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Expression of Hepatitis B Surface Antigen in *Coleus forskohlii*

Neha Guleria*[1], Paramanahally Hanumanthe Gowda Ramanjini Gowda[1], Satish Kumar Kariyaiah[1], Renu Pasricha[2]

[1]*Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, India*
[2]*National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India*

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Abstract

Hepatitis B, a common viral infection, affecting millions of people every year, leads to disability and death. It has become one of the alarming diseases in the world. Vaccination is the best possible way to prevent this deadly viral infection and its consequences. Unaffordability of the mass vaccination program due to low health budgets especially in developing countries have demanded the economical, effective and easily available vaccine production against hepatitis B virus. The expression of subunit vaccines and recombinant proteins in plants is a convenient, safe and potentially economical platform technology. Hence, development of a plant-based vaccine could be promising. Therefore, the present investigation focused on expression and large- scale production of hepatitis B surface antigen (HBsAg) in *Coleus forskohlii* for the development of vaccine. Eight transformed *C. forskohlii* plants were generated via *Agrobacterium* -mediated transformation method. The integration of 681bp of *HBsAg* gene into the plant genome was confirmed using PCR. SDS-PAGE showed the presence of ~48kDa dimer and ~24kDa monomer form of HBsAg protein and the expression of recombinant protein was further confirmed by western blot. *C. forskohlii* expressed HBs Ag was recorded 260µg/g leaf fresh weight as measured by ELISA. Transformed plants of *HBsAg* showed the accumulation of Virus Like Particles of 22 nm size using transmission electron microscopy. This study offers a great potential for the large -scale production of hepatitis B vaccine in *C. forskohlii* which provides a strategy for contributing a means to achieve global immunization for the hepatitis B prevention and eradication.

***Corresponding Author:** Neha Guleria ✉ neha.gul5@gmail.com

Introduction

Hepatitis B virus (HBV) causes transient and chronic infection of the liver ultimately leads to untreatable liver cancer (Yogendra *et al.,* 2009). In the HBV-infected people, the virus persists for the rest of their lives and can be passed on to others (Goldstein *et al.,* 2005). HBV infection has a worldwide distribution. It is estimated that more than 2 billion people have been infected. Of these, approximately 400 million are chronically infected and at risk of serious illness and death from cirrhosis and hepatocellular carcinoma (HCC), diseases that are estimated to cause 650 000–780 000 deaths each year worldwide (WHO, 2015). Therefore, Hepatitis B has become a global public health problem.Vaccination against this virus was introduced over 25 years ago (Czyz*et al.,* 2014), a proven strategy to control HBV infection. The current subunit vaccines e.g. Shanvac B, Engerix B, Genevac-B, Biovac-B etc. produced in *Pichia pastoris* (methylotrophic yeast) which express the major surface antigen of the Hepatitis B virus, is used for vaccination. But HBV is still responsible for significant morbidity and mortality in poor countries (Kong *et al.,* 2001). The situation is deteriorating, also in developing countries due to limited budget for health care as large- scale vaccination programs are required (Smith *et al.,* 2003). Hence, there is a need for large-scale production and use of relatively cheap and effective hepatitis B vaccine.

Earlier, the majority of work was focused on the production of recombinant proteins and subunit vaccines in prokaryotes, mainly in *Escherichia coli* because it offers low cost and short production timescale (Ma *et al.,* 2003). With the time, limitations of prokaryotes came into light such as low product yield and lack of post-translational modifications. Therefore, then researchers turned to eukaryotic systems: yeast, mammalian cells, insects and transgenic animals. These systems were also proven inefficient in terms of cost, production, safety, authenticity and scalability (Balen and Rasol, 2007; Ahmad, 2014). The plants obtained in recent decades synthesize great number of valuable proteins such as human serum proteins, growth regulators, antibodies, vaccines, industrial enzymes, biopolymers, and reagents for molecular biology and biochemistry.

