STUDIES ON MOLECULAR CHARACTERIZATION OF THE HEPATITIS B RECOMBINANT VACCINE PRODUCED IN PLANTS

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AFFECTIONATELY
DEDICATED TO
MY BELOVED PARENTS AND
MY BROTHER

DEPARTMENT OF BIOTECHNOLOGY
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CERTIFICATE

This is to certify that the thesis entitled "**STUDIES ON MOLECULAR CHARACTERIZATION OF THE HEPATITIS B RECOMBINANT VACCINE PRODUCED IN PLANTS"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (Agri.)** in **PLANT BIOTECHNOLOGY** of the University of Agricultural Sciences, GKVK, Bangalore, is a bonafide record of research work done by **Mr. YOGENDRA, K.N. PAK 6221** during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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Thesis Abstract

Molecular characterization of Hepatitis B recombinant vaccine produced in plants

By

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Immunization with Hepatitis B vaccine is the most effective means of preventing Hepatitis B virus infection and its consequences. As the available recombinant vaccine is expensive, cheaper and improved vaccines are urgently needed to address the global scourge of infectious diseases. Plants are a potential source of *HBsAg* that is not dependent upon process technology to ensure protein folding and particle assembly. In addition, a plant based *HBsAg* expression system makes possible the testing of an oral immunization strategy by simply feeding the plant samples. The primary means of transformation is the *Agrobacterium* mediated gene transfer which has provided a reliable means of creating transformants in a wide variety of species and also can express a wide variety of pharmaceutically important products including recombinant vaccines. The present investigation lays emphasis on study of integration and stability of the recombinant protein expressed in tobacco plants. Restriction digestion analysis of the gene construct pHB118 with restriction enzymes *Eco*RI and *Bam*HI yielded two separate bands of 9.7kb and 3.6kb size. The leaf explants of tobacco were transformed with Hepatitis B surface antigen gene along with *npt-II* as an antibiotic selection marker gene. The presences of *HBsAg* gene in putative transformants were confirmed by PCR analysis. All the putatively transformed

tobacco plants showed the presence of 900 bp band in the PCR analysis. The crude protein obtained from the transformed tobacco plants were tested by SDS-PAGE for presence of 24 kDa protein, western-blot and ELISA confirmed the antigen specificity and immunogenic nature of the Hepatitis B surface antigen. The T_1 generation seeds obtained from the transgenic tobacco plants were tested for the germination in presence of kanamycin. It was observed that the segregation ratio was 3:1 indicating Mendelian inheritance. The growth parameters of T_0 and T_1 generation transgenic and control tobacco plants showed no major variation in growth between transgenic and control plants.

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INTRODUCTION

I INTRODUCTION

Hepatitis B virus infection occurs throughout the world and is endemic in Africa, Eastern Europe, the Middle East, Central Asia, China, Southeast Asia, the Pacific Islands, and the Amazon basin of South America. In these areas, most persons become infected as infants or young children, and up to 70 per cent of the adult population tests positive for prior infection. Among those populations, 8 to 15 per cent have chronic Hepatitis B virus infection. Worldwide Hepatitis B virus causes more than 1 million deaths per year and about 350-400 million people are persistently infected with this agent. India has been placed into the intermediate zone of prevalence of Hepatitis B (2-7 per cent prevalence rates) by the WHO (Qamer, *et al*., 2004). In India, there are 43 million people who are estimated to be *HBsAg* carriers with a carrier rate of 3 per cent, which contribute nearly 10 per cent of the Hepatitis B virus carriers in the world (Chowdhury, *et al*., 2005).

Hepatitis B virus is a 42-nm coated DNA virus with a circular, partially double-stranded 3.2 kb genome that preferentially infect hepatocytes and are referred to as hepadnaviridae. Hepatotropic DNA viruses are infectious for humans and a few animal species (duck, squirrel and woodchuck). Each complete virion consists of an inner core (nucleocapsid or hepatitis core antigen, HBcAg) surrounded by an outer protein coat or envelope (the hepatitis B surface antigen, *HBsAg*). There are four overlapping open reading frames (ORF), which encode for the envelope, precore/core, polymerase, and X proteins. The envelope ORF encodes for the large, middle and small surface glycoproteins of *HBsAg* (Roberts, 2004). The precore/core ORF is translated into a precore polypeptide, which is secreted as Hepatitis B ‘e’ antigen, detectable in the blood as HBeAg and HBcAg; detected only in the liver. Upon entering the hepatocytes, the Hepatitis B virus genome is transported to the nucleus and is converted into closed circuit circular DNA. The Hepatitis B virus closed circuit circular DNA serves as a template for transcription of mRNA and the RNA pregenome. Once transferred to the

cytoplasm, Hepatitis B virus polymerase uses reverse transcription to convert the RNA genome into new circular genomic DNA. Replicative Hepatitis B virus, either during the acute or chronic stages of infection, causes liver disease because of vigorous cytotoxic T lymphocyte (CTL) and/or cytokine-mediated elimination of HBV-infected hepatocytes (Hollinger and Liang, 2001).

Seven different genotypes (A-G) of Hepatitis B virus have been recognized worldwide. Of these, A is pandemic, B and C are seen predominantly in Asia, D in Southern Europe, E in Africa, F and G in USA, and G in France(Sanchez-Tapias *et al.,* 2002). As of now, 6 kinds (A, B, C, D, E and F) of hepatitis viruses are identified to cause liver disease. There are two modes of transmission, blood-borne and fecal-oral routes. Hepatitis A, E and F viruses are mainly transmitted through fecal matter or orally whereas hepatitis B, C, D and G are transmitted mainly through blood transfusion. Vaccines for hepatitis A and B are available, but not for hepatitis C, D, E and G (Jau-Shin, 2004).

Hepatitis B virus infection results from percutaneous or mucosal exposure to the blood or bodily fluids of infected persons. Infected persons need not be symptomatic to transmit the virus. Blood contains the highest concentration of virus. Common sources of exposure include sexual contact, contaminated needles, contaminated blood or blood products, and perinatal exposure to an infected mother. In as many as one third of cases of acute Hepatitis B virus infection, the source of exposure is unknown. Nonsexual transmission has been documented in cases of long-term exposure in household settings. Although the mechanism has not been elucidated, it appears that horizontal transmission of Hepatitis B virus can occur among infants, children, and elementary school students who live with an infected household member. Hepatitis B virus is not transmitted by the fecal–oral route. Young adults (18 to 39 years of age) are at increased risk for Hepatitis B virus infection (Gregory *et al*., 2004).

Hepatitis B virus vaccine has been recommended as a routine infant vaccination worldwide since 1991 and as a routine adolescent vaccination since

1995. Although there are no federal laws requiring the vaccine for day-care and school attendance, the World Health Organization (WHO) recommends that all countries with routine Hepatitis B vaccination programs continue that practice, while countries not currently immunizing against Hepatitis B virus infection adopt such programs immediately. The vaccine is delivered in a series of three intramuscular injections over a six-month period. It requires refrigeration, and injections must be administered by a medical professional, with the total cost ranging between $100 and $150 per person. These factors, coupled with transportation and distribution issues, make mass immunization, especially in Third World countries difficult (Sharma *et al*., 1999).

Although effective immunization has been achieved by intra-muscular injection of serum derived *HBsAg* or yeast derived r*HBsAg*, there is still a need for a less expensive vaccine source in most developing countries. Most of the drugs used by man, until very recently were being derived from plants (Sharma *et al*., 1999). Plants are now gaining widespread acceptance as a general platform for the large-scale production of recombinant proteins. Over the past 10 years, several efficient plant-based expression systems have emerged. However, a number of issues remain to be addressed before plant as bioreactors can be accepted and adopted widely in preference to the established microbial and mammalian platforms. We are facing a growing demand for protein diagnostics and therapeutics, but lack the capacity to meet those demands using established facilities. A shift to plant based bioreactors may, therefore, become necessary within a next few years, making it more imperative that the technical and regulatory limitations are addressed and solved. The production of pharmaceutical proteins in plants will only realize its huge potential if the products are provided at consistent high quality levels, allowing the delivery of clinical grade proteins that will gain regulatory approval and which can be used routinely in clinical trials (Schillberg *et al.,* 2005).

The key dement to plant vaccines is transgenic plants, which can be produced through various methods. The gene coding for the vaccine candidate has to be cloned along with a specific promoter and selectable marker. The former is to make the gene express itself and the latter to identify the presence of the gene in the transgenic plant. The construct resulting from this combination can be transformed into plant cells through various methods such as biolistic method, electroporation, PEG mediated transfer or by Agrobacterium mediated transformation. The Agrobacterium mediated transformation is perhaps the best as it results in highly stable integration of target gene without compromising the transformation efficiency (Meins *et al*., 1984).

Tobacco plants transformed with the hepatitis B surface antigen gene was used to study the stability and inheritance patterns of the foreign gene in plant systems. The present investigation was conducted with the following objectives:

1. To study the integration of Hepatitis B gene with molecular techniques.
2. To study the expression of recombinant Hepatitis B protein produced in plants.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Infectious diseases are a major cause of mortality both in humans and animals especially in the developing countries. Most of these infectious diseases are preventable by suitable vaccine schedules. Hence development of suitable and potent vaccines is a major aim of modern scientific research. This chapter is intended to present a brief review of the earlier work done on hepatitis B virus and its effects, Hepatitis B vaccines, edible plant vaccine production, production of antibodies in plant system, *Agrobacterium* mediated transformation, regeneration and evaluation studies in tobacco, including the use of reporter genes, immunization with hepatitis B vaccines, western blotting, ELISA as well as other areas of research related to the present investigation.

2.1 Vaccine production in plants:

Universal childhood vaccination would have profound effects on leveling the health inequities in many parts of the world. But each year, 33 million children remain unvaccinated for vaccine-preventable diseases for various reasons including cost and difficulty in vaccination by injections. As an alternative to administration of vaccines by needle and syringe, oral vaccines offer significant logistical advantages, as the polio eradication campaign has demonstrated. Over the past decade, the expression of subunit vaccine antigens in plants has emerged as a convenient, safe and potentially economical platform technology, with the potential to provide a novel biotechnological solution to vaccine production and delivery (Thanavala et al., 2005).

This effort was stimulated by the interest in evaluating the capacity of plants to produce different classes of proteins of pharmaceutical value and the practical need for new technology in the production and delivery of inexpensive vaccines. With these targets in mind Mason and Arntzen first hypothesized the idea of plant vaccines in 1995(Mason and Arntzen, 1995).

Nagesha *et al* (2006) reported *Agrobacterium*-mediated transformation of *Cucumis melo* with a rabies virus (strain ERA) glycoprotein gene (*PRGSpRgp*). Partially purified, recombinant rabies glycoprotein from transformed cantaloupe melon fruit was injected intramuscularly and intraperitoneally into mice, and resulted in the induction of anti-PRGSpRgp antibodies. When these mice were challenged with rabies virus strain ERA they survived infection due to the accumulation of sufficient quantities of anti-rabies glycoprotein antibodies (0.6 - 1.6 IU mL^{-1}). These results demonstrated the accumulation of rabies glycoprotein in edible transgenic cantaloupe melon fruit, which offers the promise of biomedical exploitation.(Gowda *et al*., 2000)

Maria *et al.* (2001) have expressed VP60 structural protein of rabbit hemorrhagic disease virus (RHDV) successfully by making use of the PPV-NK vector. Inoculation of extracts from VP60 expressing plants induced a remarkable immune response against RHDV in rabbits, its natural host. Moreover these animals were protected against a lethal challenge with RHDV. Similarly Modelska *et al.* (1998) immunized mice with two rabies epitopes that were expressed in plant. They showed that, mice immunized intra-peritoneally or orally with engineered plant expressing rabies antigens amounted a local and systemic immune response. After the third dose of antigen, given intra-peritoneally, 40%of the mice were protected against challenge infection with a lethal dosage of rabies virus. Oral administration of antigen stimulated serum Ig G and Ig A synthesis and ameliorated the clinical signs caused by the intranasal infection with an attenuated rabies virus strain.

Tobacco plants were genetically transformed with the gene encoding hepatitis B surface antigen (*HBsAg*) linked to a nominally constitutive promoter. Enzyme-linked immunoassays using a monoclonal antibody directed against human serum-derived *HBsAg* revealed the presence of *HBsAg* in extracts of transformed leaves at levels that correlated with mRNA abundance. This suggests

that there were no major inherent limitations of transcription or translation of this foreign gene in plants. Recombinant *HBsAg* was purified from transgenic plants by immunoaffinity chromatography and examined by electron microscopy. Spherical particles with an average diameter of 22 nm were observed in negatively stained preparations. Sedimentation of transgenic plant extracts in sucrose and cesium chloride density gradients showed that the recombinant *HBsAg* and human serum-derived *HBsAg* had similar physical properties. Because the *HBsAg* produced in transgenic plants is antigenically and physically similar to the *HBsAg* particles derived from human serum and recombinant yeast, which are used as vaccines, it was conclude that transgenic plants hold promise as low-cost vaccine production systems (Mason *et al.,* 1992).

Tariq *et al.* (1995) have expressed the binding subunit of *Escherischia coli* heat labile enterotoxin (LT-B) in potato and tobacco, which was highly active as oral immunogen. The transgenic tobacco and potato plants were made with the use of genes encoding LT-B or an LT-B fusion protein with a microsomal retention sequence. The plants expressed the foreign peptides, both of which formed oligomers that would bind the natural ligand. Mice immunized by gavage produced serum and gut mucosal anti – LTB immunoglobulins neutralized the enterotoxin in cell protection assays. Feeding of mice with fresh transgenic potato tubers also caused oral immunization.

Craig *et al.* (1994) administered rabies ribonucleocapsid (RNP) orally as well as by parenteral routes and found it to prime specific T- cells and elicit nucleoprotein specific antibodies. Parenteral, oral and intramuscular immunization led to the production of antibodies of Ig A and Ig G isotypes, respectively. Mice, primed orally with RNP produced significantly enhanced the amounts of virus neutralizing antibody compared to non-immune controls upon subsequent parenteral booster immunization with inactivated rabies virus. Thus, oral immunization with rabies RNP primed the cells capable of mediating a secondary

systemic response to rabies virus. The results of experiments in which peptide and protein antigens were administered either physically coupled to or mixed with RNPO indicate that RNP has an inherent capacity to enhance the immune response.

Two animal diseases, rinderpest and peste des petits ruminants (PPR) often known as “cattle plague” and “goat plague”, respectively because of the fatal and contagious nature of the disease are caused by negative stranded RNA viruses known as rinder pest and peste des petits ruminants virus. These enveloped viruses harbor 2 surface glycoproteins haemagglutinin (H), haemagglutinin neuraminidase (HN) and fusion (F) protein which are highly immunogenic and protective. With the aim of developing edible vaccines for Rinderpest and PPR, transgenic groundnut plants expressing H/HN have been developed. Khandelwal *et al.* (2002) have reported the cloning of the haemagglutinin (H) protein gene of rinderpest virus into a binary vector containing constitutive CaMV35S promoter and *nos* terminator. The plasmid was transformed into tobacco and peanut plants via *Agrobacterium tumefaciens* system. The H protein was detected in both the transgenic lines using polyclonal mono specific antibody to H protein. The antigenic authenticity of the plant derived H/HN was confirmed by western blotting and ELISA using a series of antibodies including convalescent sera. The immunogenicity of the plant derived H/HN have been studied in a mouse model and the protective ability of these antibodies have been tested *in vitro* by mouse neutralization test. Cattle and sheep fed with transgenic peanut leaves expressing H/HN elicit high levels of antibody reacting to H/HN. The priming of T cells, which take part in cell-mediated immune response, has been shown by *in vitro* lympho proliferation assays. Plant derived H/HN when supplied orally as a part of food, are able to induce both arms of immune response.

