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Expression of hepatitis B surface antigen in transgenic banana plants

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Abstract Embryogenic cells of bananan cv. Rasthali (AAB) have been transformed with the 's' gene of hepatitis B surface antigen (HBsAg) using *Agrobacterium* mediated transformation. Four different expression cassettes (pHBS, pHER, pEFEHBS and pEFEHER) were utilized to optimize the expression of HBsAg in banana. The transgenic nature of the plants and expression of the antigen was confirmed by PCR, Southern hybridization and reverse transcription (RT)-PCR. The expression levels of the antigen in the plants grown under in vitro conditions as well as the green house hardened plants were estimated by ELISA for all the four constructs. Maximum expression level of 38 ng/g F.W. of leaves was noted in plants transformed with pEFEHBS grown under in vitro conditions, whereas pHER transformed plants grown in the green house showed the maximum expression level of 19.92 ng/g F.W. of leaves. Higher monoclonal antibody binding of 67.87% of the antigen was observed when it was expressed with a C-terminal ER retention signal. The buoyant density in CsCl of HBsAg derived from transgenic banana leaves was determined and found to be 1.146 g/ml. HBsAg obtained from transgenic banana plants is similar to human serum derived one in buoyant density properties. The transgenic plants were grown up to maturity in the green house and the expression of HBsAg in the fruits was confirmed by RT-PCR. These transgenic plants were multiplied under in vitro using floral apex cultures. Attempts were also made to

enhance the expression of HBsAg in the leaves of transgenic banana plants by wounding and/or treatment with plant growth regulators. This is the first report on the expression of HBsAg in transgenic banana fruits.

Keywords *Agrobacterium* · Edible vaccine · Embryogenic cells · Hepatitis B surface antigen · Transgenic banana

Abbreviations ADS: Adenine hemisulfate · BA: Benzylaminopurine · CsCl: Cesium Chloride · EFE: Ethylene forming enzyme · F.W.: Fresh weight · HBsAg: Hepatitis B surface antigen · NAA: α -Naphthalene acetic acid · RT-PCR: Reverse transcription-polymerase chain reaction

Introduction

Hepatitis B is one of the alarming diseases in the developing world. It is estimated that there are about 350 million chronic carriers of Hepatitis B and 20 million new infections occur annually (Joshi and Kumar 2001). Prophylaxis in the form of vaccines can control the disease spread. Recombinant vaccine derived from yeast is expensive and restricts its use in the mass immunization programs of the developing world. The plant-based production of vaccine for hepatitis B in the edible fruits may be an economical alternative. Hepatitis B surface antigen (HBsAg) expression has been reported in transgenic tobacco plants (Mason et al. 1992), tobacco cells (Sunil Kumar et al. 2003), lettuce and lupin (Kapusta et al. 1999), carrot (Imani et al. 2002) and potato (Richter et al. 2000).

The plant derived HBsAg, self assembles into subviral particles that are virtually indistinguishable from the plasma and yeast derived HBsAg with respect to the following: size, density sedimentation, antibody binding, in eliciting HBsAg specific antibodies in mice, primes T cells in vivo. This can be stimulated in vitro by tobacco

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derived rHBsAg, yeast-derived rHBsAg, and by a synthetic peptide that represents an epitope of the HBsAg (Thanavala et al. 1995). It was immunogenic in mice when administered orally through transgenic potatoes (Kong et al. 2001). Kapusta et al. (1999) reported that human volunteers fed with transgenic lettuce plants expressing HBsAg, developed protective levels of anti-HBsAg IgG at >10 IU/l. Smith et al. (2003) characterized the plant derived HBsAg for its structure and post-translational processing. Extensive disulfide bond cross linking, which is required for immunogenicity was observed in 21–37% of total HBsAg protein displayed epitopes, which correlates with the potency of the vaccine.

Banana is an ideal host for the production of edible vaccines as it offers advantages like digestibility and palatability by the infants, availability throughout the year in the tropics and subtropics where economical vaccines are required to immunize large segment of the population (Sunil Kumar et al. 2004). As many of the edible bananas does not set seeds and are vegetatively propagated through suckers, it is an ideal candidate for gene containment and there is no segregation of the transgene. Genetic transformation of banana has been achieved with considerable success; using *Agrobacterium* mediated transformation (May et al. 1995; Ganapathi et al. 2001), particle bombardment (Sagi et al. 1995) or electroporation (Sagi et al. 1994). Smith et al. (2005) has recently reviewed the progress in genetic transformation of banana. Chakrabarti et al. (2003) demonstrated enhanced disease resistance in transgenic banana plants with the expression of an anti-microbial peptide (MSI-99). Considering the success achieved in the genetic transformation of banana, it is now possible to transfer and express useful genes (Ganapathi et al. 2002). In this communication, we report the expression of HBsAg in banana and to the best of our knowledge this is the first report on the expression of an antigen in transgenic banana plants.