Plant cells possess enzymatic systems of post-translational modification, which are necessary for the assembly of synthesized monomeric proteins of vaccine into immunogenic multimers (Rukavtsova *et al.,* 2015). The target antigens causing active immune response can also be synthesized in plant cells (Mason and Arntzen, 1995). Thus, now the plants have received the most attention in molecular pharming community for the production of subunit vaccines. The expression of subunit vaccines and recombinant proteins in plants has emerged as a convenient, safe and potentially economical platforms technology (Thomas *et al.,* 2011; Xu *et al.,* 2012). Similarly, several studies have revealed that plant expression system produce many biologically active complex human proteins (Merle *et al.,* 2002; Peeters *et al.,* 2001). Therefore, with the time extensive technology advancements for glycan modification makes plants the most versatile platform for the production of recombinant protein and subunit vaccines.

Hepatitis B surface antigen (HBsAg) is a transmembrane protein consisting of S, M and L-HBsAg. All HBsAg proteins are encoded within a single reading frame and contain a common S domain. The S-HBsAg contains only the S domain and form strongly immunogenic determinant (Pniewski, 2013; Rybicki, 2014). The hydrophobic S-HBsAg carries all the necessary information for membrane translocation, the component of virus envelope and assembled into immunogenic structures known as Virus Like Particles (VLPs). These particles are self- assembled protein structures, devoid of viral DNA, unable to replicate and non-infectious (Hyun *et al.,* 2014). It has been known that the lipid molecules closely associated with S-HBsAg are responsible for the antigenicity, protein conformation and stability of the VLPs (Jadwiga *et al.,* 2014). VLPs possess repetitive high-density displays of viral surface proteins and are potentially effective in eliciting strong humoral and cellular immune responses. Hence, these characteristics provide a great platform for VLPs as an effective vaccine candidate (Natasha *et al.,* 2012).

In 1986, Federal Drug Administration (USA) approved the first recombinant protein-based Hepatitis B vaccine for humans. It is based on a recombinant HBV surface antigen (HBsAg), which upon production in yeast or mammalian cells forms 22-nm spherical VLPs (Greiner *et al.,* 2012). Similarly the structure authenticity and function of plant produced VLPs is first explained by Mason *et al.,* in 1992 who studied expression and production of HBsAg VLPs in tobacco. The same group studied the immunogenic response of VLPs produced in transgenic potato on mice. The study emphasized the importance and effectiveness of VLP vaccine. Therefore, it would be safe to say that VLPs can represent one of the most exciting new technologies to rapidly produce effective vaccines with long lasting protection (Rybicki, 2014).

The present report is the first study on the expression of HBsAg in *C. forskohlii* for the development of injection HBsAg vaccine. *C. forskohlii* is animportant medicinal herb in Indian Ayurvedic medicine considered endangered medicinal plant, the *C. forskohlii*, a member of the *Lamiaceae* family is valued for the production of labdane diterpenoid forskolin (produced only in roots) from its tuberous roots used for relief of cough, eczema, skin infections, heart failures and certain type of cancers (Boby and Bagyaraj, 2003).

The novelty and priority of the research is that *C. forskohlii* is a potential candidate to produce vaccine, as this crop is vegetatively propagated with high biomass production and can be grown in the field without any chances of genetic contamination (does not set seeds), so it offers good platform to produce novel compounds. It means, large quantities of recombinant protein can be produced quite rapidly, thereby significantly reducing the cost of production. When we have a plant which does not carry threat of gene pollution then biosafety laws of any given country can accommodate a programme to grow recombinant protein producing plant (Lou *et al.,* 2007).

Material and methods

Strains and Plasmids

Plant binary vector pHB118 contained *HBsAg* gene under the control of CaMV 35S promoter was kindly supplied by Dr. Hugh S. Mason, Arizona Biodesign Institute USA and further maintained in *Agrobacterium tumefaciens* strain LBA4404 in our laboratory. The construct contains *npt-II* as selection marker gene (Fig. 1.).