Golovkin et al. (2007) have reported the *in planta* production of the recombinant vaccinia virus B5 antigenic domain (pB5), an attractive component of

a subunit vaccine against smallpox. The antigenic domain was expressed by using efficient transient and constitutive plant expression systems and tested by various immunization routes in two animal models. Whereas oral administration in mice or the minipig with collard-derived insoluble pB5 did not generate an anti-B5 immune response, intranasal administration of soluble pB5 led to a rise of B5-specific immunoglobulins, and parenteral immunization led to a strong anti-B5 immune response in mice. Mice immunized with pB5 generated an antibody response that reduced virus spread *in vitro* and conferred protection from challenge with a lethal dose of vaccinia virus. These results indicate the feasibility of producing safe and inexpensive subunit vaccines by using plant production systems.

The use of transgenic potatoes to produce immunogenic proteins has in fact been established using the B subunit of the heat labile enterotoxin (LT) from *E. coli* (Haq *et al*., 1995). Enterotoxic *E. coli* is the major cause of diarrhoea. LT-B was expressed at low levels, upto 0.01% of total protein in potato tubers. Mice fed with raw transgenic potato expressing LT-B generated both specific serum IgG and mucosal IgA responses. Significantly this plant vaccine was tested on human beings (Tacket *et al*., 1998). The result showed that the raw potatoes containing the vaccine was generally well tolerated and indicated immunological priming of the gut mucosal immune system. A four fold increase in IgG anti LT was also detected in the volunteers.

Cholera toxin from *Vibrio cholerae* is a protein that is structurally and antigenically homologous to *E. coli* LT. The expression of this CT has been reported in transgenic potato plants (Arakawa *et al*., 1998). Expression levels of upto 0.3% of total plant proteins were achieved. The LT-B and CT antigens can act as oral immunogens particularly for mucosal antibody responses. Two further demonstrations of the immunogenecity of plant derived recombinant antigens have been reported. Mason *et al*. (1995) has expressed the capsid protein of Norwalk

virus in tobacco and potato. Norwalk virus is a common cause of an acute epidemic gastroenteritis in human. When produced in plants, a recombinant capsid protein assembled into virus like particles that were immunogenic. The expression levels were upto 0.37% of total protein, which was significantly higher than that achieved for *HBsAg*. Immunization by gavage with the plant antigen resulted again in serum and mucosal antibody responses.

For oral vaccination with transgenic plant material potato may not be ideal as the cooking process might denature the recombinant protein and raw potato is rather unpalatable. Mc Garvey *et al*. (1995) used tomato for the expression of a rabies virus glycoprotein with the view to develop an oral vaccine. They confirmed gene integration through southern blots and northern blots. They achieved 12 to 14 mg/mL of G-protein expression in the leaf and 1 to 2 mg/mL of G-protein in the fruits. They also showed localization of proteins in the golgibodies, vesicles and cell wall of the F1 plants. However the rabies glycoprotein from the plant varied slightly from the G-protein from the denatured rabies virus possibly due to differences in glycosylation in plants. Although the levels of expression were as low as 0.001% soluble protein, the rabies G-protein from tomato was recognized by specific anti-sera and a monoclonal antibody.

Plant vaccines that can be produced in large amounts can be used against autoimmune diseases also. Ma *et al*. (1997) reported the expression of glutamic acid decarboxylase (GAD), an auto antigen associated with diabetes in tobacco and potato. High levels of expression were seen. Non-obese diabetic mice, which were fed with the transgenic materials, showed delayed progression of clinical diabetes.

The production of immunogenic antigens in plants as vaccines has not been restricted to human diseases alone. Carillo *et al*. (1998) expressed structural protein VP1 of foot and mouth diseases virus in *Arabidopsis*. The mouse that was immunized intraperitonially with the leaf extract elicited immune response to

synthetic peptides carrying various epitopes of VP1 or to the complete VP1. Furthermore all the mice immunized with leaf extract were protected against challenge with virulent viruses.

The high-yield rapid production of *HBsAg* in plant leaf using a novel viral transient expression system. *Nicotiana benthamiana* leaves infiltrated with the MagnICON viral vectors produced *HBsAg* at high levels, averaging 295 μg/g leaf fresh weights at 10 days post-infection, as measured by a polyclonal enzyme-linked immunosorbent assay. Transiently expressed *HBsAg* accumulated as the full-length product, formed disulphide-linked dimers, displayed the conformational 'a' antigenic determinant and assembled into VLPs. Immunization of mice with partially purified *HBsAg* elicited *HBsAg*-specific antibodies. Furthermore, it was found that transient production of *HBsAg* using vacuum infiltration of whole plants, rather than syringe infiltration of leaves, was readily scalable, and greatly improved the accumulation of correctly folded *HBsAg* that displays the protective 'a' determinant (Zhong *et al.,* 2008).

An alternative strategy of producing plant-based vaccine is to infect the plant with recombinant viruses carrying the desired antigen that is fused to the viral coat protein. Using this strategy Modelska *et al*. (1997) have shown that immunization of mice intraperitonially or orally is possible through feeding of plants infected with the recombinant alfalfa mosaic virus (AIMV) carrying rabies peptide CPDrg24. Local as well as systemic immune response with stimulation of both serum Ig G as well as IgA was noted. Fourty percent of the immunized mice were protected against a single dose of challenge virus.

Transgenic plants are also being looked upon as a source for producing large-scale antibodies that can be used for passive immunization. Ma *et al*. (1995) have succeeded in producing multimeric secretary IgA (SIgA) molecules in plants. As SIgA not only contains heavy and light chains but is also dimerised by a J-chain, and protected from proteolysis by a secretory component (SC), its

production in mammalian cells is very complicated. So they produced four transgenic tobacco plants, each producing one of the components of this antibody. Through a series of sexual crosses all four proteins were made to express simultaneously in a single plant resulting in a functional antibody. This demonstrated the potential of plants in the assembly of antibodies.

There have been several attempts in recent times to produce rabies glycoprotein in various plants. Xioping Zhu (1998) showed the presence and expression of the rabies glycoprotein gene in tobacco, Muskmelon and sweetpotato by PCR and Southern blot analysis. Similarly Bindu (1998) reported the presence of rabies glycoprotein gene in transgenic Muskmelon produced by Electroporation. Shilpa (1999) has shown the stable integration of the rabies glycroprotein gene in tobacco through *Agrobacterium* mediated transformation. PCR and Dot blot analysis confirmed the gene integration in both the cases. The transgenic tobacco containing the rabies glycoprotein was found to induce rabies neutralizing antibody titers upto 1.5 international units per ml in later studies.

Sharma *et al*. (1999) reported that transgenic material to be used as vaccines in the form of seed or fruit can be easily stored and transported from one place to another without fear of its degradation. In addition transgenic plants capable of producing several different products can be created at any given time by crossing plants producing different products. Thus plants are increasingly being considered as a new unconventional source of vaccines and hold enormous potential (Moffat, 1995).

Mariana *et al.* (1997) investigated the expression of GAD65 in *E. coli* as an alternative to the expressing in eukaryotic systems, which is expensive and technically difficult. The results obtained provided the solution to two main problems associated with the expression of GAD65 in *E. coli* misfolding, leading to the formation of inclusion bodies; and the presence of alternative initiation sites for translation that causes the preferential production of truncated variants of

GAD65. Ultimate out come was the production of properly folded, fully active and immunochemically competent GAD65 as an N-terminal fusion protein with thioredoxin. This would replace the isolation of large amounts of GAD65 from pancreas or other tissues.

The production and characterization of transgenic tobacco and carrot plants expressing human GAD65, a major auto antigen in human insulin dependent *Diabetes mellitus* (IDDM) Andrea *et al*. (1999) has reported that Immunogold labeling and electron microscopy of transgenic tobacco tissue showed the selective targeting of human GAD65 to chloroplast thylocoids and mitochondria. *In planta,* expressed GAD65 has a correct Immuno-reactivity with IDDM-associated autoantibodies and retains enzymatic activity. In transgenic tobacco and carrot, the expression levels of human GAD65 varies between 0.01% and 0.04% of the total soluble proteins.

Linda *et al.* (2002) reported increase in expression levels of hGAD65 in transgenic tobacco (*Nicotiana benthamiana*) by targeting the enzyme to the plant cell cytosol, by mediating expression through the potato virus X (PVX) vector, and by substituting the NH2 terminal region of hGAD65 with a homologous region of GAD67. The expression levels were increased to 0.19% of the total soluble protein, compared to only 0.04% of wild type hGAD65; transient expression of wild type full length hGAD65 in *Nicotiana benthamiana* mediated by PVX infection was associated with expression levels of immunoreactive protein as high as 2.2% of the total soluble protein. The substantial improvement of expression of hGAD65 in plants paves the way for Immuno-prevention studies of oral administration of GAD65 containing transgenic plant material in animal models of spontaneous autoimmune diabetes.

Humans have been using plants for medical purposes for several thousand years; however production of biopharmaceuticals from plants with the assistance of gene engineering was introduced much later. Production of recombinant plant

proteins offers a series of potential benefits such as cheap production and storage, large-scale manufacture of biopharmaceuticals, higher health safety level in comparison with vaccines of animal origin. Further advantage of plant biopharmaceuticals is potential elimination of a purification process, in case plant tissues are used as food. Last but not least, it is noteworthy that the target protein may be inserted into a particular cell compartment (chloroplast); its stability is consequently increased or this protein may even be expressed in this compartment (Daniell *et al*., 2001).

The technology of plant bioreactors is limited by the following factors: low protein yield usually caused by its low stability, problems during protein production that are reflected in variable quality of the final product, even minor differences in post-translational modifications can potentially affect the activity and/or immunogenictity of the recombinant molecules (Miele, 1997).

Despite the majority of recombinant proteins being produced by microorganisms, transgenic plants represent an alternative system for their production. The most suitable plant for production of human vaccines (edible vaccines) seems to be the banana plant for the following reasons: it is consumed easily by children, consumption is in raw form and it occurs naturally in developing countries. However, tomato plants, maize and potato plants are rather used as model systems in typical experiments dealing with expression of foreign proteins in plants (Stirn and Lorz, 2003).

Genetically modified organisms (GMO) become a real constituent of our lives and nowadays, they are commonly introduced into the food chain of people and animals in some states. Among higher organisms, plants are used above all for genetic modifications; potatoes are a suitable model plants for this purpose. Nowadays, a number of various genetic modifications of potato plants are available, particularly those with increased resistance to biological agents and factors of the external environment or with improved nutritional value. Plants that produce

proteins of the immune system of man or animals or substances that may be used as vaccines in human or veterinary medicine are highly important. Modified potato plants that produce biomaterials for potential applications in the industry are a significant category (Pribylova *et al.*, 2006)

Plant-based expression systems offer an oral delivery alternative with low production costs, and they also encapsulate the antigen. Some plant-based systems also stabilize antigen and therefore reduce storage and distribution costs. The hepatitis B major surface antigen has been expressed in several plant systems. A variety of regulatory sequences and subcellular targets have been used to achieve expression suitable for early stage clinical trials. However, further increase in expression will be necessary for practical and efficacious products. Appropriate processing can yield palatable products with uniform antigen concentration. The antigen expressed in plant systems shows extensive disulphide cross-linking and oligomerization and forms virus-like particles. Oral delivery of the antigen in plant material can induce a serum antibody response, prime the immune system for a subsequent injection of antigen and give a boosted response to a prior injection. Small scale clinical trials in which the antigen has been delivered orally in edible plant material indicate safety and immunogenicity (Streatfield, 1999)

Hepatitis B is a global health problem. Patients with chronic hepatitis B (CHB) carry a significant risk to eventually develop cirrhotic liver disease. Recent therapeutic advances against CHB offer excellent potential for long-term suppression of hepatitis B virus replication during antiviral therapy, and occasionally a durable remission of medication. Selection of appropriate patients for antiviral therapy depends on identification of HBV replication and an elevated alanine aminotransferase level or histologic liver injury. Pegylated interferon alpha offers potent immunomodulatory and antiviral activity with the potential for durability, but also with adverse effects and significant cost (Mailliard and Gollan, 2006).

Transgenic plants are emerging as an important system for the expression of many recombinant proteins, especially those intended for therapeutic purpose. The production of foreign proteins in plants has several advantages. In terms of required equipment and cost, mass production in plants is far easier to achieve than techniques involving animal cells. Successful production of several proteins in plants, including human serum albumin, haemoglobin, monoclonal antibodies, viral antigens (vaccines), enkephalin, and trichosanthin, has been reported. Particularly, the demonstration that vaccine antigens can be produced in plants in their native, immunogenic forms opens exciting possibilities for the "bio-farming" of vaccines. If the antigens are orally active, food-based "edible vaccines" could allow economical production (Okada, 1997).

2.2 *Agrobacterium* mediated transformation:

The *Agrobacterium* mediated transformation is one of the most suitable and efficient methods of transferring cloned genes into plants. The gram negative soil bacterium, *A. tumefaciens* and *A. rhizogenes* are natural genetic engineers capable of transforming a range of dicotyledonous plants by transferring plasmid encoded genes into recipient plant genomes (Binns and Thomashow, 1988).

The plasmid DNA has two important regions namely the T-DNA and the vir region. The T-DNA has a 25 base pair direct repeat sequence flanking it on either side. This helps in the transfer of any foreign gene cloned into the T-DNA region (Zambryski, 1992, Stachel *et al*., 1986). *Vir* genes, labeled *Vir* A to *Vir* G, are required for the virulence and infective capacity of the *Agrobacterium*. Normally the *Vir* genes are not expressed in *Agrobacterium* until they become activated by certain plant factors. These factors have been identified as phenolic compounds viz., Acetosyringone (AS) and hydroxyl acetosyringone (De Block *et al*., 1984). Protoplast derived cells, callus and a variety of explants taken from leaves, cotyledons, hypocotyls and roots have been transformed through *Agrobacterium* mediated transformation.

The first transgenic plants were produced via *Agrobacterium* mediated transformation of tobacco protoplast (Horsch *et al*., 1985). Since then a large number of crop plants have been transformed with a variety of genes by this method. Although several novel methods for the transformation of plants have been developed in recent times, this method is still the most preferred one.

2.2.1 Regeneration and transformations in tobacco:

Tobacco is the most commonly used plant for introducing novel genes (Gerstel and Sisson, 1995). This is because extensive research has been carried out with respect to regeneration protocols, somatic embryogenesis, rooting, callusing and transformation.

Murashige and Skoog, (1962) first standardized their media using Tobacco. This media is used almost universally in tissue culture of plants now. A large number of reports exist on the use of different combinations of growth regulators in achieving regeneration for this crop because of the diverse varieties available.

Malcolm *et al* (1973) showed the use of the MS media; 10^{-5} M of Indole 3-acetic acid and Kinetin, produce shoots from the initiated callus. Marcotrigiano (1986) as well as Schiff and Bennice (1992) reported that adventitious shoots could be regenerated from cultured tobacco leaf tissue on Murashige and Skoog (MS) medium. Shoots were derived from leaf midrib callus cultured on MS medium with 20mg/l IAA, 2.0mg/l Kinetin and 160mg/l Adenine sulfate within 8 days (Yadav *et al* 1996).

Generally auxins in high concentrations favour rooting. However auxins and cytokinins with high auxin ratios (1-20mg/l) has never favoured rooting. Purushotham (1999) reported that both high and low concentrations of NAA and IAA did not produce rooting. Only IBA at low concentrations of 0.6mg/l has resulted in a low degree of rooting.

Horsch *et al*, (1984) reported that the Tobacco is the earliest transformed plant. Since then it has become the most extensively transformed plant. It has been transformed with a variety of genes for disease resistance, insect resistance and for value added novel compounds in its role as an experimental system.