Materials and methods

Establishment of banana embryogenic cell cultures and transformation

Embryogenic cells of banana cultivar Rasthali (AAB genome) were used for transformation. The establishment of the embryogenic cell cultures and *Agrobacterium* mediated transformation were carried out essentially as described earlier (Ganapathi et al. 2001). The embryogenic cells were co-cultivated with *Agrobacterium tumefaciens* strain EHA 105 (Hood et al. 1993) harboring one of the four expression vectors pHBS, pHER, pEFEHBS, and pEFEHER. The cells after co-cultivation were transferred to M₂ medium (Cote et al. 1996) supplemented with Cefotaxime (400 mg/l) and after a period of 3 days the cells were transferred to RR medium (Ganapathi et al. 2001) with Cefotaxime (Smith Kline Beecham) (400 mg/l) and Ge-

neticin (G418, Sigma; 5 mg/l) for somatic embryo development. The embryos developed shoots upon transfer to MS basal medium (Murashige and Skoog 1962) supplemented with BA (0.5 mg/l). These shoots were rooted on MS + NAA (1 mg/l) + Geneticin (5 mg/l) and Cefotaxime (400 mg/l). Fifty transgenic plants were regenerated for each construct and hardened in the green house. Two plants for each construct and un-transformed control were grown up to maturity in the green house and the fruit bunches were harvested.

Construction of plant expression vectors

The 's' gene of hepatitis B surface antigen with endoplasmic reticulum (ER) retention signal (HER) and without signal (HBS) was cloned as described previously (Sunil Kumar et al. 2003). Hepatitis B virus genome (adw2 subtype) cloned in pBR322 was used as a template (obtained from ATCC, USA). HER and HBS were sequenced and further sub-cloned into plant expression vectors with *ubq3* promoter from *Arabidopsis* (Norris et al. 1993) or Ethylene forming enzyme (EFE) promoter (May and Kipp 1997) from banana to construct four expression vectors namely pHBS, pHER, pEFEHBS and pEFEHER. pHBS and pHER were constructed as described previously (Sunil Kumar et al. 2003). pEFEHBS and pEFEHER were constructed by cloning HBS and HER fragments in to the *Bam*HI and *Sac*I sites of pEFEGUS (a pBI 121 based plant expression vector with *EFE* promoter of banana) by replacing the GUS with HER or HBS. The T-DNA portions of the plant expression cassettes are depicted in Fig. 1.

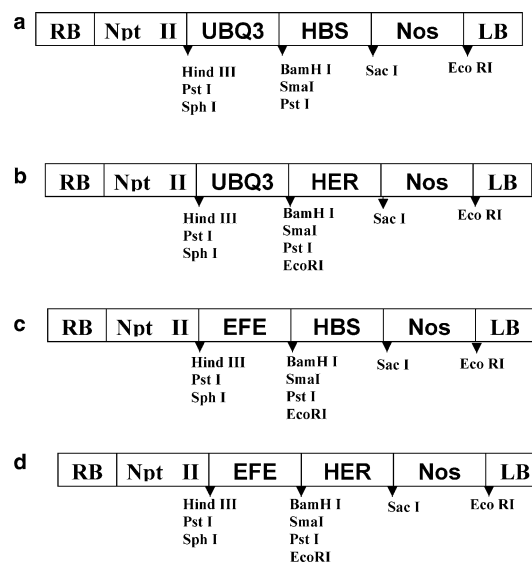


Fig. 1 T-DNA region of **a** pHBS, **b** pHER, **c** pEFEHBS and **d** pEFEHER. RB and LB are right and left borders, Npt-II is neomycin phosphotransferase, UBQ3 is ubiquitin promoter from *Arabidopsis*, EFE is ethylene forming enzyme promoter of banana, HBS is HBsAg 's' gene, HER is HBsAg 's' gene with ER retention signal and Nos is nos terminator

Analysis of the transgenic plants by polymerase chain reaction (PCR)

Total genomic DNA was extracted from the putatively transgenic plants and control un-transformed plants using modified CTAB method (Stewart and Via 1993). A 50 µl of PCR mix contained the primers (100 ng each), Taq DNA polymerase (1.0 unit), 200 µM of each dNTP, 1X 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 of 10 min. The amplified products were analyzed on a 1% agarose gel. The primer sequences used to amplify a 681 bp HBsAg 's' gene are as follows:

- (a) 5'-TACTGGATCCACCATGGAGAACATCAACA-3' and
- (b) 5'-TCTAGAGCTCTTAAATGTATACCCAGAGACAAAAGAA-3'

Southern analysis of transgenic plants

Ten micrograms of each genomic DNA sample was digested with *Bam*HI and *Sac*I to release the 's' gene of HBsAg from the T-DNA region, electrophoresed on 1% agarose gel and transferred to Hybond N + membrane (Amersham Pharmacia Biotech, UK). Southern analysis was carried out as described (Sambrook et al. 1989). These DNAs were hybridized against a random primed radiolabeled probe of 681 bp fragment obtained by digesting pBS HER with *Bam*HI. Results were visualized by autoradiography.