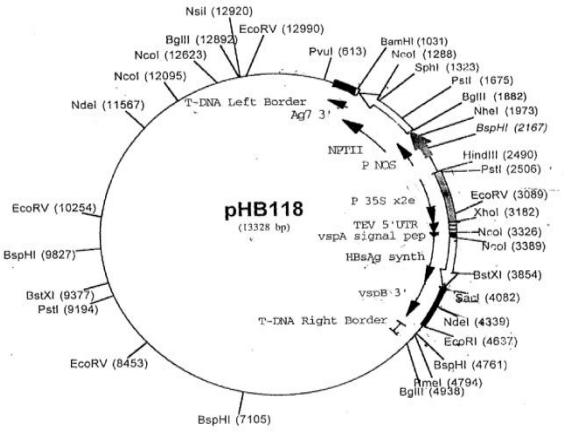


Fig. 1. Structure of plasmid pHB118. The *HBsAg* coding region lies downstream of CaMV 35S promoter and is followed by the nopaline synthase (NOS) terminator.

Plant Regeneration and Transformation studies

As a pre-requisite for plant transformation studies, a regeneration protocol of *C. forskohlii* was standardized. Different combinations of Benzyl Amino Purine (BAP) and Naphthalene Acetic Acid (NAA) were used along with MS salts (Sigma-Aldrich, USA). The leaf explants were kept under running tap water to remove adhering dust particles and surface sterilized with 0.8% (w/v) bavistin and 0.1% (w/v) mercuric chloride for 1-2 minutes, followed by 3-4 washing with sterile distilled water, under aseptic conditions. After four rinses in sterile distilled water, leaves were trimmed into pieces of about 1 $cm^2$and then inoculated onto culture media prepared using Murashige and Skoog,1962 salts, added with MS micronutrients, vitamins, 3% (w/v) sucrose 0.8% agar and supplemented with different concentration of BAP (0.2 & 0.3 mg /L) along with NAA (0.4,0.5, 0.6, 0.7 & 0.8 mg/L). The pH of the media was adjusted to 5.8 with 0.1 N NaOH and autoclaved at 121°C and 16 pounds/square inch for 15 min.

The cultures were maintained at 25 ± 2°C in the growth chamber under a 16 h photoperiod using white fluorescent tubes. All cultures were kept for 4 weeks without subculture to fresh medium. Regenerated shoots were sub-cultured in every 15 days of interval and rhizogenesis was obtained on same media.

The pHB118 plant binary vector carrying *HBsAg* gene was mobilised into *Agrobacterium tumefaciens* LBA4404 strain which was then further used to transform *C. forskohlii* using leaf discs. The *Agrobacterium* culture was grown in yeast extract peptone (YEP) broth media at 28°C and 200 rpm speed for overnight. Further, the grown culture was centrifuged at 6000 rpm for 5 minutes at room temperature and pellet was suspended in half strength MS broth. The OD_{600} of *Agrobacterium* suspension maintained was 0.75 and then used for transformation experiment. The co-cultivation was done for two days and then explants were washed with cefatoxime 250 mg/L dissolved in autoclaved distilled water to remove the *Agrobacterium* overg-rowth. The transformed shoots were selected on standardized regeneration MS medium containing kanamycin 50mg/L and cefatoxime250mg/L. Shoots were rooted on the same medium and transplanted into the cocopeat: sand (1:1) pots and well maintained in the greenhouse.

Genomic DNA isolation and PCR analysis

Plant genomic DNA was isolated by Cetyl Trimethyl Ammonium Bromide (CTAB) method from young leaves of transformed plants and also from the non-transformed plant as a control (Doyle and Doyle, 1987). The integration of the *HBsAg* gene was confirmed by PCR using a pair of gene specific primers 5' GCG AATT CATGGA GAG CAC AAC ATC AGGA TTC3' forward and 5'TGAAGCTTT CAAATGTA TACCCAAAGACAAAAG3' reverse. The genomic DNA (100 ng) from each transformed plant was used as a template DNA. The amplification was done using PCR machine model Rcorbett research CG1-96.