Yoneyama *et al.,* (1993) reported that the Tobacco has been transformed to induce resistance against *Pseudomonas syringae* pv. *tabaci* causing Tobacco wild fire disease thus obtaining disease resistant transgenic plants. An engineered cercopin gene cassette, consisting of a sequence from barley alpha amylase proteinase inhibitor II gene promoter has been introduced into Tobacco by *Agrobacterium* mediated transformation (Huang *et al*, 1997). Tobacco has also been one of the most commonly used plants to test the production of vaccines in plants. The first plant vaccine to be produced, the spa A Streptococcal antigen against tooth decay, was produced in Tobacco (Curtiss and Cardineau, 1990).

A number of plant vaccines such as those against hepatitis B, gastroenteritis and even rabies have since been produced in Tobacco (Mason *et al.,* 1992, Mason *et al.,* 1995 and Shilpa, 1999, Devaiah, 2000, Nagesha, 2001).

2.3 Inheritance of transgene in transgenic plants:

The evaluation of transgenic progeny has been mostly restricted to the transgenics produced against disease and herbicide resistance. The segregation ratio reflects the number of loci at which the foreign gene is present (Firoozabady *et al.,* 1999)

Becker *et al*., (1994) reported that the histochemically stained pollen grains of a GUS gene transformed wheat plants showed a 1:1 segregation of the uid A gene in all the plants tested. A 3:1 segregation of the introduced gene was demonstrated by enzyme sensitivity test and southern blot analysis of R1 generation plants.

In wheat plants transformed with the gene coding for phosphinothricin acetyl transferase (PAT), PAT activity was detected in a 3:1 ratio in R1 generation plants following cross or self pollination. Both male and female transmission of the PAT gene and its segregation as a dominant Mendelian ratio in R1 and R2 plants were demonstrated (Vasil *et al*., 1993).

Rusell *et al*., (1993) reported that the *Phaseolus vulgaris* transformed with a bean golden mosaic virus coat protein characterized by 5 generations of self-fertilization did not show any loss of the introduced gene or its expression. In addition several families were crossed with non-transgenic parents and these plants also showed expected inheritance patterns.

R1 generation rice transformed through PEG mediated DNA uptake with a Bar gene for herbicide resistance and a Gus reporter gene was evaluated for the inheritance of the foreign gene through southern analysis. Both genes expressed in T1 and T2 progeny. Enzyme assays on T1 progeny plants also showed a gene dose response reflecting their homozygous and heterozygous status (Rathore *et al*., 1993).

Burza *et al*., (1995) reported that, the R1 generation progeny of cucumber meristem protoplasts transformed with GUS gene through electroporation was evaluated for the presence of a gene. The transfer and expression of the gene in F1 transgenic plants were confirmed by southern blot analysis.

Seed progeny of transgenic sorghum plants obtained after microprojectile bombardment with a bar gene was screened through southern analysis. These diploid transgenic plants demonstrated homozygous integration of multiple copies of the transgene at one locus in one plant and heterozygous integration at two loci in another plant (Casas *et al.,* 1997).

Chowda reddy, (1999) showed that the seeds obtained from the transgenic tobacco plants show the glucane-chitinase gene segregation in the ratio of 3:1 in R_1 generation.

2.4 Molecular techniques for confirmation of the vaccines produced in plants:

2.4.1 Polymerase Chain Reaction:

PCR is used to amplify specific regions of a DNA strand (the target DNA). This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size.

PCR or *Polymerase Chain Reaction* was first popularly thought to have been conceived by Dr. Kerry Mullis in 1983 while working at the Cetus Corporation in Emeryville, CA, along with other researchers at Cetus Corporation. Cetus Corporation discovered a method to start and stop DNA polymerase enzyme activity at specific points along a single strand of DNA. On the other hand, some pioneering research was also done by Gobind Khorana, who described a basic principle of replicating a piece of DNA using two primers (1971). Cell-free DNA amplification by PCR was able to simplify many of the standard procedures for DNA cloning, DNA analysis, and the modification of DNA. PCR, as Kerry Mullis stated "lets you pick the piece of DNA you're interested in and have as much of it as you want".

Polymerase chain reaction can be used in isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA, DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid or the genetic material of another organism. Bacterial colonies (E.coli) can be rapidly screened by PCR for correct DNA vector

constructs (Pavlov *et al*., 2006) PCR may also be used for genetic fingerprinting; a forensic technique used to identify a person or organism by comparing experimental DNAs through different PCR-based methods. PCR allows early diagnosis of malignant diseases such as leukemia and lymphomas, which are currently the highest, developed in cancer research. PCR also permits identification of non-cultivatable or slow-growing microorganisms such as mycobacterium, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.

Zhong *et al*., (2008) reported the presence of *HBsAg* transgenes in Ginseng callus cells was confirmed by PCR. *HBsAg* gene linked with omega leader and amplified with the primers specific to forward omega leader and *HBsAg* gene was identified by the presence of a product of *750* bp, while it was absent in control.

PCR analysis was carried out with randomly selected four rooted plantlets of tomato for each construct to detect the presence of 's' gene of hepatitis B virus in pEFEHBS and pEFEHER transformed plants. A 681-bp fragment amplification specific to's' gene was noted in both the transformed plants, while it was not detected in the untransformed control plants. The expression of *HBsAg* was confirmed in these plants by RT-PCR analysis. The gene-specific fragment (681 bp) of expected size was observed in all the leaves and fruits of transformed plants, while being absent in untransformed control plants. (Srinivas *et al.*, 2008).

Zhong *et al*., (2005) reported the presence of *HBsAg* gene in *Nicotiana benthamiana* leaf by PCR confirmation. They used four plant binary vectors pHB 117, pMHB, pNV3M110 and pNVT 110. here MHB transgenic lines yielded the same 0.96 kb band as plasmid pMHB, while pHB 117 produced 0.8 kb amplified product while no PCR product was found in nontransformed plants.

A 700 bp fragment amplified from the coding region of the *HBsAg* gene was found in 14 out of 40 colonies by PCR. The results show that the *HBsAg* gene is stably integrated into the *D. salina* genome. (Degui *et al*., 2003)

Four putative transformed plants from potato hairy roots were analysed for their transgenic nature by PCR. Transgenic plants showed the amplification of 681 bp fragments by PCR of genomic DNA with *HBsAg* 's'gene specific primer, while it is absent in nontransformed plants. They also confirmed this gene by RT-PCR analysis. (Sunil kumar, *et al.,* 2006).

Sunil kumar, *et al.,* (2006) reported the expression of hepatitis B surface antigen in transgenic tobacco (*Nicotiana tabacum*) by PCR analysis. Transgenic plants showed the 681 bp fragments of genomic DNA with *HBsAg* specific primer which was absent in nontransformed plants.

The genomic DNA was isolated from putative transformants and nontransformed Muskmelon plant. The plasmid DNA of pHB118 carrying *HBsAg* gene was used as positive control. The genomic DNA from control plant was used as negative control. Integration of the gene was confirmed by using the *HBsAg* specific primers. Out of 28 putative transformed plants chosen for the analysis, four have shown the presence of single 900 bp amplified product, which is the size of the *HBsAg* gene. (Hanumantappa, 2006).

PCR analysis of the genomic DNA from the leaves taken from putative transformed plants of *coleus forskohlii*, plasmid DNA isolated from *Agrobacterium* and the genomic DNA from control plants have shown the presence of 900 bp amplified band of *HBsAg* gene on both putative transformed plants and the plasmids, but it was absent in control plants when primers specific to *HBsAg* gene were used. All of the five plants subjected for PCR analysis have shown positive results. (Sandesh, 2007).

2.4.2 Southern hybridization:

A Southern blot is a method routinely used in molecular biology to check for the presence of a DNA sequence in a DNA sample. Southern blotting combines agarose gel electrophoresis for size separation of DNA with methods to transfer the size-separated DNA to a filter membrane for probe hybridization.

Southern Blotting is a molecular analysis technique which was developed in 1970 by Dr. Edward Southern of Edinburgh University. This technique can locate particular fragments of DNA in a complex mixture and can quickly identify a gene or gene segment within the genome. Southern blotting can be used in either direct or indirect detection strategies. Due to its versatility and accuracy, this technique has become an indispensable molecular genetics tool for medicine, research, and public health. This can be used during prenatal screening and diagnosis, Use in oncology and cancer research, Use in evolutionary biology, Screening of food supply with the help of Southern Blot and Testing for emerging infectious diseases. (Southern, 1975).

Sandesh (2007) reported the Southern hybridization confirmation of *HBsAg* gene in coleus plants. He used non radioactive labelled probes to determine the integration of the transgene in two out of five putative transformants of coleus that were positive for PCR. The plasmid DNA was used as positive control and the genomic DNA from control coleus plant was used as negative control. The results confirmed that single copy of hepatitis B surface antigen encoding *HBsAg* gene has been integrated in two out of three putative transformants subjected for southern hybridization. The results also confirmed that *HBsAg* gene encoding *HBsAg* S-protein was intact in the plasmid. The control *C. forskohlii* plant did not show any hybridization signal

Hanumanthappa (2006) reported presence of single copy of the transgene *HBsAg* in muskmelon by southern hybridization. The results confirmed that

hepatitis B surface antigen encoding *HBsAg* S- protein has been integrated in one out of three putative transformants subjected for southern hybridization.

Sunil kumar *et al.,* (2006) reported the expression of hepatitis B surface antigen in transgenic tobacco *(Nicotiana tabacum*) by southern blot analysis. The amplified PCR products were hybridized to the radioactive labelled 681 bp *HBsAg* fragment confirming their transgenic nature.

Sunil kumar, *et al.,* (2006) reported the expression of hepatitis B surface antigen in potato hairy roots by southern blot analysis. The amplified PCR products were hybridized to the radioactive labelled 681 bp *HBsAg* fragment confirming their transgenic nature. While no hybridization signal was detected in nontransformed plants.

2.4.3 SDS-PAGE and Western blot analysis:

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their electrophoretic mobility.

The western blot (immunoblot) is a method to detect a specific protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide or by the 3-D structure of the protein. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein. This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines. The method originated from the laboratory of George Stark at Stanford. The name western blot was given to the technique by W. Neal Burnette

Western blotting was performed to confirm the integrity of the *HBsAg* antigen expressed in transgenic plants. Under reducing condition, yeast-derived S protein was observed as a 24 kDa band, a band at the same position occurred in

pHB117 transgenic plants. A 30 kDa band, as expected for M protein, was detected from transgenic MHB line 28 extracts indicating that the M protein accumulated as full-length product. For detection of *HBsAg*, they were probed membranes with goat anti-*HBsAg*, followed by rabbit anti-goat IgG conjugated to horseradish peroxidase (Zhong, *et al*., 2005).

Expression of rabies glycoprotein was detected by SDS-PAGE and western immunoblotting in transformed cantaloupe fruit. The successful expression of the rabies glycoprotein gene in transgenic cantaloupe fruit shown positive results of the 66 kDa antigenic rabies glycoprotein. (Nagesha *et al.,* 2006).

The protein was extracted from transformed and untransformed *C. forskohlii* plants and subjected to SDS-PAGE to see the expression of *HBsAg* gene. The SDS-PAGE gel revealed the presence of the band around 24 kDa in three putative transformants and the absence of same band in control plants indicating the over expression of the gene in the transgenic Coleus plants. (Sandesh, 2007).

SDS PAGE was carried out by extracting the proteins from the leaves of transformed and control Muskmelon plants. The transgenic plants containing the recombinant protein have shown a thick band at around 24 KDa revealing the over expression of the hepatitis B surface antigen gene present in the transgenic. (Hanumanthappa, 2006).

Degui *et al*., (2003) showed that the *HBsAg* gene is successfully transcribed and translated into protein in *D. salina*. Protein extracts from transformants were further analyzed by Western blot analysis. After hybridization, a band at about 24 kDa in transformants was found, which was at a position similar to that of standard *HBsAg*.

Western blotting of the secreted *HBsAg* from pEFEHBS- and pSHER-transformed tobacco cell cultures confirmed the presence of *HBsAg* specific band, while it was absent in the untransformed cell cultures. The results demonstrated

that denatured *HBsAg* secreted by tobacco cell cultures was similar to yeast-derived r*HBsAg* having 24 kDa. (Sunil Kumar, *et al.*, 2007).

2.4.4 Enzyme Linked Immunosorbant Assay (ELISA):

Enzyme Linked Immunosorbant Assay is based on the ability of low molecular weight antibodies to couple with enzymes to produce enzymatically active immunological conjugates. This allows the detection of immune reaction with histochemical staining techniques, because the antibody component is involved in immune reaction and the conjugated enzyme can be used for staining reaction utilising appropriate substrate.

Using this principle ELISA was initially developed in 1971 independantly by 2 groups – Engvall and Perlmann; Weeman and Schuurs. Later Vollar *et al.*, (1976) and Clark and Adams (1977) utilised ELISA for detection of virus infection. Several modifications of original ELISA are known and it is widely used in diagnosis.

The popularity of ELISA is due to its high specificity and sensitivity at low cost, amplification system which allows detection of nanogram quantities, adaptability to automation, elimination of hazardous reagents, use of colour reactions that can be evaluated visually and simultaneous screening of many samples in the same microtitre plate with as little as 50µl total volume (WHO manual 1995).

Perrin *et al.*,(1995) reported the development of a solid phase enzyme linked immunosorbant assay called rapid rabies enzyme immunodiagnosis (RREID) for the diagnosis of rabies. It was based upon the detection of rabies virus nucleocapsid antigen in the brain tissue. This test involves the capture of nucleocapsid antigen present in the tissue homogenate by the polystyrene wells coated with anti nucleocapsid antibody followed by reaction with peroxidase tagged anti nucleocapsid antibody and colour development by

orthophenyldiamine. It was found easy to perform and showed high precision and reproducibility.

ELISA is used in the diagnosis of hepatitis B. The polystyrene wells are coated with purified glycoprotein. The test is based on binding of glycoprotein antigen with the anti glycoprotein antibody present in the test sera followed by reaction with anti human IgG peroxidase conjugate.

ELISA analysis of the putatively transformed plants showed the maximum expression level of 255.5 ng/g D.W. in the pEFEHER-transformed plants maintained under in vitro conditions. The same plant grown in the greenhouse expressed *HBsAg* at the levels of 161.4 ng/g D.W. The monoclonal antibody-based ELISA indicated the expression levels of 160.1 ng/g D.W. and 62.64% of monoclonal antibody reactivity in pEFEHER-transformed plants grown in tissue cultures. Low levels of *HBsAg* expression in the range of 60–70 ng/g D.W. were noted in the transgenic fruits of pEFHBS/HER transformed plants. (Srinivas *et al*, 2008)

Total protein was isolated from non-transformed control and transgenic cell lines as described. The extracts were assayed for levels of expression of *HBsAg* with the Auszyme kit (Abbot), human serum- derived *HBsAg* as a positive standard, and protein extracted from non-transformed cells as a negative control. (Zhong *et al.*, 2008).

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. Expression levels in the hairy root derived plant were almost similar to that of transgenic plant. (Sunil Kumar, *et al*., 2006).

2.5 Purification of Recombinant protein produced in plants:

Perez *et al* (1998) reported a new method for the isolation of glycoprotein G from viral haemorrhagic septicimia virus (VHSV), a fish rhabdovirus, by using affinity chromatography with immobilised concanavalin A. The glycoprotein G was isolated from detergent solubilised concentrated virions. The purity achieved was higher than 85%. The estimated recovery of the initial glycoprotein G present in the virions was between 20% to 50%. These glycoprotein G preparations showed the presence of about 30% of trimers by ultracentrifugation, reacted with antibodies to the phosphatidyl serine binding domain (p2) in a pH dependant manner by solid phase binding assays. These data suggested that concanavalin A puirfied glycoprotein G conserved most of its native properties and confirmation.

Affinity chromatography was used for purifying soluble glycoprotein from virion depleted, rabies infected tissue culture fluid, for chemical and immunological analysis. A purified monoclonal antibody specific for rabies virus glycoprotein was coupled to cyanogen bromide activated sepharose. The crude solubilised viral glycoprotein was passed through the antibody column. Of the fractions eluted, peak fractions were pooled, dialized and used (Bernard Dietzschold *et al.,* 1983).