Reverse transcription (RT)-PCR of transgenic banana plants

Total RNA was isolated from the leaves of transgenic plants as well as from the un-transformed control using Rneasy Plant Mini Kit (Qiagen, USA) following manufacturers instructions. A 1 µg aliquot of total RNA was used for cDNA synthesis using cMaster RT PCR system (Eppendorf, USA). Five microliters of this cDNA was used as template for PCR and the PCR conditions and the primers used were same as mentioned in PCR analysis. Total RNA was isolated from transgenic banana fruits as well as untransformed control fruits essentially as described (Hasan et al. 2000). cDNA synthesis and RT-PCR were carried out as described above. PCR with total RNA extracted from leaves and fruits of banana was also carried out to check genomic DNA contamination.

Extraction of total protein from banana leaves and ELISA analysis

Total protein was extracted from the leaves of un-transformed control as well as transgenic plants as mentioned previously (Mason et al. 1992). The extracts were clarified and assayed in triplicates for the levels of HBsAg expression and mean values were calculated. ELISA analysis was carried out using monoclonal antibody based (mAb ELISA) HBsAg EIA Plus Kit (Anilab systems, Finland) and polyclonal antibody based (pAb) Biokit HBsAg ELISA (Spain). The positive control (human serum derived HBsAg) as a standard and negative control (protein extracted from un-transformed control) were used. The percentage of monoclonal antibody binding was calculated as follows:

$$\begin{aligned} &\% \text{ of monoclonal antibody reactivity} \\ &= \frac{\text{Amount of HBsAg as estimated by mAb ELISA}}{\text{Amount of HBsAg as estimated by pAb ELISA}} \times 100 \end{aligned}$$

Extraction of total proteins from transgenic banana fruits and ELISA analysis

Total protein was extracted from the 2 g F.W. of the transgenic fruits by homogenization in 20 ml of the extraction buffer (20 mM Sodium phosphate pH 7, 20 mM L-cysteine and 1% Triton×100). The homogenate was clarified by centrifugation at 10,000g for 20 min. The total proteins in the resulting supernatant was precipitated by adding three volumes of chilled acetone, followed by incubation at -20°C for 4 h and centrifugation at 10,000g for 10 min. The protein pellet was washed thrice with chilled acetone, air dried, dissolved in 100 µl of extraction buffer and used for ELISA analysis (Shan Test HBsAg elisa, Shantha biotechnics Ltd., India).

Cesium chloride density gradient analysis of HBsAg from leaves of transgenic plants

Total protein was extracted from pEFEHER transformed banana leaves and CsCl density gradient centrifugation was carried out as described previously (Sunil Kumar et al. 2003). The gradients were centrifuged in a Beckman Nvt 65 rotor at 55,000 rpm for 18 h. The gradient fractions (0.5 ml each) were collected and assayed for the presence of HBsAg by ELISA (Shan TestHBsAg elisa). The density of the gradient fractions with HBsAg was measured by refractometry (Erma Hand Refractometer, Tokyo, Japan).

Wounding and/or treatment with plant growth regulators

One gram F.W. of banana leaves from transgenic plants of all the four constructs were harvested from the plants grown under in vitro conditions. For unwounded experiments the entire leaves were maintained in 10 ml of 0.4 M mannitol solution and for wounding experiments, these were cut into 1 cm² pieces, wounded with the help of a stainless steel surgical blade and incubated for 6, 12, 24, and 48 h. An amount of 250 mg of tissue was taken at each interval for total protein extraction. Similarly for Absciscic acid (ABA) or Indole acetic acid (IAA) treatment, wounded and unwounded 1 g F.W. leaves were maintained in 10 ml of 0.4 M mannitol supplemented with 100 μ M ABA or IAA and incubated for 24 h and total protein was extracted as described above. The ELISA analysis was carried out to estimate the levels of HBsAg. All these experiments were carried out in triplicates.

Multiplication of transgenic plants from floral apex cultures

Two transgenic banana plants with highest HBsAg expression for each construct were grown up to maturity in the green house. After the transition from female to male flowers the terminal bud was excised and swabbed with 70% ethanol and outer bracts were removed under aseptic conditions till it reached 5 mm in length. These buds were used to initiate the cultures on MS medium supplemented with BA (2 mg/l) and ADS (30 mg/l). The multiple shoots thus obtained were multiplied on the fresh medium of same composition and excised shoots were rooted individually on MS medium supplemented with NAA (1 mg/l). The rooted plantlets were hardened in the green house.