The PCR programme was set at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min, with a final extension at 72°C for 10 min. The PCR products were analysed by electrophoresis on a 1.0% agarose gel.

SDS-PAGE and Western blot

Total protein from both transformed and non-transformed *C. forskohlii* plants were isolated using a protein extraction buffer (pH 7.8, 2M K_2HPO_4, 0.5M EDTA, Triton X-100, 80% Glycerol, 1.0M DTT). The total protein was isolated from 1 g of young leaves with 2 ml of 1X protein extraction buffer. The samples were centrifuged at 10,000 rpm for 10 min at 4°C. The crude protein collected was partially purified by using 10kDacentricon column (Pall Corporation). The crude sample (2ml) was pipetted out into sample reservoir of centricon and centrifuged at 10,000 rpm for 30 min at 4°C. The desired protein sample was collected into a sterile Eppendorf (2ml) from the sample reservoir.

Protein expressed by transformed plants carrying *HBsAg* gene was detected by 12% Sodium Dodecyl Sulfate – Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using discontinuous buffer system followed by Western immunoblot as per standard procedures (Sambrook *et al.,* 1989).

The protein ladder (Pure-gene, Genetix) was used as marker. The membrane was probed consecutively with a primary antibody (mouse anti-hepatitis B surface antigen, Invitrogen, 1:1000 dilution) and secondary antibody (goat anti-mouse IgG horseradish peroxidase conjugate, Invi-trogen, 1: 20,000 dilution). The protein produced by non-transgenic plant was used as a negative control and commercial vaccine (Shanvac B, manufactured by Shantha Biotechins, India) as a positive control.

ELISA

The protein samples containing expressed HBsAg were detected by ELISA and quantified with standard curve established by serial dilutions of bovine serum albumin (BSA) standards.

The partially purified protein samples of transgenic *C. forskohlii* were used as an antigen source for ELISA. They were incubated in blocking solution for 1 h at 37°C, followed by incubation with the primary(mouse anti-hepatitis B surface antigen, Invitrogen, 1:1000 dilution) and secondary antibody (goat anti-mouse IgG horseradish peroxidase conjugate, Invitrogen, 1: 20,000 dilution) for 1 h each at 37 °C. The washing steps were performed after every incubation with 1X PBST (phosphate buffer saline tween -20) buffer for ten minutes. Detection involved 0.4 mg/mL of chrom-ogen tetra methyl benzidine liquid substrate system (Sigma-Aldrich, USA).

The blue coloration was deve-loped in all the *HBsAg* transformed plant samples. The absorbance was recorded at 450 nm. The nega-tive control (protein extracted from non-transformed plants) and positive control (commercial vaccine) were used.

Transmission Electron Microscopy

Young leaves of transformed and non- transformed plants were excised, cut into 1-mm squares, with razer blade, and fixed in 2% paraformaldehyde + 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH6.8, for 0.5h at 23°C followed by 1 h at 4°C. The tissue was post-fixed in 2% osmium tetraoxide at 23°C for 1h, washed and dehydrated in a graduated ethanol series at 4 °C, 1:1 ethanol and 100% ethanol.

The tissue was infiltrated with spurr's resin at 23°C and cured at 60°C overnight. Sections of about 60nm in thickness were obtained with an ultra RMC microtome.

The sections were viewed on a FeiTecnai T_{12} Biotwin (Philips, Eindhoven, The Netherland) at 100KV.

Statistical Analysis

Each treatment of *C. forskohlii* regeneration experiment had 5 replicates with 15 explants and all the experiments repeated twice to see the reproducibility of the results. The data were reported as mean standard error and means were analysed by Analysis of variance (one way ANOVA) and significant differences between means were compared by least significant difference (LSD).