Rajshekar Baru Tulasi and Nadimpalli (2002) report the isolation of 2 distinct lectins which differ in their sugar specificities i.e., the glucose/mannose specific lectin and the galactose specific lectin from *Dolichos lab lab* . The protein extracted from stem and leaves of *Dolichos lab lab* was subjected to ammonium sulphate precipitation and pellets was dissolved in Tris-HCl buffer. The protein was subjected to affinity chromatrophy through sepharose-divinyl sulphone-galactose and sepharose –divinyl sulphone lactose gel. The gels were washed extensively with Tris-HCl buffer and sequentially eluted with 0.2 M galactose, 0.2 M lactose in column buffer and 0.1 M acetic acid respectively.

The process of growing pharmaceutical proteins in plants, extracting, and purifying is a hard task considering the lack of available information. In this work, a recombinant murine monoclonal antibody specific for the hepatitis B surface antigen, expressed in stably transformed transgenic *Nicotiana tabacum* plants, was purified by means of a recombinant protein A Streamline chromatography as the main purification step. The antibody expression level varied with the age of the plants and the number of harvests from 40 to 15 lg/ml and the maximum process yield was about 25mg of plantibody/kg of biomass. Protein A Streamline chromatography was successfully used in the purification process yielding a recovery of about 60% and a plantibody SDS–PAGE purity of over 90% but unexpectedly; previous clarification steps could not be totally avoided. (Rodolfo, *et al*., 2003).

Sophie *et al*., (2007) reported purification of *HBsAg* recombinant protein produced in yeast (*Pichia pastoris*).The yeasts were resuspended in buffer containing protease Inhibitors and lysed by passing through a high pressure cell disrupter to break the membranes. After cell lyses, recovery of immunoreactive *HBsAg* forms in the lysate was improved by addition of non-ionic detergent. The cell debris was removed by centrifugation. *HBsAg* in the supernatant corresponded at this step to 1–3% of the total soluble protein fraction. The supernatant was further clarified by microfiltration using 0.2 lm filter and subjected to ultrafiltration using 300,000 or 500,000 NMWC hollow fiber system allowing buffer change, fivefold concentration of *HBsAg* and elimination of at least 60% of contaminant proteins. At this step, *HBsAg* corresponded to 2.5–7.5% of total proteins. Negligible *HBsAg* immunoreactivity was lost in the ultrafiltrate (less than 1% on 300,000 NMWC fibers and less than 5% on 500,000 NMWC fibers), suggesting as expected, production of *HBsAg* mainly under high oligomeric structures and probably virus-like particles.

Mason *et al.,* (1992) reported the Immuno Affinity purification of *HBsAg* from the transgenic tobacco using monoclonal antibody raised against human

serum derived *HBsAg*. The antibody bound to Affi-Gel Hz hydrazide gel. The bound *HBsAg* was eluted with 0.2 M glycine and immediately neutralized with Tris base.

Nadia, *et al*., (2003) reported the protein purification in tobacco leaves. The plantibody was easily and efficiently purified from fresh leaves of tobacco by affinity chromatography in protein A sepharose, the column was washed with the citric acid buffer and protein eluted in the same buffer.

Nagesha, *et al.*, (2006) reported transgenic Tobacco leaf protein and also muskmelon fruit protein samples were subjected to affinity chromatography through a Concanavalin A agarose column. Purification of the protein was achieved, where all the glycoproteins were separated out from the crude sample.

MATERIAL AND METHODS

III MATERIAL AND METHODS

In the present study, *Agrobacterium* mediated transformation of Tobacco was carried out with Hepatitis B recombinant protein (*HBsAg*).Transgenic plants of T_0 and T_1 tobacco were analyzed for the morphological characters as well as integration and expression of Hepatitis B recombinant protein (*HBsAg*) through SDS-PAGE, protein dot blot and western blot.

3.1 Location:

The present investigation involving the transformation of Tobacco with Hepatitis B recombinant protein (*HBsAg*) gene was carried out at the Plant Vaccine Lab, Department of Biotechnology, UAS, GKVK, Bangalore during the year 2007-2008.

3.2 Tissue culture of Tobacco:

3.2.1 Chemicals:

All the salts, which were used for the preparation of the tissue culture media, were of analytical reagent grade obtained from Himedia Co., Bombay (India) and Merk (India). The vitamins used were also from Himedia and Merk. Growth hormones used were of cell culture grade obtained from Sigma-Aldrich Co., USA. The gelling agent used for the media was certified agar or phytagel obtained from Himedia.

3.2.2. Glassware and chemicals:

Glassware like culture tubes, conical flasks, petriplates, beakers etc., were of Borosil make. All the chemicals and plant growth regulators were of analytical grade and were procured from standard chemical manufacturing companies like Himedia, Sigma Aldrich, and Merck.

3.2.3. Cleaning of glassware:

Glassware were rinsed in water and then soaked in 0.15% chromic acid overnight. The chromic acid was drained out and the glassware were washed with cleansol. The thoroughly washed glassware were rinsed in distilled water and dried in a hot air oven. The instruments like forceps, scalpels etc., were also cleaned and dried.

3.2.4. Sterilization of glassware:

Clean glassware were rinsed in double distilled water and dried in oven at 80°C and sealed with aluminum foil, petriplates placed in autoclavable covers, instruments like scalpel, forceps, and blade holders wrapped in aluminum foil were autoclaved at 121 °C and at 15 lbs. pressure for 15 min. The glassware were then transferred to sterile inoculation chamber for further use.

3.2.5 MS media composition:

The basal media used for all the experiments was MS medium (Murashige and Skoog, 1962). The composition in which different stock solutions used for the preparation of the media is as follows,

Stock solutions:

Stock solutions were prepared by dissolving the chemicals of analytical grade in double distilled water and storing them in brown bottles.

A. 10X MS macro nutrient stock (per liter);

H_2O	800mL
KNO_3	19.0g
$NH_4 NO_3$	16.5g
$CaCl_2.H_2O$	4.4g
$MgSO_4.7H_2O$	3.7g
KH_2PO_4	1.7g

All the chemicals were mixed well on a magnetic stirrer and the volume was made up to one liter using double distilled water, sterilized at 121^0C at 15lbs for 15 min. and refrigerated at 4^oC.

B. 1000X MS micro nutrient stock (per liter);

H_2O	800mL
H_3BO_3	6.22g
$MnSO_4.H_2O$	22.40g
$ZnSO_4$	8.60g
KI	0.83g
$Na_2MoO_4.2H_2O$	0.25g
$CuSO_4.5H_2O$	25mg
$CoCl_2.6H_2O$	25mg

All the chemicals were mixed on a magnetic stirrer and the volume was made up to one liter using double distilled water, sterilized at 121^0C at 15lbs for 15 min. and refrigerated at 4^oC.

C. 200X MS Iron stock (per liter):

$FeSO_4.7H_2O$	2785mg in 250mL H_2O
Na_2 EDTA	3725mg in 250mL H_2O

Both the dissolved solutions were combined and boiled for a few min until it turned clear and was then stored in a brown bottle at 4^0C.

D. 1000X MS Vitamin stock;

H_2O	100mL
Glycine	200mg
Nicotinic acid	50mg
Thiamine HCl	10mg

Pyridoxine 10mg

All the vitamins were dissolved, sterilized at 121^0C at 15lbs for 15 min. and refrigerated at 4^oC.

E. Preparation of plant Hormone stock:

2, 4 –D ($2mgl^{-1}$) stock solution:

100mg of 2, 4 D was dissolved in 5 ml of 0.2 N NaOH and volume was made up to 50 ml using sterile distilled water and stored at 4^0C.

BAP (6-Benzylaminopurine) ($2mgl^{-1}$):

1 N KOH was added drop by drop to 100 mg of BAP until dissolved and then volume was made up to 50 mL with Milli Q water and Stored at 4^0C.

NAA (Naphthalene Acetic Acid) ($2mgl^{-1}$):

100 mg of NAA was dissolved in 1 mL absolute ethanol and 3 mL of 1 N KOH. Volume was made up with 40 mL with Milli Q water and pH was adjusted to 6.0. Then volume was made up to 50 mL with Milli Q water and stored at 4^0C.

3.2.6 Preparation of culture medium:

Composition of MS basal medium (per liter);

Double distilled water	800mL
10X MS Macro nutrient stock	100mL
1000X MS Micro nutrient stock	1.0mL
200X MS Iron stock	5.0mL
Myo-inositol	100mg
Sucrose	30g
Agar	8.0g

All the ingredients were dissolved and the volume was made up to 1000mL. The required concentrations of hormones were added and the pH was adjusted to 5.8 with 1N NaOH and 1N HCl. Agar was added and melted by

heating. The medium was then distributed into conical flasks making sure that each flask was filled with not more than half its capacity to ensure proper autoclaving. The flasks were plugged with cotton and autoclaved at 121^0C at 15lbs pressure for 15 min.

3.2.7. Preparation of explants

A. Materials

1. Leaves
2. Sterile forceps, scalpels
3. 0.1% Bavistin
4. 0.1% $HgCl_2$
5. Sterile blotting papers
6. Sterile double distilled water
7. Glass bead sterilizer
8. Laminar flow chamber

B. Procedure:

1. The first and second pair of Tobacco leaf from the top were harvested and brought in water from green house grown plant. They were then washed under running tap water overnight.
2. The leaves were then treated with 0.25% bavistin for 2 hrs, rinsed with sterile water and then transferred into a sterile laminar airflow chamber.
3. The leaves were treated with 0.1% $HgCl_2$ for 30 seconds twice
4. The treated leaves were thoroughly washed with sterile distilled water 3-4 times.
5. Then the leaves were made into pieces of 1 cm^2 - 1.25 cm^2 size.
6. These explants were then placed on plates containing MS media with different concentrations of growth regulators.
7. Then the plates were placed in the culture room by maintaining 16 hrs photoperiod and temperature at 25^0C

3.3 Transformation:

Experimental details:

Preparation of Antibiotics:

Kanamycin stock of 100mg/mL was prepared in sterile water, filter sterilized and kept at 4^0C.

Cefotaxim stock of 250mg/mL was prepared in sterile water within the laminar air flow chamber and sealed before bringing it out. Then stored at 4^0C.

3.3.1 Selection media preparation:

When antibiotics were to be added to the media, the media was autoclaved first as described earlier, cooled to 45^0C and required amount of the selection agent was added. This media was poured into autoclaved bottles and allowed to solidify. This operation was done within a sterile laminar air flow chamber.

3.3.2 Bacterial strains:

The complete genome of Hepatitis B virus has been illustrated in the figure 1. The disarmed *Agrobacterium tumefaciens* strain LBA4404 was used for the transformation. The plant binary vector pHB118 was mobilized into the *Agrobacterium* strain LBA4404. The plasmid pHB118 contains the complete plant optimized Hepatitis B surface antigen gene from the Hepatitis B virus strain under the control of a modified CaMV 35S promoter called as a super promoter which gives the constitutive expression. This is to enhance the expression levels of the gene. The plasmid also contains sequences coding for intracellular targeting signals aimed at the endoplasmic reticulum, vacuoles and chloroplasts, with 5′ UTR and 3′ UTR signals and *vspB* gene. The *npt* II gene is included in the vector as a selectable marker. This construct was provided by Dr. Hugh S. Mason, Arizona Biodesign institute, USA (Figure 1).

3.3.3 Maintenance of bacterial culture:

The *Agrobacterium* culture was grown in YEP broth containing 50mg/L kanamycin at 28^0C overnight. An equal volume of glycerol was added to this and this glycerol stock was stored at -80^0C as a mother culture. For routine use, solid YEP medium containing 50mg/L of kanamycin was streaked with the mother culture once in a month and stored at 4^0C.

Preparation of YEP medium (per litre):

Peptone	10.0g
Yeast extract	10.0g
NaCl	5.0g
Agar Agar	16.0 g

The agar was excluded when YEP broth was required.

The above media was autoclaved at 121^0C at 15lbs pressure for 15min. After autoclaving, the media was cooled and 50mg/L of kanamycin was added under sterile conditions within the laminar air flow chamber.

3.3.4 Preparation of culture for co-cultivation:

A single *Agrobacterium* colony was taken from the YEP plate containing *Agrobacterium* culture. It was inoculated onto 20mL YEP broth containing 50mg/L kanamycin in a sterile conical flask. This was kept in orbital shaking incubator at 28^0C overnight. The culture was ensured to have 0.6-0.8 OD at 600 nm before using it for transformation. The overnight grown culture was centrifuged at 6,000 rpm for 10 min at 4^0C. The supernatant was discarded and the bacterial pellet was re-suspended half MS media using the following treatments and this was used for co-cultivation.

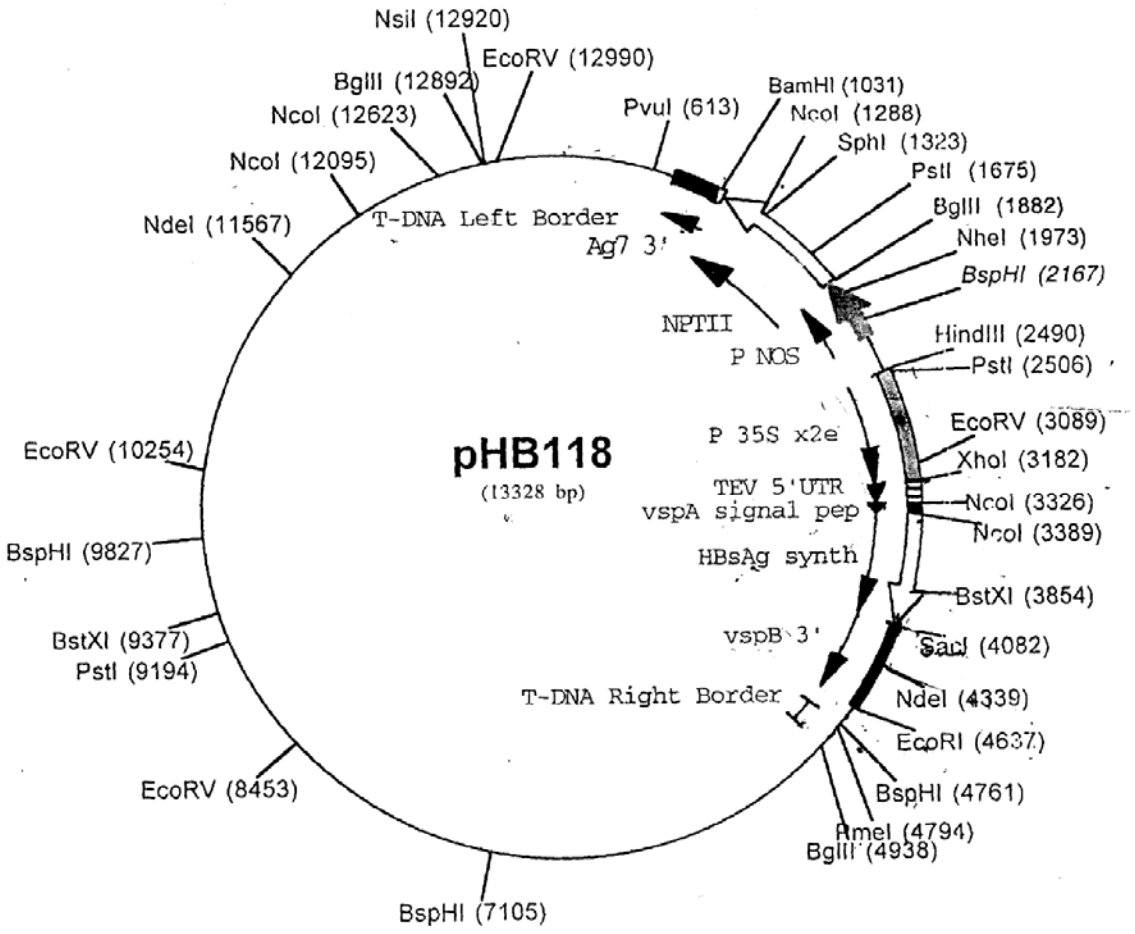


Figure 1: Plasmid construct of pHB118.