Results

Transformation of embryogenic cells and regeneration of plants

Banana embryogenic cells were transformed with the four different expression cassettes. All the transformed lines developed into embryos on the banana embryo induction medium supplemented with geneticin (5 mg/l). Whitish embryos developed from the transformed tissues in 3 weeks whereas un-transformed tissues turned brown and necrosed. These embryos produced secondary embryos upon subculturing on the fresh medium of same composition. After three rounds of subculture, the embryos were regenerated into plantlets on embryo germination medium (RR medium). Germinated embryos were transferred to MS+NAA (1 mg/l) and Geneticin (5 mg/l) for complete plantlet development. The transformed plantlets grew vigorously with a

profuse root system. About 50 plants were regenerated for each construct and were hardened in the green house. Almost 100 of the plants survived hardening in the green house and all the plants were morphologically similar. Transgenic banana plants were grown up to maturity in the green house and the fruit bunches were harvested (Fig. 2a–c). The transformed plants showed the expression in the range of 0.5–1.0 ng/g F.W. of the fruits.

Multiplication of transgenic plants from floral apex cultures

The floral apices of transgenic plants for all the four constructs showed the development of one or two shoots in 3 months on a medium supplemented with BA



Fig. 2 Transgenic banana plant with a fruit bunch in the green house. **a** Plant grown up to fruiting, **b** harvested bunch and **c** ripened fruits

(2 mg/l) and ADS (30 mg/l). These shoots, when sub-cultured on the same medium produced multiple shoots (4–6 shoots/culture) in another 4–6 weeks. This way the multiplication was continued for three to four passages and each time they developed into multiple shoots. The shoots were separated and rooted on MS + NAA (1 mg/l). The plantlets thus obtained were hardened in the green house (Fig. 3a–f). The presence of transgene in these plantlets was confirmed by PCR (Fig. 4).

PCR and Southern analysis of transgenic plants

PCR analysis of the five randomly selected transformed plants for each construct showed the amplification of 681 bp fragment corresponding to the expected size of the 's' gene of HBsAg, while it was absent in un-transformed control plants (Fig. 5). Three PCR positive plants of each construct were confirmed for stable integration of the transgene by Southern hybridization. *Bam*HI and *Sac*I digested genomic DNA released the

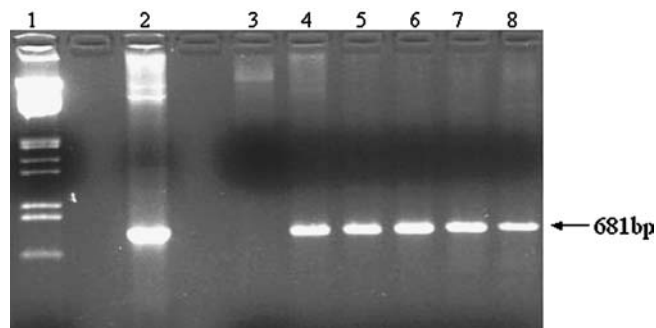


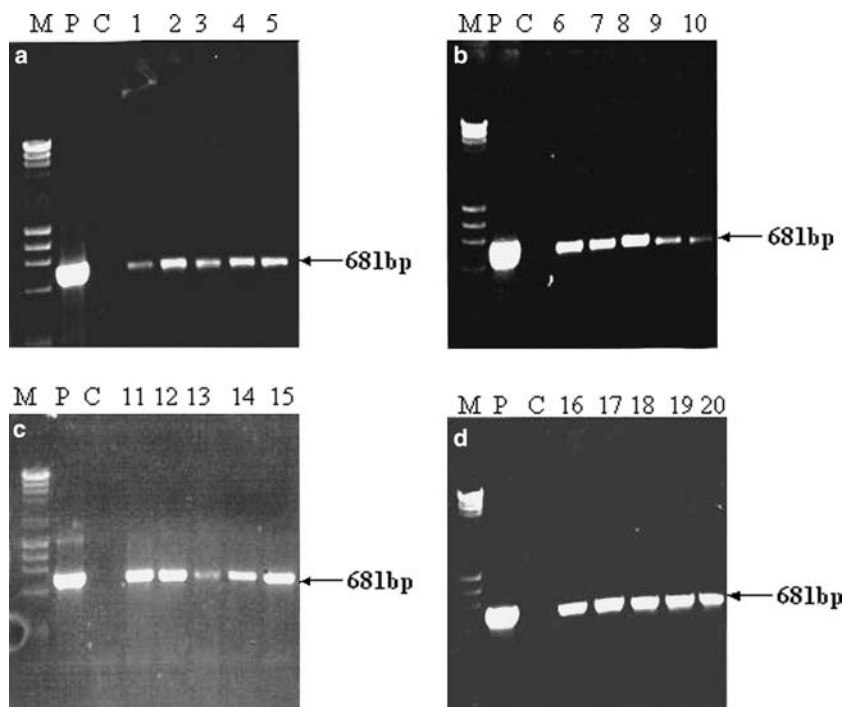
Fig. 4 PCR amplification of transgene using genomic DNA from transgenic plants obtained from floral apex culture. Lanes: 1, λ HindIII and *Eco*R I marker; 2, positive control (pHBS); 3, negative control (genomic DNA from un-transformed plant); 4, pHBS transformed plant; 5, pHER transformed plant; 6, pEFEHBS transformed plant; 7, pEFEHER transformed plant