Results and discussion

Regeneration and transformation of HBsAg gene in C. forskohlii

Plant regeneration protocol of *C. forskohlii* was standardized using leaf explant. The optimized media for regeneration was found to be MS supplemented with 2.00 mg/L BAP along with 0.5 mg/L NAA (Table1).

The regenerated shoots were found to be healthy with well-developed root system (Fig. 2A., B.& C.). In the development of any regeneration protocol, growth hormone combination and choice of explant play a major role.

In the present study, combination of BAP (2.0 mg/L) and NAA (0.5 mg/L) proved to be the best. These results are in consistent with the data obtained earlier in our laboratory by Guleria and Gowda, 2015; Khin *et al.,* 2006 reported the combination involving high concentration of cytokinin and low concentration of auxin promote adventitious shoot formation in *C. forskohlii* leaf explant.

Table 1. Effect of BAP and NAA on shoot regeneration from leaf explants of *Coleus forskohlii.*

MS media with(mg/L)		Frequency of callus regenerated into shoots *(%)	No. of shoots per explant *	Average No. of days taken for shoot initiation	Shooting observation	Average No. of days taken for rooting	Root number/shoot*	Root Characters
BAP	NAA							
2.0	0.4	60.4^{c}	14.8^{b}	35	Short, healthy	20	5.2^{b}	Thin, longer
2.0	0.5	93.4^{a}	35^{a}	30	Long, Healthy	14	8.8^{a}	Thick, longer, healthy
2.0	0.6	57.8^{d}	10.2^{c}	42	Long, vitrified, weak	22	4.4^{c}	Thin, shorter
2.0	0.7	77.2^{b}	14.6^{c}	45	Short, not healthy	25	4.0^{c}	Thin, shorter
2.0	0.8	53^{e}	10^{c}	49	Short, vitrified	29	1.8^{d}	Long, thin

15 explants cultured per treatment.

*Different letters within the column indicate significant differences according to LSD (P≤0.05) following ANOVA.

Fig. 2. Standardization of regeneration of *C. forskohlii* from leaf explants (BAP 2.0mg/L+NAA 0.5mg/L). (A) Adventitious shoots (B) Well developed rooting system (C) Hardened transformed plant (D) Transformed *C. forskohlii* plant in greenhouse.

In transformation studies, 500 leaf explants were co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 containing pHB118 plasmid and eight transformed plants of *C. forskohlii* were generated (Fig. 2D.). Genetic transformation in plants mediated via *Agrobacterium tumefaciens* has been known as most common and reliable method (De la Riva *et al.,* 1998). It has the advantage for allowing stable integration of defined gene into the plant genome that generally results in single copy number, fewer rearrangements and more stability expression over generations than other transfection methods (Hu *et al.,* 2003). Therefore, micropropagation combined with *Agrobacterium* transformation, provides a method for routine genetic transformation of many important medicinal plant species (Khan *et al.,* 2009).

Polymerase Chain Reaction (PCR) Analysis

The integration of *HBsAg* gene into plant genome was confirmed by using PCR amplification. Presence of expected amplicon size of 681bp in all transformed samples, confirmed the integration of *HBsAg* gene whereas there was no amplification in non-transformed samples (Fig. 3.). The plasmid pHB118 which contained *HBsAg* gene showed amplicon size of 681bp used as positive control. When a gene is transformed into plant cells/organisms, PCR is a quick and simple technique to detect the presence of the gene in the transformed plant.

The present results are in line with the study where expression of HBsAg studied in tobacco Yogendra *et al.* (2009, 2013) and in banana (Elkholy *et al.* 2009). This confirms the stable integration of gene of interest and successful transformation. However tobacco as a model crop could not be used for commercial production. Banana had lot of problems like less accumulation of protein, long duration of the crop, difficult to regenerate. Hence *C. forskohlii* has better advantage over other crops.