.3.3.6. Hardening of putative transformants

When the rooted shoots produced 2-3 leaves, they were transformed to a hardening medium after thoroughly washing the agar away from roots. The hardening media consisted of autoclaved peat sand mix in the ratio of 3:1. After the plants were planted in this medium, 5 mL of half strength MS medium diluted 100 times was added to the peat whenever it turned dry. The bottles were sealed and placed in the growth room. When the plants grew to 6 cm, the cap was removed and a polythene bag was covered loosely over the bottle. Once the plants were acclimatized they were shifted to a green house and polythene bag removed.

3.4 Confirmation of gene integration:

Integration of the Hepatitis B surface antigen gene:

To confirm integration of the hepatitis B surface antigen gene, the control and the transformed coleus plants were tested by PCR. For this purpose both the plasmid and plant genomic DNA were isolated.

3.4.1 Isolation of plasmid DNA from *Agrobacterium:*

a. Materials

1. Sterile eppendorf tubes
2. Micropipette and autoclaved microtips
3. Vortex mixer
4. Microcentrifuge
5. Laminar air flow chamber.
6. Inoculation needle
7. Bacterial culture plate
8. YEP liquid medium
9. Solution I
   50mM glucose
   25mM Tris-HCl
   10mM EDTA
10. Lysozyme 10mg/mL

11. Solution II
    1% SDS + 0.2N NaOH
12. Tris saturated phenol
13. Solution III
    3M sodium acetate + acetic acid (pH 4.8)
14. Absolute alcohol and 70% alcohol
15. Chloroform
16. TE buffer
    10mM Tris-HCl (pH 7.5-8.0)
    1mM EDTA (pH 8.0)
17. Sterile double distilled water.

b. Procedure:

1. A single *Agrobacterium* colony was picked up aseptically using a sterile inoculation needle and was grown overnight in 1.0mL YEP broth containing kanamycin (50mg/L) within a sterile microfuge tube.
2. Overnight grown 1.0mL culture was added to 5.0mL YEP broth containing kanamycin and again grown overnight.
3. 1.5 mL of this culture was taken and centrifuged at 5,000 rpm for 2min.
4. The supernatant was poured off and the cell pellet was resuspended in 100µl of solution I + 25µl of lysozyme and incubated for 15-20minutes at 37^0C.
5. 200µl of freshly prepared solution II was added, mixed by inverting 5 times and kept on ice for 3-5 minutes.
6. 150µl of solution III was added to the viscous solution and mixed by inverting several times and kept on ice for 5 min.
7. The bacterial lysate was centrifuged for 10 min at 13,000 rpm.
8. The supernatant was poured out into a new tube.
9. The tube was filled with equal volumes of phenol: chloroform: isoamylalcohol (25:24:1) mixture.
10. The tube was centrifuged for 5 min at 13,000 rpm.
11. The supernatant was carefully removed and transferred to a fresh tube. To that 0.6 volumes of isopropanol was added.

12. The DNA was pelletted in a microfuge for 10 minutes at 13,000 rpm.
13. The pellet was dried at 36^0C for 1.0hr.
14. The pellet was suspended in 50 µl sterile water and 1 µg/mL of pancreatic RNase was added and stored at 4^0C (Sambrook *et al*., 1993).

3.4.2 Restriction analysis of the gene construct:

Restriction digestion was carried out for plant binary vector pHB118 to confirm the specificity of the plasmid carrying the *HBsAg* gene along with the CaMV 35S promoter and the *npt*II gene for kanamycin resistance.

a. Materials

1. Sterile1.5mL eppendorf tube
2. Plasmid DNA
3. Enzymes

   *Eco*RI

   *Bam*HI
4. Assay buffer
5. Sterile water
6. Water bath
7. Micropipette.

b. Procedure

1. Sterile 1.5mL eppendorf tube was taken and 50.0µl of the plasmid DNA was added to it.
2. Using micropipette 1.5µl each of *BamHI* and *EcoRI* (NEB,USA) were added to the eppendorf tube.
3. 10.0µl of Eco RI buffer (NEB, USA)was added to it.
4. The volume was made upto 80.0µl by adding 17µl of sterile water.
5. A short spin was given for settlement of the reaction mixture.
6. It was then kept in the water bath at 37^0C for 4hrs. followed by incubating at 65^0C for 15min. to inactivate the enzymes.

7. The digested product was run on 1.0% agarose gel electrophoresis along with the marker.

3.4.3 Isolation of plant genomic DNA:

CTAB method:

Cetyl trimethyl ammonium bromide is a detergent which is used along with other reagents to liberate nucleic acids from the cell. This is an efficient method for isolating plant genomic DNA from leaf tissues. The high molecular weight DNA obtained is purified by phenol: chloroform method to remove proteins and other plant debris.

a. Materials

1. -20^{0}C freezer
2. Extraction buffer
   2% CTAB
   1.4M NaCl
   20mM EDTA
   100mM Tris-HCl (pH8.0)
   0.2% 2-mercaptoethanol.
3. Phenol:chloroform:isoamyl alcohol mix (25:24:1 v/v)
4. Isopropanol
5. 70% ethanol
6. TE buffer 10mM Tris
   1mM EDTA (pH 8.0)
7. Sterile double distilled water
8. Pestle and mortar
9. 50mL screw-cap polypropylene tubes-autoclaved
10. Vortex mixer
11. Water bath

12. Ultracentrifuge and microfuge
13. Microfuge tubes
14. Vacuum dessicator
15. Incubator
16. Micropipette and autoclaved microtips
17. Leaf tissue from transformed and non-transformed plants
18. Balance.

b. Procedure:

1. The leaf tissue was washed in water and the excess water was blotted with blotting paper and air dried briefly.
2. 2.0g of leaf tissue was weighed from each transformed plant and control plants.
3. The leaves from individual plants were cut into pieces, placed in -20^{0}C freezer overnight and ground well using mortar and pestle with 6.0mL of prewarmed extraction buffer.
4. The extract was poured into 50.0mL polypropylene tubes and incubated at 65^{0}C in a water bath for 15-20 min with gentle shaking.
5. Equal volume of chloroform:isoamyl alcohol (24:1) mixture was added and mixed well by inverting the tubes.
6. The contents were centrifuged at 13,000 rpm for 10 min. at room temperature.
7. The supernatant was taken and to this 0.6 volume of chilled isopropanol was added.
8. Centrifuged at 10,000 rpm for 5 min.
9. The pellet was dissolved in 600µl of TE.
10. The DNA was purified by phenol: chloroform cleaning as described in the earlier experiments and further recovered by ethanol precipitation.
11. The resulting DNA pellet was dissolved in 600µl TE buffer, treated with RNase and stored at -20^{0}C (Sambrook *et al.,* 1993)

3.4.4 Spectrophotometric assay of DNA concentration:

It is critical to know exactly how much DNA is present in the solution before carrying out any experiments with it. DNA concentrations can be accurately measured by ultraviolet absorbance spectrophotometry. The amount of UV-radiation at 260nm absorbed by the solution of DNA is directly proportional to the amount of DNA present in the sample. Usually the absorbance is measured at wavelengths of 260nm and 280nm. The reading at 260nm allows the calculation of DNA concentration in the sample. An OD of 1 at 260nm corresponds to 50µg/mL of double stranded DNA.

UV-absorbance can also be used to check the purity of the DNA preparation. With a pure sample of DNA, the ratio of the absorbance at 260nm and 280nm (OD_{260}/OD_{280}) is 1.8. Ratios less than 1.8 indicate that the preparation is contaminated either with phenol or proteins. Ratios higher than this indicates the presence of RNA in the preparation.

a. Materials

1. UV-Spectrophotometer
2. 3.0mL Quartz cuvettes (1 pair)
3. 1x TE buffer (pH 8.0)
4. DNA samples to be tested
5. Micropipette and autoclaved microtips

b. Procedure

1. The spectrophotometer was first turned on for 20 min. for the equipment to stabilize.
2. 3.0mL TE buffer was taken in the quartz cuvette and placed in the reference slot as the reference sample and the equipment was auto zeroed.
3. 10.0µl of the DNA sample was taken in 2,990µl of TE buffer (1x) in the other cuvette and mixed well. This was placed in the main reading slot and

the absorbance at 260nm and 280nm wavelength was measured. Three readings were taken and the average was calculated. Using the relationship, 1 OD at A_{260}=50µg/mL, the DNA concentration per mL was calculated. The ratio A_{260}/A_{280} was also calculated to check the purity of the sample (Sambrook *et al*., 1993).

3.4.5 Confirmation of the presence of *HBsAg* gene in the transformants by PCR:

The integration of the *HBsAg* gene in the putative transformants was confirmed by polymerase chain reaction. In this study, primers specific to *HBsAg* were used to confirmation of the presence of *HBsAg* gene in the putative transformants.

DNA amplification is a very simple method for *in vitro* amplification of specific nucleic acids using *Taq* DNA polymerase and a minimum of two oligonucleotides specific to the DNA to be amplified. This helps in identification of the presence of particular gene in the transformants.

The technique involves repeated rounds of DNA synthesis, which is based on three simple steps for any DNA amplification reaction. The three steps involve: -

1. Denaturation of the template to single strands at 94^0C.
2. Annealing of primers to each original strand for new strand synthesis at 54^0C.
3. Extension of the new DNA strands from the primers at 72^0C.

a. Materials

1. DNA amplification reagent kit (Bangalore Genei Pvt Ltd, India)
2. *Taq DNA polymerase* (250 units, 3U/µl)
3. dNTP's (2.5mM solution)
4. 10x *Taq DNA polymerase* buffer with 15mM $MgCl_2$.

5. *HBsAg* specific primers-
    i. Forward primer- (5' GCATTCTACTTCTATTGCAGC3') and
    ii. Reverse primer- (5' ACGTGGTAACTTAGATGTACACCCAAAG-3') for specific amplification of *HBsAg* gene.
6. DNA samples to be amplified -plasmid DNA as positive control, DNA from transformed plant samples and DNA from control plant samples as negative control.
7. Mineral oil.
8. Sterile PCR tubes.
9. Micropipeter and sterile tips.
10. Thermocycler.

b. Procedure

1. Autoclaved double distilled water of volume 38.0µl was added into the 0.5mL PCR tubes.
2. Add 5.0µl of *Taq* DNA polymerase assay buffer with 15mM $MgCl_2$.
3. Add 3µl of 2.5mM dNTP mix solution into the mixture (which was obtained by diluting each 10mM dNTP stock to 2.5mM concentration).
4. Add 2µl of sample DNA (template) (125ng/µl)
5. Add 0.5µl each of forward and reverse *HBsAg* specific primers (5pM/µl).
6. Then 1.0µl of *Taq DNA polymerase* (3units/µl) was added and the solution was mixed gently by pipetting up and down.
7. The reaction mixture was then covered with a layer of mineral oil to avoid any evaporation (two-three drops) and kept in a thermocycler.

PCR was performed initially for 2 min at 95^0C followed by 30 cycles of melting at 94^0C for 30 sec., annealing at 54^0C for 30 sec. and strand synthesis at 72^0C for 30 sec. with a final extension at 72^0C for 5 min. and final hold at 4^0C after all the cycles were completed (Hanumantappa, 2006).

After the reaction was over 20µl of the mixture was taken out and agarose gel electrophoresis was conducted on 1.0 % agarose.

3.4.6 Agarose gel electrophoresis of DNA

a. Materials

1. TBE buffer (5x stock):
    a. Tris base (54g/L)
    b. Boric acid (27.5 g/L):
    c. EDTA (0.5 M, pH 8.0) 20.0mL/L.

   The pH of the buffer was adjusted to 8.0.
2. Loading buffer 0.25 percent bromophenol blue + 30% glycerol.
3. 1.4 % agarose gel (horizontal).
4. DNA sample after PCR reaction.
5. Gel frames and comb (with a teeth size of 5mm and spaced at a distance of 3mm).
6. Ethidium bromide stock (10.0mg/mL concentration).
7. UV-transilluminator (230-280nm).

b. Procedure

1. The frame of the gel casting unit was cleaned, dried and sealed with a tape to form a mould.
2. The frame was placed on a flat platform to ensure a flat and level base. The comb was then positioned parallel to the open edge of the frame about 2mm above the surface. Agarose powder was added to TBE buffer (1x) and dissolved by heating it to 100^0C. The solution was cooled to 50^0C and ethidium bromide was added to the gel to achieve a final concentration of 0.5µg/mL. It was then poured into the gel frame and allowed to set. After setting, the gel was transferred to the gel tank such that the wells were positioned near the negative pole. The gel tank was filled with TBE buffer (1x) just enough to cover the surface of the gel.

3. 20µl of amplified DNA samples (plasmid, plant and other control) were mixed with 4µl of loading dye (6x), bromophenol blue and loaded into the wells of the submerged gel using a micropipeter. 10µl of a 100 bp marker DNA was also mixed with loading buffer as a marker and loaded into one of the wells.
4. The electrophoresis apparatus was connected to the power supply and electrophoresis was carried out at 50V for 3hrs or when the bromophenol blue dye had migrated to the end of the gel.
5. It was then visualized on an UV-transilluminator (Sambrook *et al*., 1993).

3.5 Determination of Hepatitis B Surface antigen small protein expression through SDS -PAGE:

The molecular weight of the Hepatitis B surface antigen small protein (*HBsAg* S-protein) encoded by the hepatitis B surface antigen gene is known to be 24kDa. To find if the gene was being expressed in the plant, the total protein from the plants was extracted, concentrated and subjected to SDS- PAGE to obtain a profile of the proteins present in it based on their molecular weight. The separated proteins were visualized by silver staining the gel.

3.5.1 Extraction of total proteins from leaf samples:

a. Materials

1. Liquid nitrogen
2. Extraction buffer:
    i. 50mM Tris-HCl (pH 8.4)
    ii. 150mM NaCl
    iii. 1mM $CaCl_2$
    iv. 1% insoluble polyvinyl pyrrolidone (PVP)
    v. 1mM phenyl methyl sulphonyl fluoride (PMSF)
3. Pestle and mortar
4. Cheese cloth

5. Centrifuge
6. Micropipeter and autoclaved pipette tips

b. Procedure

1. 10g leaf sample was washed and blotted dry.
2. It was then frozen in liquid nitrogen.
3. This frozen sample was powdered with a pestle and mortar.
4. 50mL of cold extraction buffer was mixed with the leaf powder and stirred for 15min.
5. The solution was filtered through cheese cloth.
6. The filtrate obtained was centrifuged at 13,000 rpm for 10min to remove any debris.
7. The supernatant was collected and stored at 0^0C.

3.5.2 Desalting of the protein:

a. Preparation of Dialysis tubing

The dialysis tube with a molecular weight cutoff of 12 kDa, was cut into convenient lengths and boiled for 10min in 2% w/v sodium bicarbonate and 1mM EDTA (pH 8.0) solution. The tube was rinsed with distilled water and boiled again in 1mM EDTA. Before use the tubing was washed in double distilled water. The tube was always handled with gloves.

The protein sample (5 mL) was loaded into this dialysis bag which was then sealed with a closure clip at either end. This was then dialyzed against double distilled water for 14hrs. at 4^0C. Fresh water was replaced once in every 4-5hrs.

3.5.3 Concentration of the protein sample

a. Dialysis:

The proteins in the dialysis bag was covered with sucrose and kept at room temperature for 3-4hrs, until the sample volume in the bag was reduced to 2mL. Then the bag was washed with double distilled water and opened. The sample was collected in a microcentrifuge tube and labeled.

b. Tri chloro acetic acid (TCA) precipitation of proteins:

For further concentration, the protein sample was precipitated with TCA as follows.