681 bp HBsAg 's' gene internal to the T-DNA region and hybridized to 681 bp probe generated by *Bam*HI digestion of pBS HER (Fig. 6).

Fig. 3 Multiplication of transgenic plants by floral apex culture. **a** Transgenic plant with a fruit bunch **b** excised floral apex **c** floral tip culture **d** multiple shoots developed from floral tip culture **e** rooted shoots **f** hardened plants in the green house



Fig. 5 PCR analysis of banana plants. **a** Lanes: 1–5, pHBS transformed plants; **b** 6–10, pHER transformed plants; **c** 11–15, pEFEHBS transformed plants; **d** 16–20, pEFEHER transformed plants. *M*, 1 kb ladder; *P*, positive control (pHBS); *C*, negative control (genomic DNA from un-transformed plant)



Reverse transcription-PCR analysis

RT-PCR was used to confirm the transgene expression. The gene-specific band (681 bp) of the expected size was observed in the leaves (Fig. 7a) and fruits (Fig. 7b) of transgenic plants of the four constructs while being absent in the un-transformed control plants. PCR with total RNA extracted from the banana leaves and fruits was carried out, and no amplification of HBsAg 's' gene was observed indicating that total RNA preparations are free from genomic DNA contamination.

Expression analysis of in vitro and green house hardened plants

Twelve transgenic lines for each construct were assayed in triplicate for expression levels of HBsAg in the leaves

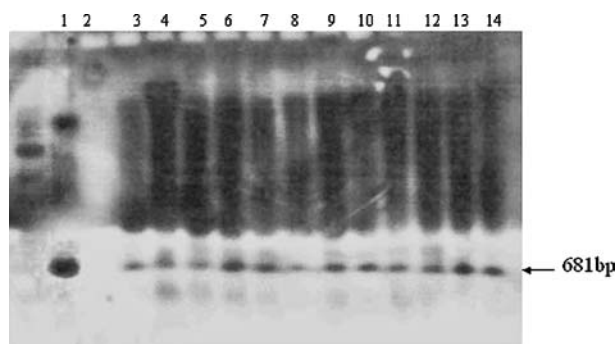


Fig. 6 Genomic Southern analysis of banana plants. Lanes: 1, positive control (pHBS); 2, negative control (genomic DNA from un-transformed plants); 3–5, pHBS transformed plants; 6–8, pHER transformed plants; 9–11, pEFEHBS transformed plants; 12–14, pEFEHER transformed plants

by pAb and mAb ELISA. The expression levels varied among the plants grown in vitro and green house. The pAb ELISA indicated the maximum expression of 38 ng/g F.W. was noted in pEFEHBS transformed plants grown in vitro. Under green house conditions, maximum expression of 19.92 ng/g F.W. was observed in pHER transformed plants. Transgenic plants of pEFEHBS/HER plants grown in the green house showed lower expression levels of the antigen than those grown under in vitro conditions (Table 1). A maximum of 67.87 monoclonal antibody binding of the antigen was noted in pHER-transformed plants (Table 2).

Buoyant density of HBsAg expressed in banana leaves

The formation of 22 nm particles is critical for the immunogenicity of HBsAg. The particulate nature of the HBsAg was studied using isopycnic density gradient centrifugation of banana leaf extracts. Total soluble protein extracted from the banana leaves was separated by gradient centrifugation and 25 fractions (0.5 ml each) were collected. The fractions were assayed by ELISA and HBsAg was fractionated into 4 out of 25 fractions collected (Fig. 8a). The buoyant density of banana leaf derived HBsAg was found to be in the range of 1.1536–1.1385 g/ml (Fig. 8b).