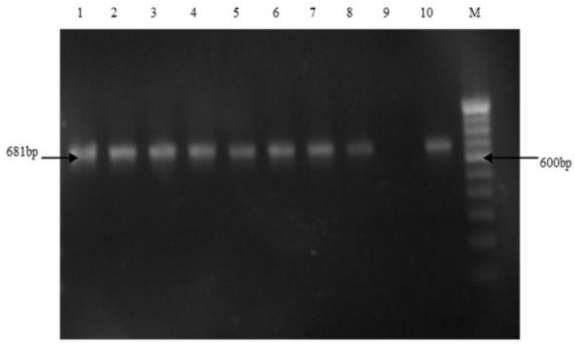


Fig. 3. PCR analysis of *HBsAg* gene into the transformed *C. forskohlii* using gene specific primers. Lane M: Marker (100bp); lane 1-8 transformed plant samples with amplicon size of 681bp; lane 9: non-transformed plant sample (negative control); lane 10: plasmid pHB118 (positive control).

Expression of HBsAg in C. forskohlii leaves

Total protein was isolated from young leaves of transformed *C. forskohlii* plant and partially purified using 10kDa centricon. Further, the protein was estimated by ELISA using standard curve. The protein concentration was found to be 260µg/g of fresh leaf weight, whereas Kapusta *et al.,* 1999 recorded 5.5ng/g of fresh weight in lettuce leaf, Li *et al.,* 2011 found HBsAg protein 100.36 ng/g of fresh weight in leaves and 127.54 ng/g of fresh weight in fruits of cherry tomato, Gao *et al.,* in 2003 reported HBsAg expression level at 300ng/g fresh weight of leaves and 10ng/g fresh weight of fruit in cherry tomato,0.01% total soluble HBsAg protein in tobacco leaf was recorded by Mason *et al.,*1992 and Ehsani *et al.,*1997 observed 0.008% total soluble HBsAg protein in potato leaf. Rukavtsova *et al.,* 2015 has recorded HBsAg protein in tubers of various potato lines upto 1 µg/g of wet weight.

In our study rHBsAg protein expressed at higher level than other reports. The possible explanation for this higher expression level could be the use of different plant host system. As *C. forskohlii* is vegetatively propagated (does not set seeds) and produce large biomass 2.5 tons per hectare, the expressed protein can be isolated at large scale. Therefore, the recombinant protein expressed in the transformed *C. forskohlii* plants could be sufficient enough to make a vaccine economical. The ELISA analysis also suggests that *C. forskohlii* expressed rHBsAg protein has an excellent immune-oreactivity. The non-transformed *C. forskohlii* did not express any recombinant protein that react with specific antibody of HBsAg.

The partially purified HBsAg protein samples were subjected to 12% SDS-PAGE analysis. The results showed the expression of protein of interest of ~48 kDa size which is dimer form of HBsAg protein. Monomer form ~24kDa was also reported (Fig. 4A.). Further, westernblot was performed to confirm the integrity of the antigen/protein expressed in transformed plants under denaturing conditions. *P. pastoris* produced commercial vaccine was used as positive control. *C. forskohlii* produced HBsAg protein showed a band size of ~48 kDa (dimer) and ~24 kDa (monomer) but not in non-transformed plants (Fig. 4B.). A typical western blot relies upon a protein samples containing target protein that can be detected by antibodies. Therefore, antibodies specific to HBsAg protein made a complex via specific binding of antibodies to proteins immobilized on a membrane and expressed a dimeric form of ~48kDa suggesting the formation of disulphide bond and strong immunogenicity.

The results are in line with previous studies on plant derived HBsAg showed that the HBsAg expressed in plant system accumulated as dimeric form with strong immunogenic behavior (Sojikul *et al.,* 2003; Zhong and Mason, 2004; Zhong *et al.,* 2008). Mishiro *et al.,* 1980 and Wampler *et al.,*1985 also showed that the dimeric form of HBsAg retain all antigenic determinants and full immunogenicity in comparison to monomeric form of HBsAg.

Therefore, correct folding of plant expressed protein is necessary for full biologically active protein.