1. Half ml of the sample was taken in a micro centrifuge tube to which 250µl of 10% TCA (2:1 v/v) was added.
2. This was kept on ice for 1hr.
3. The samples were then centrifuged at 10,000 rpm for 10min at 4^0C.
4. The supernatant was discarded and to the pellet, 50µl of 90% aqueous acetone was added.
5. This was then centrifuged at 12,000 rpm for 5min at 4^0C.
6. The supernatant was discarded and the pellet was washed with 100µl of 100% acetone.
7. The tube was once again centrifuged at 10,000 rpm for 5 min at 4^0C. The supernatant was decanted out.
8. The pellet was dried and dissolved in 50µl of SDS-PAGE loading buffer.

3.5.4 Quantification of the crude protein by FCR method:

a. Materials

1. Solution A: 2.0g NaOH + 10.0g Na_2CO_3 in 500mL of deionized water.
2. Solution B: 1.0mL of 1.35% sodium potassium tartarate + 0.1mL of 5% $CuSO_4.5H_2O$.
3. Solution C: 50mL of solution A + 1.0mL of solution B.
4. 1.0N FCR reagent

5. Standard bovine albumin solution
6. Deionized water
7. 5mL cuvettes
8. UV spectrophotometer
9. Tissue paper
10. Micropipette
11. Glass tubes

b. Procedure

1. Different aliquots ranging from 0.2mL to 1.0mL standard albumin solution was added into the glass tubes.
2. The volume was made upto 1.0mL with distilled water.
3. Blank was prepared without adding standard albumin solution
4. Simultaneously samples were also dispensed into the tubes (10µl sample + 990µl distilled water).
5. 5.0mL of solution C was added into each tube and mixed well.
6. The tubes were incubated at room temperature for 10min.
7. 0.5mL of FCR was added, mixed well and incubated in dark for 30 min.
8. Absorbance value was taken at 660nm.

3.5.5 Protein estimation by Bradford's method:

Principle:

The assay relies on the binding of the dye Coomassie-blue G250 to protein. The quantity of the protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 625/595 nm.

Kit components:

1) Protein standard, BSA (10 x 5 mg)
2) Bradford's reagent (125 ml x 4)
3) Micro pipettes

4) Spectrophotometer

Precautions:

Presence of the following compounds in the sample are known to interfere in the assay and they should be avoided.

1) High molarity of the buffers of low, high pH or strong bases
2) Detergents
3) Ammonium sulfate 3%
4) EDTA 10 mM

Standard protein:

Reconstitute one vial containing 5 mg BSA with 1 ml distilled water to get 5 mg/ml. This is stable at 4°C for 2 weeks. Dilute 0.2 ml of 5 mg/ml BSA solution with 0.8 ml distilled water to get 1 mg/ml just before use.

Procedure:

1) Pipette out standard BSA 1 mg/ml, samples and distilled water referring to the protocol given in the following table and adjust the volume to 0.2 ml.
2) 2 ml of Bradford's reagent was added, mixed and kept for 10 minutes.
3) The optical density / the intensity of the color was measured at 625 nm/595 nm or on a colorimeter using suitable filter.
4) The calibration curve is constructed by plotting optical density reading on 'Y' axis against standard protein μg/tube.
5) The value 'X' from the graph corresponding to the optical density reading for individual samples were recorded.
6) The sample concentration was calculated using the following formula.
7) Samples concentration = X/V

Where,

X = value from graph in μg

V = volume of sample in μl

BSA 1 mg/lt

Sl No.	Std/ samples µg/tube	Amount in µl	Water in µl	OD reading	X is µg
1	BLANK	0	200		
2	10	10	190		
3	20	20	180		
4	40	40	160		
5	60	60	140		
6	80	80	120		
7	Sample	'v'	200-v		

3.5.6 SDS- PAGE:

The protein samples from the transgenic and control plants were electrophoresed on 8% polyacrylamide gel as follows

a. Materials

1. Power cards
2. Power supply unit
3. Gel plates
4. 90% acetone
5. Silica gel
6. Clips
7. Gel running casting
8. Coombs and spacers

b. Buffers:

1. Loading buffer (2x): 0.1M Tris HCl (pH 6.8); 0.002% bromophenol blue
   2% v/v glycerol
   1% w/v SDS
   10% v/v 2-mercaptoethanol
2. Resolving gel buffer: 1.5M Tris HCl (pH 8.8)
3. Stacking gel buffer: 1.0 M Tris HCl (pH 6.8)
4. Electrode buffer (pH 8.3): 0.025M Tris base
   0.192M glycine
   0.1% SDS
5. Resolving gel solution (10mL):
   2.5mL of 1.5M Tris HCl (pH 8.8), SDS 0.4%
   2.7mL of acrylamide and bis acrylamide mix (36.5:1) w/w in 100mL

4.7mL of deionised water
100µl of 10% APS
6µl of TEMED

f. Stacking gel solution (2.5mL):

0.315mL of 1.5M Tris HCl (pH 6.8), 0.8% SDS
0.415mL of acrylamide and bis acrylamide mix
25µl of 10% APS
1.725mL of deionised water
3µl of TEMED

c. Procedure

The glass plates (10.5cm x 10.5cm) and spacers were cleaned with acetone and assembled into a cassette as described by Sambrook *et al*., 1993. The resolving gel solution (10mL) was poured into the cassette and a layer of distilled water was filled over it. It was allowed to polymerize at room temperature. After polymerization the water was poured out and the cassette was blotted dry. The stacking gel was then poured into the plates and the comb was inserted into it. It was allowed to polymerize. The distance between the bottom of the wells and the resolving gel was maintained at 1cm. After polymerization, the glass sandwich containing the gel was fixed on to the vertical electrophoresis unit, taking care to remove the bottom spacer and the comb. Electrode buffer was poured into the unit and the level of the buffer was maintained above the wells. Anode and cathode were connected to negative and positive poles of the power pack respectively. The wells were loaded with samples mixed 1:1 with the loading buffer. Electrophoresis was carried out at 20 mA for 4hrs. After the run was completed the gel was removed from the glass plates and visualized by silver staining (Laemmli, 1970)

3.5.7 Coomasie blue staining of SDS-PAGE electrophoresis proteins

Coomassie blue staining is one of the important methods of visualizing proteins in a gel that can detect even nano gram of proteins. The principle involved is that the coomassie blue will bind to the protein molecules and upon

reduction of the coomassie blue, the proteins become visible. It was done as follows.

a. Materials:

1. Staining solution

0.1g of coomasie brilliant blue R-250

40mL methanol

10mL glacial acetic acid

50mL water

2. Destaining solution

40mL methanol

10mL glacial acetic acid

50mL water

b. Procedure

1. The gel was placed in staining solution for 5-6 hrs or overnight with gentle shaking.
2. The staining solution was removed and the destaining solution was added.
3. The gel with destaining solution was kept on a shaker for 4-5 hrs.
4. After this, destaining solution was removed and the gel was stored in polythene cover with proper sealing.

3.5.8 Silver staining of SDS- PAGE electrophorased proteins:

Silver staining is a highly sensitive method of visualizing proteins in a gel that can detect even nano grams of proteins. It is based on the principle of silver nitrate binding to the protein molecules. On reduction of the silver nitrate to metallic silver, the proteins become visible. Extreme care must be taken to avoid

background staining caused due to the residual antibodies in the polyacrylamide. It was done as follows;

a. Materials

1. Solution A(Fixer): 50mL methanol
   12.5mL acetic acid
   50µL formaldehyde
   37.45mL distilled water
2. Solution B: 20mg sodium thiosulphate in 100mL water
3. Solution C: 40mL Silver nitrate 0.2% solution (w/v).
4. Solution D (Developer): 50mL 6% Na_2CO_3 + 0.5mL solution B
6. Stop solution: 5% acetic acid

b. Procedure

1. The gel was left to stand in solution A for 2 hrs.
2. It was then washed with 50% methanol for 20min with fresh methanol replaced every 6 min.
3. The gel was then kept in solution B for 60 sec.
4. It was then washed with distilled water three times for 20 seconds each time.
5. The gel was then transferred to solution C and allowed to stand for 15min.
6. Step 4 was repeated again.
7. Finally the gel was developed with solution D with gentle rocking until the bands gained sufficient intensity, turning black or dark brown.
8. The developer was immediately drained and replaced by the stop solution to stop further development of color.

3.6 Immunodetection of Hepatitis B surface antigen gene:

Immunodetection uses a specific antibody to detect and localize a protein blotted to the membrane. The specificity of antibody-antigen binding permits the identification of a single protein in a complex sample. Immunodetection can be done by Protein dot blotting and Western Blotting detection methods.

3.6.1 Protein Dot Blotting

Procedure:

1. Preparing the membrane:

a) The Membrane was wetted in methanol for 15 sec. The membrane should uniformly change from opaque to semi-transparent.
b) The membrane was carefully placed in Milli-Q water and soaked for 2 min.
c) The membrane was carefully placed in buffer and allowed equilibration for at least 5 min.

2. Protein transfer by spotting:

1. Each 5-10 µL of transgenic and control plant samples were spotted onto the membrane. Sample should wick into the membrane. Note: Membrane should be wet enough to absorb sample, but not so wet that sample spread across membrane.
2. After the sample was absorbed, the membrane was placed on clean filter paper for drying.

3. Antibody incubations

1. The membrane was placed in the blocking solution and incubated with agitation for 1 hr.
2. The blot was placed in the primary antibody solution and incubated with agitation for one hr. The solution should move freely across the surface of the membrane.

3. The blot was placed in PBST and wash for 10 min. Repeated twice with fresh buffer.
4. The blot was placed in Secondary antibody solution and Incubate with agitation for 1 hr.
5. The blot was placed in PBST and wash for 10 min. Repeat twice with fresh buffer.
6. After washing, the membrane was placed in DAB substrate solution which provides colour only where there is binding of the primary and secondary antibody. Usually colour develops within 1 –2 min. The reaction should be carried out in dark conditions.
7. The reaction was stopped by rinsing the membrane with water and dried under dark conditions. The membrane should not be exposed to light as the dots fade out.
8. Photograph was taken for permanent record.

3.6.2 Western blotting confirmation of hepatitis B surface antigen gene by Semi – dry transfer:

A western blot defined as the electrophoresis of the antigen followed by its subsequent transfer to nitrocellulose paper and incubation with specific antibody and then with labelled secondary antibody.

Materials :

1) Transfer buffer : 300 ml

25 mM Tris GR – 0.909 gms

192 mM Glycine – 4.32 gms

20% methanol - 60 ml

0.1% SDS – 0.3 gms

Blocking buffer : 50 ml

2) 5% skimmed milk powder dissolved in 1x PBS

3) Wash solution PSBST

1 x PBS + 0.05% Tween 20

4) Ponceau S solution : 0.5% Ponceau S in 1% acetic acid

5) Primary antibody solution

1 x PBST

1% skimmed milk powder

1:1000 dilution of primary antibody (Mouse anti Hepatitis B surface antigen)

6) Secondary antibody solution

1 x PBST

1% skimmed milk powder

1% normal goat serum

1:1000 dilution of goat anti-mouse IgG horse radish peroxidase conjugate

7) DAB (Diamino benzidine) substrate solution

5 mg of DAB powder

15 µl of H_2O_2

Dissolved in 10 ml of 1 x PBS. H_2O_2 should be added just before putting the membrane into the substrate solution. The solution should be prepared in a brown bottle.

8) Whatman 12 mm filter paper

1) Nitrocellulose membrane filter (0.45 µm)
2) Semi dry electroblotting apparatus or transblot apparatus

11) Deionised milli Q water

Procedure:

1. The protein samples were prepared and separated by SDS-PAGE. The length and width of the resolving gel was measured.

2. Once SDS-PAGE was completed, Whatmann no 1 filter paper was cut to the same dimension as that of the gel. Usually 12 such pieces were cut out and pre soaked in the transfer buffer.
3. The PVDF membrane was also cut exactly to the same size as that of the gel and soaked in transfer buffer prior to use. The gel was soaked in the transfer buffer for equilibration.
4. Preparation of the transfer stack: 6 pieces of filter paper were stacked over one another followed by the nitrocellulose membrane, gel and over the gel another 6 pieces of filter paper were placed one over the other.
5. Before placing the gel, suitable marks are made to indicate the position of the marker lane.
6. Eletroblotting should be done for 1 hr and 30 min at 50 volts and 45mA current.
7. After blotting, place the PVDF filter in Ponceau S solution for 15-20 min, to stain the proteins, which have got transferred.
8. Destain the membrane for one minute by using distilled water and mark the protein marker bands with permanent maker.
9. Then completely destain the membrane in distilled water until bands disappear.
10. The membrane is then placed in the blocking solution and kept at 4°C overnight (The blocking is done so that the area of the nitrocellulose where no protein has been transferred is occupied by milk powder, which in turn prevents non-specific binding of the antibody).
11. Next day blocking solution was poured out and washed the nitrocellulose membrane with PBST wash solution. 3 washes of 5 min each should be done.
12. The nitrocellulose membrane was placed in the primary antibody solution and kept at room temperature on a rocker for 2 hrs.
13. The membrane was washed with PSBST wash solution. 5 washes of 5 min each was carried out, changing the wash solution after each wash.

14. After washing, the membrane was placed in the secondary antibody solution for 2 hrs at room temperature on a rocker.
15. Later the membrane was washed with PSBT wash solution. 5 washes of 5 min each was carried out, changing the wash solution after each wash.
16. After washing, the membrane was placed in DAB substrate solution which provides colour only where there is binding of the primary and secondary anti body. Usually colour develops within 1 –2 min. The reaction was carried out in dark conditions.
17. The reaction was stopped by rinsing the membrane with water and dried under dark conditions. The membrane should not be exposed to light as the bands fade out.
18. Photograph was taken for permanent record.

3.6.3 Enzyme linked immunosorbant assay confirmation of immunogenic nature of the recombinant hepatitis B surface antigen gene.

ELISA is based on the ability of low molecular weight antibodies to couple with enzymes to produce enzymatically active immunological conjugates. This allows the detection of immune reaction with histochemical staining techniques, because the antibody component is involved in immune reaction and the conjugated enzyme can be used for staining the reaction utilizing appropriate substrates.

Materials and reagents for ELISA:

1) Tarson's 96 well flat bottom ELISA plates
2) Multichannel micropippette
3) Autoclaved microtips
4) Trough
5) 0.05 M carbonate buffer pH 9.6

Solution A : Dissolve 0.53g Na_2CO_3 (anhydrous) in distilled water and make up the final volume to 100 ml.

Solution B : Resolve 0.42 g Na HCO_3 in distilled water and make up the final volume to 100 ml.

To 32 ml of solution a, slowly add solution B till pH is 9.6 (approximately 68 ml).

6) Phosphate citrate buffer, pH 5.0

Solution A : Dissolve 11.9 gms of NO_2 $HPO_4.2H_2O$ in distilled water and make up the volume to 1 litre.

Solution B : Dissolve 7 gms of citric acid in distilled water and make up the volume to 1 litre.

Mix 55 ml of soution 'a' with 45 ml of solution 'b'. Adjust pH to 5.0 with either 'a' or 'b' if necessary.

7) 0.01 M Phosphate buffer saline (pH 7.2), 1000 ml

NaCl – 8 gms

Kcl - 0.2 gms

KH_2PO_4 – 0.2 gms

$Na_2HPO_4.2H_2O$ -1.5 gms

The pH is adjusted to 7.2 with 0.1 N Na0H or 0.1 N Hcl if necessary.

8) Wash solution PBST

0.5 ml of tween 20 (Polyoxyethylene sorbitan monolaurate) was added per litre of 1 x PQS to prepare PBST.