Enhanced expression of HBsAg by wounding and/or treatment with plant growth regulators

The wounded transgenic banana leaves showed the enhanced expression of HBsAg. The pHBS transformed plants 48 h after wounding of the leaves accumulated

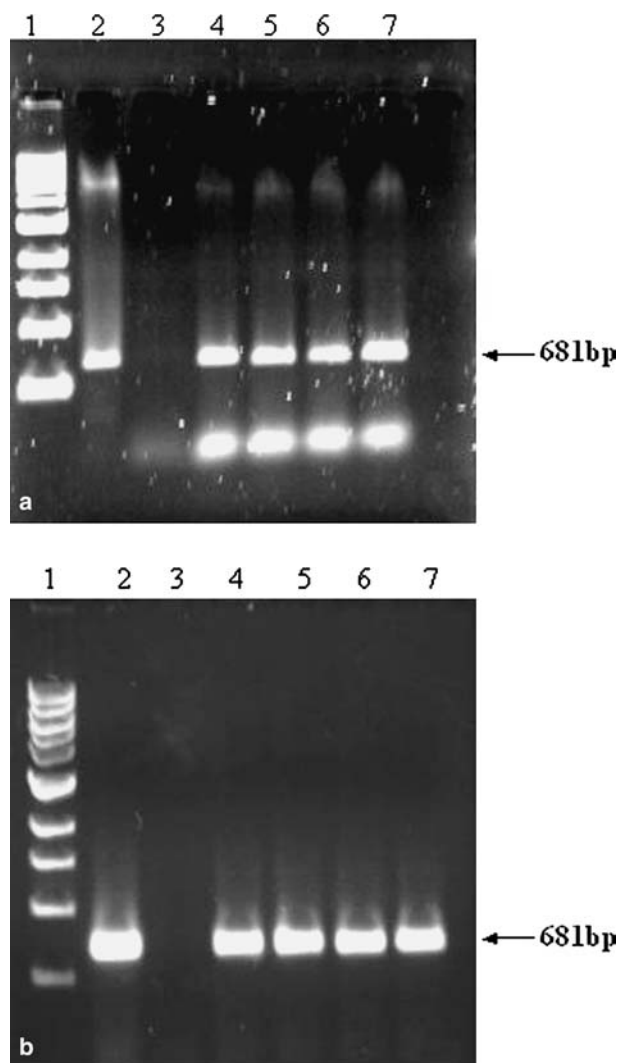


Fig. 7 RT-PCR analysis of transgenic banana plants. **a** Leaves, **b** fruits. Lanes: 1, 1 kb ladder; 2, positive control (pHBS); 3, total RNA from un-transformed plant; 4, pHBS transformed plant; 5, pHER transformed plant; 6, pEFEHBS transformed plant; 7, pEFEHER transformed plant

Table 1 Polyclonal antibody based ELISA analysis of transgenic banana plants grown in in vitro and in green house

Construct	Maximum amount of HBsAg expressed in in vitro plants (ng/g F.W. of leaves) ^a	Maximum amount of HBsAg expressed in green house plants (ng/g F.W. of leaves) ^a
pHBS	12.5 ± 0.03	12.72 ± 0.02
pHER	10.6 ± 0.01	19.92 ± 0.01
pEFEHBS	38.0 ± 0.02	9.76 ± 0.05
pEFEHER	25.0 ± 0.01	13.28 ± 0.04

^a12 transgenic lines were screened for each construct

2.5-fold higher antigen levels (Fig. 9). The antigen levels were further enhanced by treating unwounded or wounded leaves with 100 μ M IAA or ABA (Fig. 10). A maximum of 3 fold increase was noted in unwounded

Table 2 Expression levels of monoclonal antibody reactive HBsAg in transgenic banana plants in green house

Construct	Maximum amount of HBsAg expressed (ng/g F.W. of leaves) using monoclonal antibody based ELISA ^b	Percentage of monoclonal antibody reactive HBsAg ^a
pHBS	6.46 ± 0.02	50.786
pHER	13.52 ± 0.04	67.87
pEFEHBS	5.26 ± 0.02	53.89
pEFEHER	8.26 ± 0.03	62.19

^aLevels of HBsAg binding to monoclonal antibodies relative to polyclonal antibodies reactive antigen

^bThe maximum expression noted for each construct

leaves of pHBS transformed plants and 1.8 fold increase in wounded leaves of pHER transformed plants treated with IAA for 24 h. ABA treatment for 24 h enhanced the antigen levels by a maximum of 1.63 fold in the wounded leaves of pEFEHER transformed plants.

