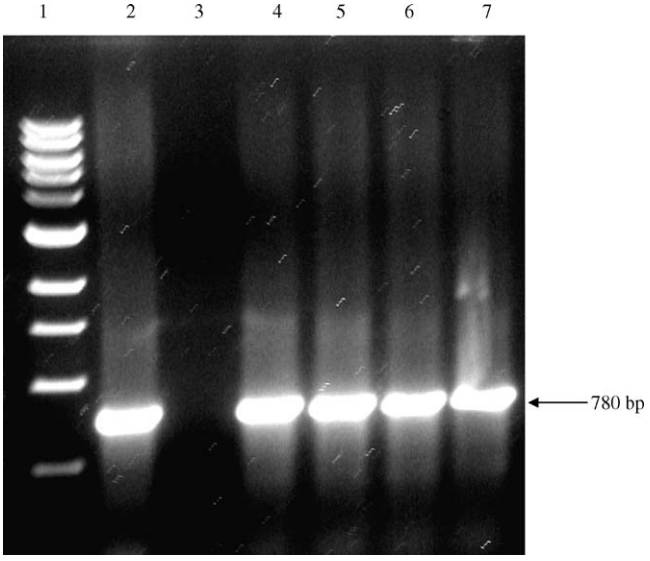

Fig. 9. PCR amplification of a 780 bp fragment of the *rolB* gene using genomic DNA derived from potato hairy roots. Lane 1: 1 kb ladder; lane 2: positive control (*Agrobacterium rhizogenes* culture); lane 3: negative control (genomic DNA extracted from non-transformed roots of transgenic potato plants); lanes 4 and 5: potato hairy roots with pEFEHBS expression cassette; lanes 6 and 7: potato hairy roots with pEFEHER expression cassette.

The objective of this study was to demonstrate the expression of HBsAg in Indian cultivar (Bahar) of potato and induce microtubers and hairy roots. The advantages of hairy root cultures can be exploited for the production of HBsAg. Recently there is a growing interest in using hairy roots as expression systems in addition to the production of secondary metabolites. Potato hairy roots were established by infecting tuber discs with *Agrobacterium rhizogenes* for enhanced production of anti-microbial sesquiterpenes and lipoxygenase metabolites [21]. In the present study, hairy roots were initiated from the stem inter-nodal segments using *A. rhizogenes* strain ATCC 15834. Among the different media tried for the optimum growth of hairy roots, B5 medium was found to be better for maximum biomass accumulation. However, the expression levels of HBsAg were similar in hairy roots cultured on different media. As doubling time observed in this study was only 2.32 days suggesting that these can be multiplied rapidly to obtain required biomass.

Recently, Borisjuk et al. [22] developed a rhizosecretion system that could secrete three functional recombinant proteins at seven times higher from hairy roots than adventitious roots of tobacco plants. We have noted six times higher expression levels of HBsAg in potato hairy roots. This could be because of active protein synthesis in hairy root tissue compared to the adventitious root tissues, as mRNA levels in both the roots were similar [8].

The secretion of HBsAg by NT-1 cells of tobacco into the spent medium using the ER retention signal was reported earlier by us [11,23]. The use of secretory signal sequences could direct the HBsAg to secretary pathway of the hairy root cultures. As hairy roots utilizes the culture medium the secreted protein gets accumulated can be harvested continuously by taking out the spent medium and replacing with the fresh medium. The stability of the secreted protein can be increased by using certain protein stabilizers [7]. Further attempts are required in this direction.

Hepatitis B vaccine production has been optimized in potato [24] and oral immunogenicity studies of these potatoes were conducted in mice, which resulted in priming and boosting of serum anti-HBsAg IgG responses [4]. Recently, Thanavala et al. [5] reported Immunogenicity studies of potato based hepatitis B vaccine in humans.

It is demonstrated in the current study that EFE promoter from monocot like banana is functional in potato (dicot). The preliminary studies of the banana genome structure and organization suggested that it is closer to *Arabidopsis* than to rice and places it in a unique position with more affinity to dicots than other monocots [25]. This creates new opportunities to use banana promoters in potato/dicots for expression of recombinant proteins in transgenic plants as well as in microtubers as similar and comparable expression levels were noted in transgenic banana plants [11].

HBsAg expression levels were higher, when it was expressed with a C-terminal ER retention signal. As noticed in other recombinant plant systems (tobacco cells, transgenic plants of banana) [11,23]. ER facilitates proper folding and disulfide bond formation is promoted by an oxidizing environment and is facilitated by an enzyme protein disulfide isomerase [26]. The recombinant proteins when targeted either to endoplasmic reticulum or secretory pathway showed proper folding of proteins, thereby increasing the functional protein level expressed in plants [27]. ER targeting is essential for the glycosylation and disulfide bridge formation [28]. Similarly, in the present study also, higher levels of HBsAg were obtained when it was expressed with a C-terminal ER retention signal.

The expression levels noted in the present study are lower, use of tuber abundant protein promoters, which include patatin [29] and granule bound starch synthase (GBSS) promoter [30], may enhance the expression levels in tubers. The expression levels in hairy roots can be further enhanced by using root specific promoters like *rolD* gene promoter [31].

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