9) Primary antibody solution: 10 ml of 1 x PBST

2 µl of primary antibody i.e., Mouse anti hepatits b surface antigen (1:5000 dilution).

10) Secondary antibody solution

10 ml of 1 x PBST

10 ul of secondary antibody i.e goat antimouse IgG horse radish peroxidase conjugate (1:1000 dilution).

11) Blocking solution

15 ml of 1% skimmed milk powder in PBST

12) Substrate solution

4 mg of orthophenyl diamine was dissolved in 10 ml of phosphate citrate buffer (pH 5.0) and to this 10 µl of H_2O_2 was added. The substrate solution was prepared just before use in a brown coloured bottle.

Procedure:

1) The transgenic and control protein samples being used as the antigen were diluted using carbonate buffer such that the concentration is 1 µg per 100 µl.
2) 100 µl of the sample was dispensed into each well of the ELISA plate and kept overnight at 4°C or at 37°C for 2 hours for antigen coating.
3) The ELISA plate was then washed thrice with wash solution.
4) The plate was then tap dried on a dry filter paper.
5) The ELISA plate was then blocked by dispensing 150 µl of blocking solution per well. The plates were incubated at 37°C for 2 hrs.
6) The ELISA plate was washed thrice with wash solution.
7) 100 µl of primary antibody solution was then dispensed per well.
8) The ELISA plate was incubated at 37°C for 2 hrs.
9) The plate was then washed thrice with wash solution. It was tap dried on a dry filter paper.
10) The secondary antibody solution was dispensed at 100 µl per well.
11) The plate was then incubated at 37°C for 2 hrs
12) After incubation the plate was washed thrice with wash solution.
13) The ELISA plate was tap dried and 100µl of substrate solution was added per well.
14) The ELISA plate was incubated at room temperature for 20 minutes.
15) 50 µl of 4 N sulphuric acid was added per well to stop the reaction.
16) The ELISA plate was read at 492 nm using an ELISA reader.

3.7 Study of growth and yield parameters of transgenic and control Tobacco plants:

The control and transgenic plants were obtained through co cultivation using *Agro bacterium* strain with CaMV-35S promoter with Hepatitis B surface antigen gene. These plants were used for the growth parameters of control and transgenic plants.

The observations recorded were:

1. Height of the plant
2. Number of leaves
3. Leaf area
4. Number of flowers

3.8 Inheritance of the transgene:

Materials

l. Seeds of transgenic tobacco plants and control plants.

2. Petriplates

3. Distilled water

4. 100 ppm of Kanamycin

5. Blotting paper

Procedure

1. The blotting paper was cut into round shape and placed in Petri plates.

2. They were moistened with 100 ppm of kanamycin.

3. The seeds from different transgenic tobacco plants and non transgenic tobacco plant were placed on the blotting paper (32 seeds number in each plate).

4. These Petri plates were sufficiently moistened for favorable germination of seeds and development of the seedlings.

5. The observations were taken after 10 days of sowing by counting the germinated seeds and ratio between germinated and non germinated seeds in presence of kanamycin was calculated.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Plants have emerged as an excellent alternative to other expression systems for the production of complex pharmaceutical proteins, including recombinant subunit vaccines. It was shown that some plant-derived antigens can induce systemic and mucosal immune responses and in some cases, confer protection against challenges (Goldstein *et al*., 2005). Despite some difficulties with the expression of certain recombinant proteins, especially those of viral origin, plant biotechnology holds the promise for producing recombinant proteins to be used in vaccine formulations Mason *et al*. (1992). Expression of *HBsAg* in transgenic tobacco in the form of virus like particles (VLPs) through stable transformation with foreign DNA has been reported. Subsequently, Thanavala *et al*. (1995) demonstrated that the plant derived *HBsAg* was immunogenic in mice.

A detailed study was conducted on the *Agrobacterium* mediated transformation of Tobacco with Hepatitis B recombinant protein (*HBsAg*).Transgenic plants of T_0 and T_1 generation were analyzed for the morphological characters as well as integration and expression of *HBsAg* through SDS-PAGE, Protein dot blot and Western blot. This study helped in understanding the inheritance and stability of the Hepatitis B surface antigen in transgenic tobacco.

4.1 Restriction analysis of the plasmid DNA

Confirmation of presence of *HBsAg* gene in the vector pHB118 (13.3 Kb) was carried out by restriction digestion analysis of the plasmid with *Eco RI* and *Bam HI.* The insert between *Eco* RI and *Bam* HI was of 3.6 kb, consisting of *npt II* marker gene, nos terminator gene and *HBsAg* gene along with 5! UTR driven by double CaMV 35 S promoter. The restriction digestion with these enzymes released two bands each of 3.6 Kb and 9.7 Kb (Plate 1) when run on 1.2 % agrose gel at 50 V. This has given the primary confirmation of the presence of *HBsAg*

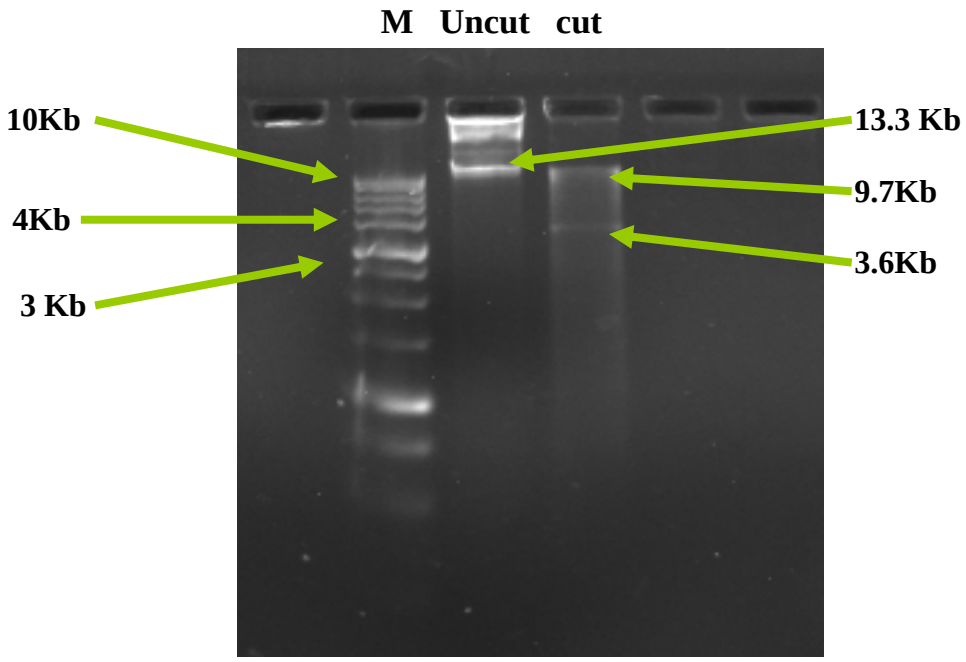


Plate 1: Restriction digestion analysis of the plasmid construct of HBsAg (pHB118).

Legend
M- 1 kb DNA Marker
Uncut – Undigested plasmid DNA
Cut –Digested plasmid DNA with *EcoRI* and *Bam HI*

gene in the vector. For further confirmation of presence of the gene, gene specific primers were used to amplify the 900 bp *HBsAg* gene.

4.2 Confirmation of gene integration

4.2.1 PCR analysis of *HBsAg* gene

The *npt-II* gene was used as a selectable marker for the study. There are reports that even though plants seem to be transformed on a selection medium, they might not have been transformed, having escaped on the selection pressure. Therefore, it is necessary to look for the integration of these genes.

PCR analyses of the genomic DNA from the leaves of putative transformed Tobacco plants of T_0 and T_1 generation, plasmid DNA isolated from *Agrobacterium* and the genomic DNA from control plants have shown the presence of 900 bp amplified band of *HBsAg* gene on both putative transformed plants and the plasmids, but it was absent in control plants when primers specific to *HBsAg* gene were used. In T_0 generation all the three putative transformants plants shown the positive results (Table 1 and Plate 2). In T_1 generation seeds from plant1 were sown, eight plants have shown the positive results (Table 2 and Plate 3).

4.2.2 SDS PAGE Analysis

SDS PAGE was carried out by extracting the proteins from the leaves of transformed and control plants of the T_0 and T_1 Tobacco plants. The transgenic plants containing the recombinant protein have shown a thick band at around 24 KDa revealing the over expression of the hepatitis B surface antigen gene present in the transgenics. In T_0 generation three putative transformants have shown the over expression of *HBsAg* gene (Table 1 and Plate 4) and in T_1 seven putative transformants have shown the over expression of *HBsAg* gene (Table 2 and Plate 5).

4.2.3 Confirmation of antigenicity of the hepatitis B surface antigen expressed in transgenic tobacco plants:

4.2.3.1 Protein Dot Blotting

Protein dot blotting is the quick method of confirming the presence of specific proteins in the total proteins. This was carried out by spotting 5-10 μL of each transgenic and control plant samples onto the membrane. After the sample was absorbed, the membrane was incubated with blocking solution followed by incubation in primary and secondary antibody for one hour each. The detection of brown spot was done by using the substrate DAB (Diamino benzidine). All the three putative transformants of the T_0 generation of Tobacco plants showed the formation of brown dot which was not detected in the untransformed control (Plate 6) and in T_1 generation seven putative transformants have showed the formation of brown dot which was not detected in the untransformed control (Plate 8 and Table 2).

4.2.3.2 Western blotting:

The crude protein samples extracted from the T_0 and T_1 generation and control tobacco plants were used as antigens for the western blotting. The successful expression of the hepatitis B surface antigen gene in transgenic tobacco plants was summarized in the Table 1 and Plates 7. Following immunostaining, transgenic tobacco plant samples indicated positive results of the 24 kDa antigenic Hepatitis B surface antigen, which was not detected in the untransformed control plant samples. The results indicated that the transgenic tobacco plants positively contain the immunogenic hepatitis B surface antigen.

4.2.4 Testing the immunogenic nature of the transgenic plant protein samples by ELISA:

Enzyme linked immunosorbant assay is based on the ability of low molecular weight antibodies to couple with enzymes to produce enzymatically active immunological conjugates. This allows the detection of immune reaction,

because the antibody component involved in the immune reaction is conjugated with an enzyme which can be used for staining reaction utilising appropriate substrate.

The crude protein samples of control and transgenic T_0 and T_1 generation tobacco leaf were used for ELISA. The mouse anti-Hepatitis B Surface Antigen was the primary antibody and goat anti mouse IgG horse radish peroxidase was the conjugate used. In case of T_0, all the transgenic samples gave an absorbance significantly higher than the control (Table 3 and Figure 2). The transgenic leaf protein sample gave an absorbance value of 1.10. This was significantly higher than the negative control which gave an absorbance of 0.59 and was comparable with the absorbance value of the commercial vaccine which was at 1.21.

In case of T_1 generation, all the transgenic samples gave an absorbance significantly higher than the control (Table 4 and Figure 3). The transgenic leaf protein sample gave an absorbance value of 0.16 which was significantly higher than the negative control (0.09) and was comparable with the absorbance value of commercial vaccine (0.223).

Table 1: Molecular analysis of T_0 generation transgenic tobacco transformed with Hepatitis B surface antigen

Character	Transgenic plants			Control plants
	1	2	3	
PCR	+	+	+	_
SDS-PAGE	+	+	+	_
Western blot	+	+	+	_

Legend:

+ Positive result

- Negative result

Table 2: Molecular analysis of T_1 generation transgenic tobacco transformed with Hepatitis B surface antigen

Character	Transgenic plants								Control plants
	1	2	3	4	5	6	7	8	
PCR	+	+	+	+	+	+	+	+	–
SDS-PAGE	+	+	+	+	+	+	+	–	–
Protein dot blot	+	+	+	+	+	+	–	–	–

Legend:

+ Positive result

- Negative result

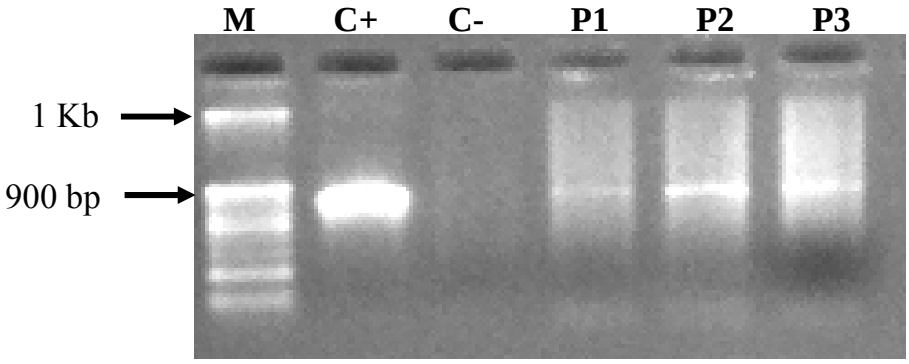


Plate 2: PCR amplification of Hepatitis B surface antigen in T_0 generation transgenic Tobacco plants with *HBsAg* gene specific primers

Legend:

M : 100 bp DNA marker
C+ : Positive control (Plasmid DNA)
C- : Negative control (non transgenic tobacco plant)
P1- P3: Putative transformants

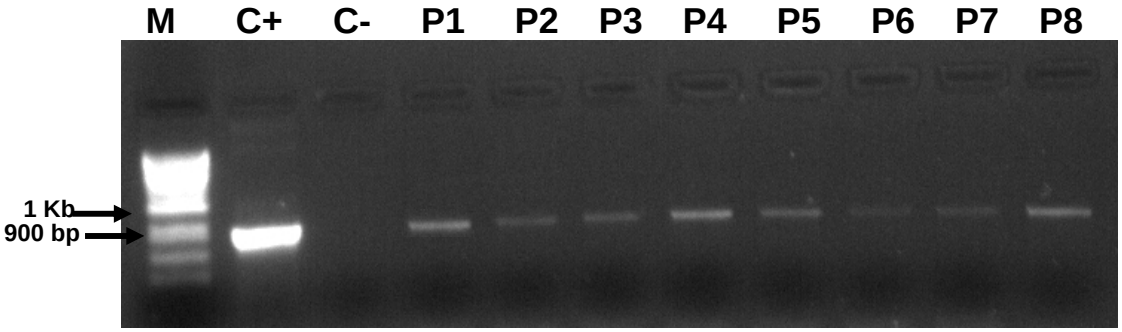


Plate 3: PCR amplification of Hepatitis B surface antigen in T_1 generation transgenic Tobacco plants with *HBsAg* gene specific primers

Legend:
M : 1 Kb DNA marker
C+ : Positive control (Plasmid DNA)
C- : Negative control (non transgenic tobacco plant)
P1- P8: Putative transformants

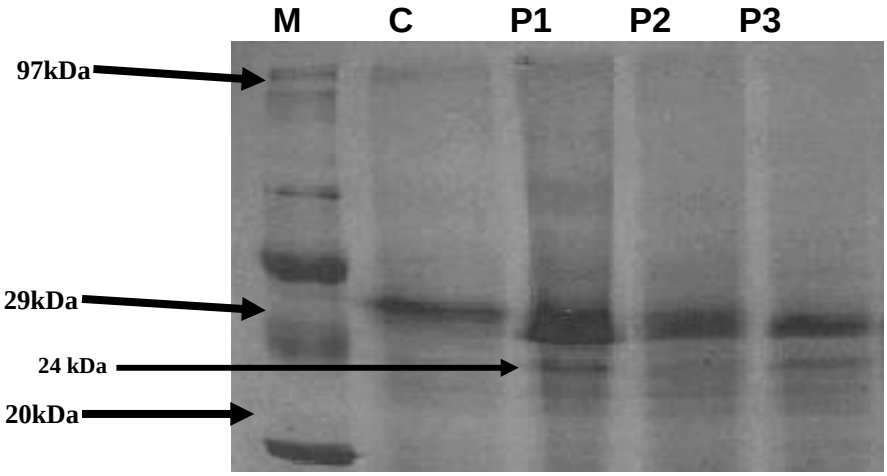


Plate 4: Confirmation of expressed HBsAg protein in T_0 generation tobacco plants by SDS- PAGE