Discussion

In recent years there has been a growing interest to produce therapeutic proteins and vaccines in the transgenic plants (Twyman et al. 2003). Production of vac-

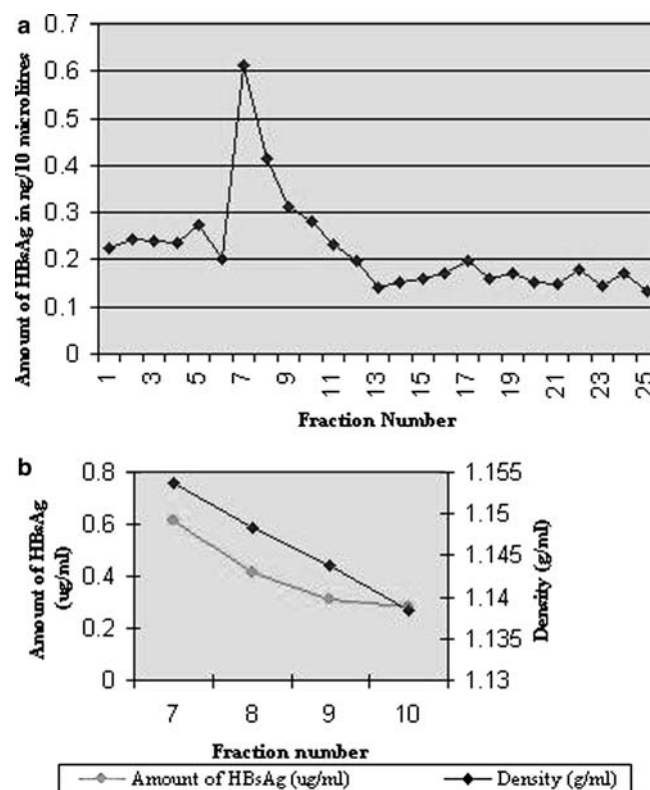


Fig. 8 Buoyant density studies of HBsAg expressed in transgenic banana plants. **a** ELISA analysis of the CsCl gradient fractions of pEFEHER transformed banana plants (Note the fractionation of HBsAg in fractions 7–10); **b** buoyant density of HBsAg from the transgenic banana plants in CsCl gradient fractions

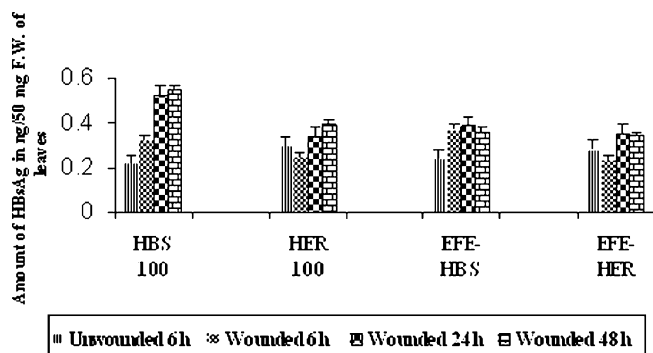


Fig. 9 Effect of wounding on HBsAg expression in the leaves of transgenic banana plants

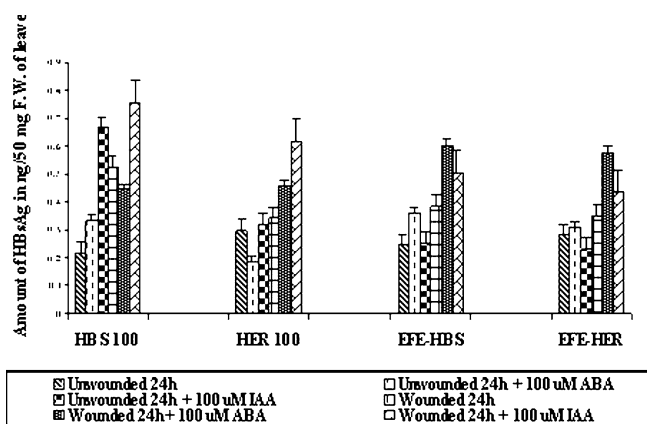


Fig. 10 Effect of IAA and ABA on HBsAg expression in the wounded or un-wounded leaves of transgenic banana plants

cines in the edible parts of the plant offers several advantages like induction of mucosal immunity, ease of delivery, patient compliance etc. (Sunil Kumar et al. 2004). Banana is an ideal crop for the production of edible vaccines, as it is grown in the tropical and sub-tropical regions of the world, where cheaper vaccines are needed. Genetic transformation of banana by *Agrobacterium* mediated transformation using apical meristem and corm slices has been reported (May et al. 1995). However, this protocol resulted in generation of chimeric plants. The use of embryogenic cells and regeneration of transgenic plants via somatic embryogenesis alleviates this problem. Ganapathi et al. (2001) described transformation of embryogenic cells via *Agrobacterium* mediated method and demonstrated the expression of GUS in the transgenic fruits. However, there is no report on the expression of vaccines in banana. This is the first report on the expression of an antigen in banana fruit.

Hepatitis B is one of the serious diseases in the world. It spreads through blood transfusion and sexual contact. Mucosal immunity is important for protection against transmission by sexual contact. Mucosal immunity is best achieved by oral rather than parenteral route. Oral delivery of vaccines, especially in the form of edible

plant tissues may induce IgA response resulting in protection against infection through mucosal route. The plant derived HBsAg induced specific antibody response in mice and human volunteers (Thanavala et al. 1995; Kapusta et al. 1999).