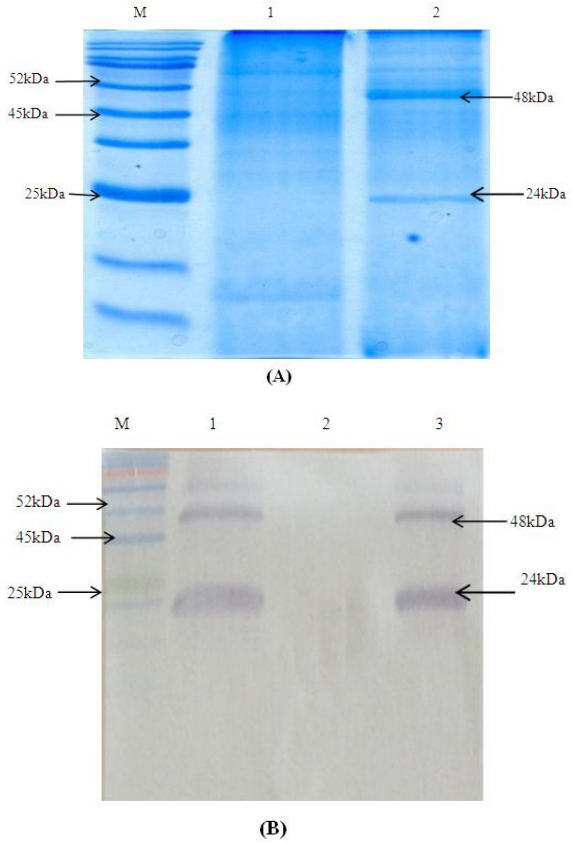
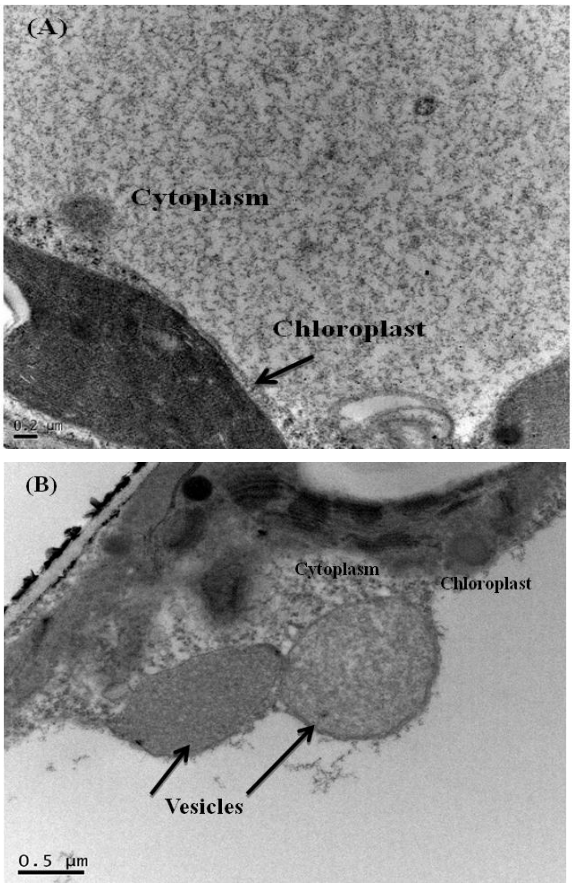


Fig. 4. SDS-PAGE and western immunoblot analysis of recombinant HBsAg protein expressed in leaf of *C. forskohlii.* (A) Lane M: Protein ladder; lane 1: protein sample of non-transformed plant (control); lane 2: protein samples of transformed plants expressing both dimeric (~ 48 kDa) and monomeric (~24 kDa) form of HBsAg. (B) Western immunoblot analysis. Lane M: Protein ladder; lane 1: *P. pastoris* derived commercial vaccine (positive control); lane 2: protein sample of non-transformed plant (negative control). lane 3: protein samples of transformed plants confirmed both dimeric (~ 48 kDa) and monomeric (~24 kDa) forms of HBsAg.

rHBsAg assembled into VLPs

The rHBsAg viral proteins have the ability to assemble into highly immunogenic virion-like structures without genetic material (Zhong *et al.,* 2005). Such particles are known as virus like particles. It had been reported that HBsAg gets assembled into virus like particle with diameter of 22 nm and stabilized by multiple disulphide bonds (Jadwiga *et al.,* 2014).