In this study, we report the expression of HBsAg in transgenic banana plants. Four different expression cassettes were employed to optimize the HBsAg expression levels. These cassettes included either *ubq3* gene promoter of *Arabidopsis* or *EFE* gene promoter of banana. 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 (Dickman 2004). Chakrabarti et al. (2003) reported the use of *ubq3* gene promoter to drive the expression of MSI-99, an antimicrobial peptide in transgenic banana plants and obtained enhanced disease resistance. In light of these results, *ubq3* promoter was utilized to drive HBsAg expression in banana plants. *EFE* promoter constructs gave threefold higher expression levels in the plants grown under in vitro conditions than *ubq3* gene promoter constructs. On the contrary, the green house hardened plants showed enhanced expression when *ubq3* promoter was used. The expression levels were higher in transgenic plants grown under in vitro conditions, in which *EFE* promoter drives HBsAg expression. This could be due to the production of ethylene during tissue cultures (Biddington 1992). Amino-cyclopropane 1-carboxylic acid oxidase (Ethylene forming enzyme) transcript levels increases with increase in ethylene production of the tissue (Gomez et al. 1997). In our studies the expression of HBsAg is driven by *EFE* promoter (pEFEHBS and pEFEHER), therefore higher expression levels of HBsAg were noted in the pEFEHBS and pEFEHER transformed plants grown under in vitro conditions. In contrast, it was reduced in the greenhouse plants. This result suggests that ethylene enhances the expression of the antigen from the ethylene forming enzyme promoter. HBsAg expression was higher, when *EFE* promoter was used compared to *ubq3* promoter in in vitro conditions.

The enhanced transgene expression by ethylene, wounding and/or treatment with plant growth regulators could be due to the presence of yet uncharacterized response elements in the *ubq3* or *EFE* promoters. This could be developed as a useful technology for post harvest enhanced expression of the foreign proteins, to avoid the deleterious effects of the recombinant protein on the growth and development of transgenic plants. This approach may also increase the stability and the yield of the target protein in fully active form. It also reduces the effects of environmental factors on protein yield and quality (Cramer et al. 1999).

Immunologically, important epitopes of HBsAg are dependent on disulfide bonding. It is known that monoclonal antibodies are specific to the epitopes in the highly conformational, immuno-dominant 'a' determinant region of HBsAg and will not detect

HBsAg on non-reducing or reducing Western blots. Attempts were also made earlier to increase the proportion of HBsAg displaying the monoclonal antibody reactive epitopes by altering the concentration of sodium ascorbate in the HBsAg extraction procedures from recombinant plant systems. (Smith et al. 2002). In the ER, disulfide bond formation is promoted by an oxidizing environment and is facilitated by an enzyme protein disulfide Isomerase (Lodish et al. 2000). In the present study, the incorporation of C-terminal ER retention signal enhanced the proportion of HBsAg displaying the monoclonal antibody reactive epitopes. We could obtain 67.87 monoclonal antibody reactive HBsAg in pHER transformed banana plants, which is higher than previous reports wherein a maximum of 37% was reported without the use of ER retention signal (Smith et al. 2003). The higher monoclonal antibody reactive HBsAg noted in the present study could be due to the retention of HBsAg in the ER, which facilitates the proper folding and disulfide bond formation. 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 (Schooten et al. 1996). ER targeting is essential for the glycosylation and disulfide bridge formation (Ituriaga et al. 1989).

The particulate nature of the plant-derived HBsAg was analyzed by buoyant density studies (Mason et al. 1992; Smith et al. 2003; Sunil Kumar et al. 2003). In our studies, the banana plant derived HBsAg has similar buoyant density and monoclonal antibody binding properties to that of human serum derived one. The average buoyant density of banana leaf derived HBsAg was found to be 1.146 g/ml. This is in confirmation with the previous report (Mason et al. 1992). The HBsAg derived from the human serum showed a density of about 1.2 g/ml (Valenzuela et al. 1982). Thus, the transgenic banana plant derived HBsAg exhibits density properties that are very similar to those obtained from human serum.

Although expression levels of the antigen are low in banana fruits, the expression levels of the vaccine antigens can be increased by the use of promoter of abundant pulp protein (Clendennen et al. 1998), or promoters of the proteins found in abundance in the ripe banana fruits (Peumans et al. 2002). Codon optimization and use of banana UTRs (un-translated regions) may also enhance foreign gene expression in the fruits. The results of our investigation demonstrate that an antigen like HBsAg can be expressed in the required particulate form in banana plants. However, the expression levels have to be increased for immunogenicity studies and to develop banana as an edible vaccine production system.

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