The cells of *HBsAg* transformed and non-transformed *C. forskohlii* plants harboured unusual membrane bound vesicles in the cytoplasm (Fig. 5B.). These vesicles contained circular structures ranging in diameter 13 to 40 nm. Max-imum number of particles were in range of 15 and 29 nm diameter. Therefore, the average diameter was found to be 22 nm in size indicated by arrows (Fig. 5C.). Microscopic examination of non-transformed cells did not show any vesicles containing circular structures (Fig. 5A.). VLPs of different diameters ranging from 13 to 40 nm and average of 22nm were observed in the transformed *C. forskohlii* plant cells which were evident in case of HBsAg expression in tobacco (Mason *et al.*,1992). The formation of HBsAg VLPs has also been observed in potato (Richter *et al.,* 2000) and in soyabean (Smith *et al.,* 2002). Thus, the present work showed the correct folding of *C. forskohlii* expressed HBsAg. Thus, plant derived rHBsAg antigen appears to possess all the necessary antigenic properties which are considered as major aspects of any effective vaccine.



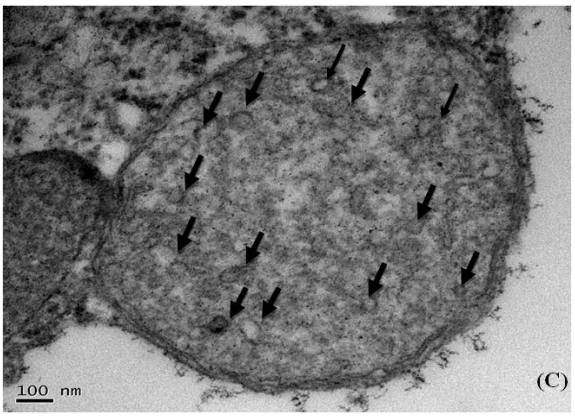


Fig. 5.TEM of Non-transformed & HBsAg expressing *C. forskohlii:* (A) Thin section of non-transformed *C. forskohlii* cells (Bar=0.5µm) (B) Thin section of transformed *C. forskohlii* cells (Bar=0.5µm. (C) Enlarged image of membrane bound vesicles, 22nm VLPs indicated by arrows. (Bar=100nm).

The number of VLP vaccines are in process and available in the market (Dwarakaprasad and Rao, 2013). When a person is injected with the VLPs, the immune system will mimic VLPs as authentic virus and ultimately give strong immune response for the production of antibodies, which are recognized readily by the immune system and presents viral antigen in a more authentic conformation.

Furthermore, the compact highly ordered structures of VLPs may provide resistance to degradative enzymes in the gut; the particulate nature of VLPs allows them to be efficiently sampled by the 'M' cells of gut epithelium that transport antigens across the mucosal barrier (Santi *et al.,* 2006).

The potency of VLPs can elicit a protective response at lower doses of antigen, which significantly reduces vaccine cost (Roldao *et al.,* 2010). VLPs have therefore shown dramatic effectiveness as a vaccine candidate.

In conclusion, HBsAg protein expressed at higher level (260µg/g) in *C. forskohlii* leading it to potential candidate for the production of vaccines.

The plant derived HBsAg assembled into VLPs of 22nm size. These results demonstrated that the rHBsAg VLPs expressed in *C. forskohlii* could provide potential, promising, economic and large scale materials for the development of diagnostic kit and vaccine to prevent the HBV infection.

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