Legend:

M : Protein molecular weight marker
C : Crude protein from control tobacco
P1-P3: Crude protein from putative transformants

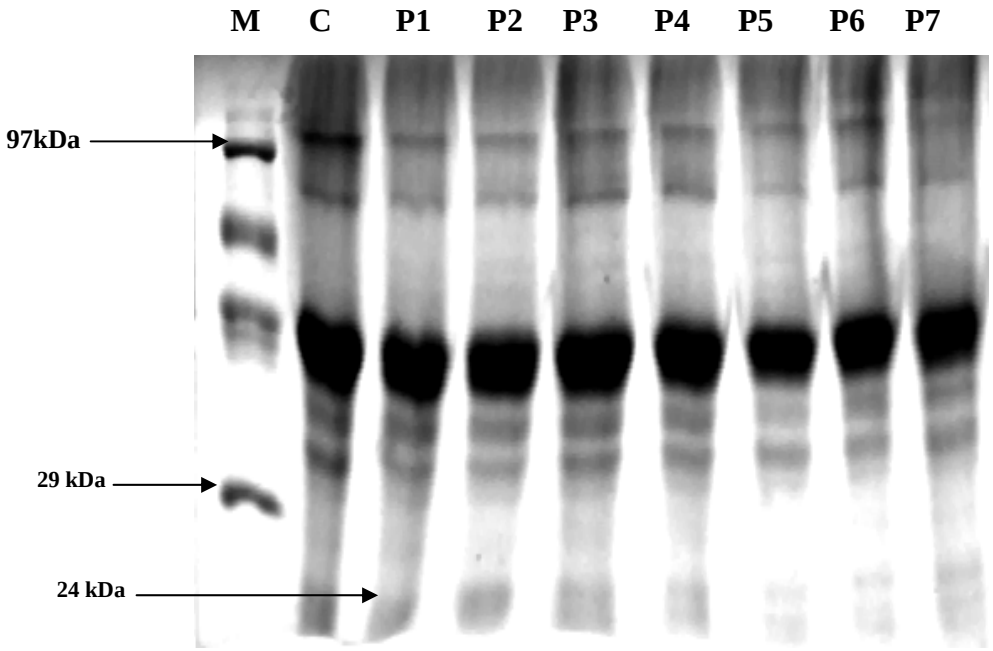


Plate 5: Confirmation of expressed HBsAg protein in T_1 generation tobacco plants by SDS- PAGE

Legend:
M : Protein molecular weight marker
C : Crude protein from control tobacco
P1-P7: Crude protein from putative transformants

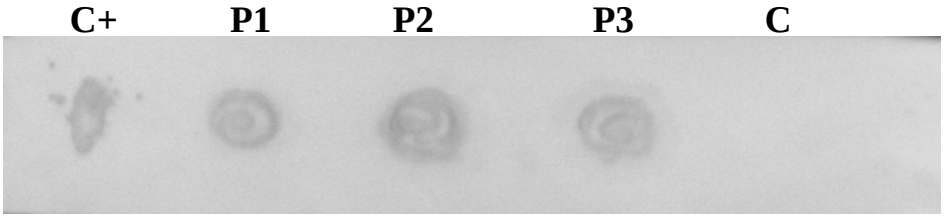


Plate 6: Protein dot blot analysis showing the expression of the Hepatitis B surface antigen from the T_0 generation transgenic tobacco.

Legend:
C+ : Positive control (commercial vaccine)
P1-P3: Crude protein from putative transformants
C - : Crude protein from control tobacco

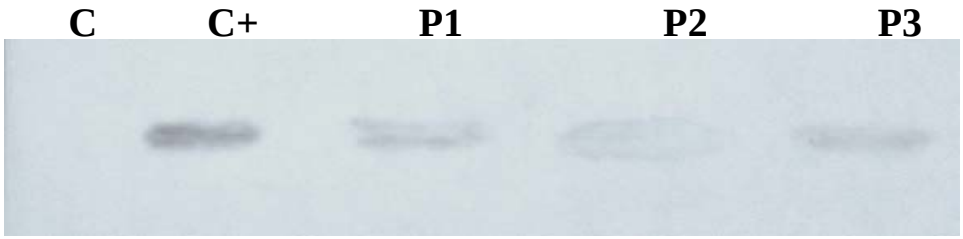


Plate 7: Western blot analysis showing the expression of the 24 kDa Hepatitis B surface antigen from the T_0 generation transgenic tobacco

Legend:

C+ : Positive control (commercial vaccine)
P1-P3: Crude protein from putative transformants
C - : Crude protein from control tobacco

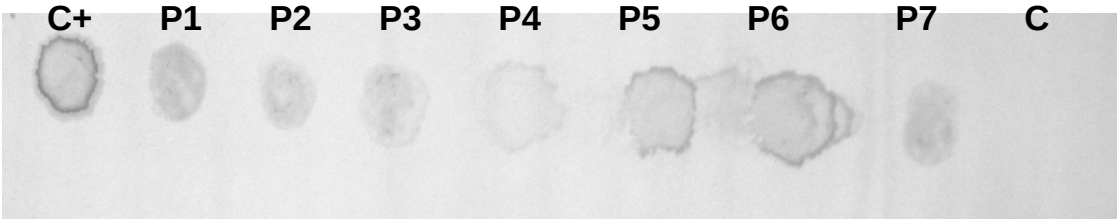


Plate 8: Protein dot blot analysis showing the expression of the Hepatitis B surface antigen from the T_1 generation transgenic tobacco.

Legend:
C+ : Positive control (commercial vaccine)
P1-P7: Crude protein from putative transformants
C : Crude protein from control tobacco

Table 3: ELISA absorbance values taken at 492 nm for transgenic and control tobacco (T_0) leaf protein.

Samples	**OD_{492nm}**
T1	1.052
T2	1.10
T3	1.022
C	0.59
CV	1.212

Legend:
T1-T3 = Transgenic tobacco leaf protein samples..
C = Control tobacco leaf protein sample.
CV= Commercial vaccine.

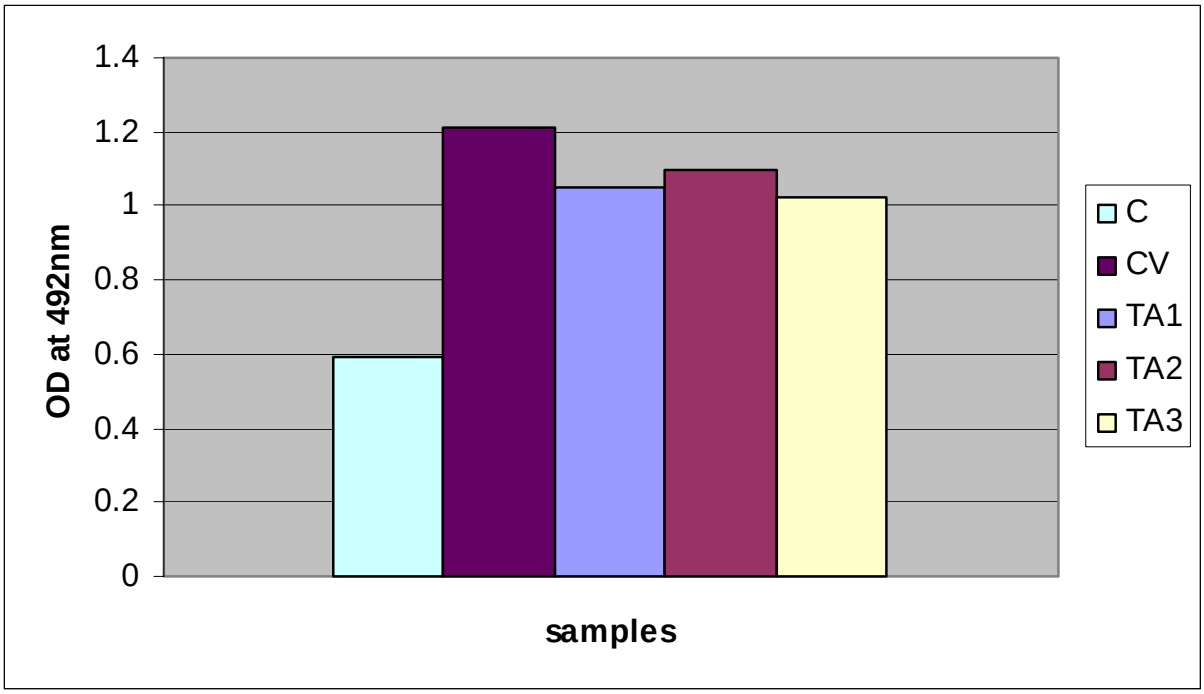


Figure 2: ELISA absorbance values recorded at 492nm for T_0 generation of transgenic and control tobacco leaf protein (crude) samples

Table 4: ELISA absorbance values taken at 492 nm for transgenic and control tobacco (T_1) leaf protein.

SAMPLES	OD_{492NM}
T1	0.160
T2	0.149
T3	0.137
T4	0.142
T5	0.158
T6	0.145
T7	0.138
T8	0.138
C	0.090
CV	0.223

Legend:

T1-T8 = Transgenic tobacco leaf protein samples.

C = Control tobacco leaf protein.

CV= Commercial vaccine.

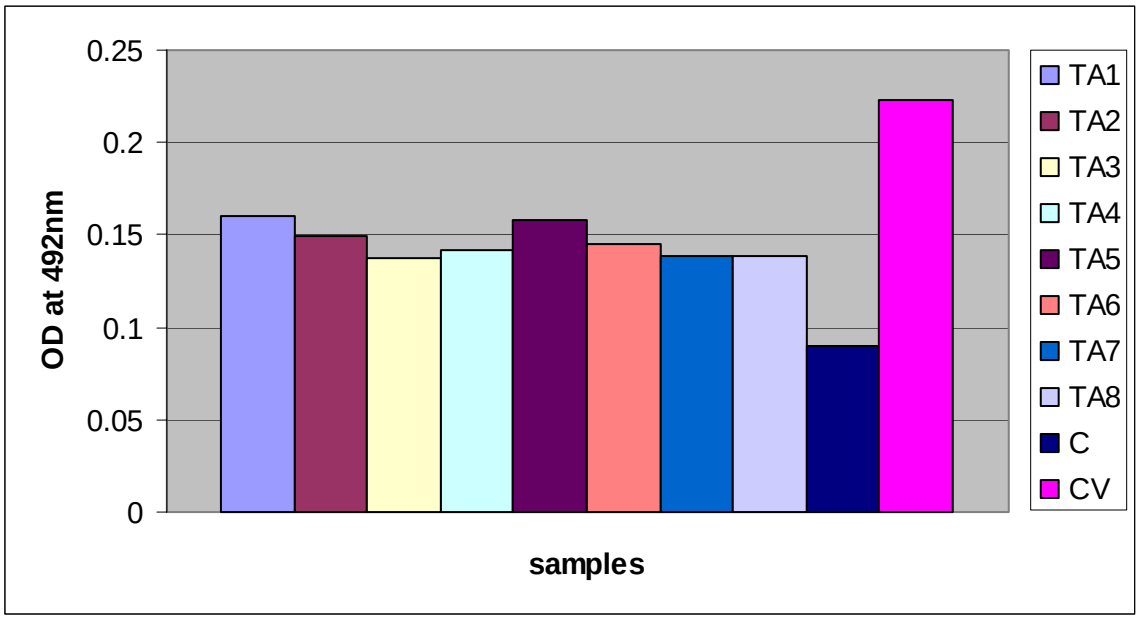


Figure 3: ELISA absorbance values recorded at 492nm for T_1 generation of transgenic and control tobacco leaf protein (crude) samples.

4.3 Observations on growth and yield parameters of T_0 and T_1 generation transgenic and control tobacco plants:

Insertion and expression of a foreign gene into the plant genome may lead to insertional mutagenesis. This may silence some of the constitutive gene of the plant genome essential for the normal growth of the plant. Hence, it may cause abnormalities in the plant growth. To study the effect of *HBsAg* gene integration and expression on growth and yield parameters of tobacco, the growth and yield parameters of transgenic plants (T_0 and T_1) and non transgenic tobacco were recorded and compared.

The growth parameters of both transformed and control tobacco plants were recorded after 90 days of sowing (Table 5). The data was analysed using single factor ANOVA. No significant difference was observed on the plant height of both transgenic and non transgenic plants. However, the average height of the transgenic plants was higher (70.29 cm) than the average height of the control plants (67.85 cm). No significant difference was observed in number of leaves per plant. But the average number of leaves per plant in case of transgenic (18.68) was higher than in the control plant (18.32). All the transgenic plants showed better growth when compared to the control plants (Plate 9).

To conduct similar studies on second generation (T_1) of transgenic tobacco plants, the T_0 generation seeds of transgenic tobacco plants with hepatitis B surface antigen producing gene and non transgenic tobacco seeds were sown. Growth parameters were recorded after 30 days of sowing (Table 6). For second generation also, no significant difference in the growth parameters was observed. The higher plant height and more number of leaves in transgenic plants were observed in second generation compared to the non transgenic tobacco plants. The average height of the transgenic plant (3.67 cm) which was higher than the

average height of the control plants (3.63 cm). The average number of leaves per plant in case of transgenic (4.69) was higher than in the control plants (4.54).

4.4 Studies on inheritance of Hepatitis B surface antigen gene in the seeds obtained from transgenic tobacco:

npt II gene was used a selectable marker gene in transformation of tobacco with *HBsAg* gene. The expression of *npt* II gene in transgenic plants confers resistance to kanamycin and aid in selection of putative transformants in tissue culture. This trait can also be used to study the stable gene expression in transgenics over generations. This is done by germinating seeds of transgenic and non transgenic plants on optimum concentration of kanamycin.

In this study, the seeds obtained from the transformed tobacco plants with hepatitis B surface antigen gene were tested for their germination in presence of kanamycin. The seeds were allowed to germinate on the moist blotting paper containing 100 ppm of kanamycin. It was observed that out of 32 transgenic tobacco seeds studied, 25 to 26 seeds were able to germinate in 100 ppm of kanamycin and remaining seeds were unable to germinate. Where as seeds from the nontransgenic tobacco seeds, none of them germinate under similar conditions. (Table7 and Plate 10). This study also reveals the Mendelian pattern of inheritance as the number of transgenic seeds germinating on the 100 ppm kanamycin and non germinating seeds were in 3:1 ratio.

Table 5: Comparison of growth parameters of non transgenic Tobacco with T_0 generation of transgenic tobacco with Hepatitis B Surface Antigen (90 Days after sowing).

Trait	**Average plant height (cm)**	**Average leaf length (cm)**	**Average leaf breadth (cm)**	**Average leaf area (cm^2)**	**Average no. of leaves**	**Average no. of flowers**
Control	67.85	21.23	9.24	196.11	18.38	11.85
Transgenic	70.29	21.35	9.32	198.92	18.62	11.77
CD	4.97	0.27	0.11	2.8	0.48	0.041
SEm	2.32	0.12	0.056	1.3	0.22	0.019
CV (%)	8.57	1.51	1.53	1.6	3.15	5.46

* T test

Table 6: Comparison of growth parameters of non transgenic Tobacco with T_1 generation of transgenic tobacco with Hepatitis B Surface Antigen (30 Days after Sowing).

Trait	Average plant height(cm)	Average leaf length(cm)	Average leaf breadth(cm)	Average leaf area(cm^2)	Average no. of leaf
Control	3.63	3.23	2.19	7.06	4.54
Transgenic	3.67	2.81	2.08	5.85	4.69
CD	0.132	0.27	0.22	0.827	0.419
SEm	0.061	0.128	0.104	0.386	0.196
CV (%)	4.31	1.84	5.43	4.28	6.84

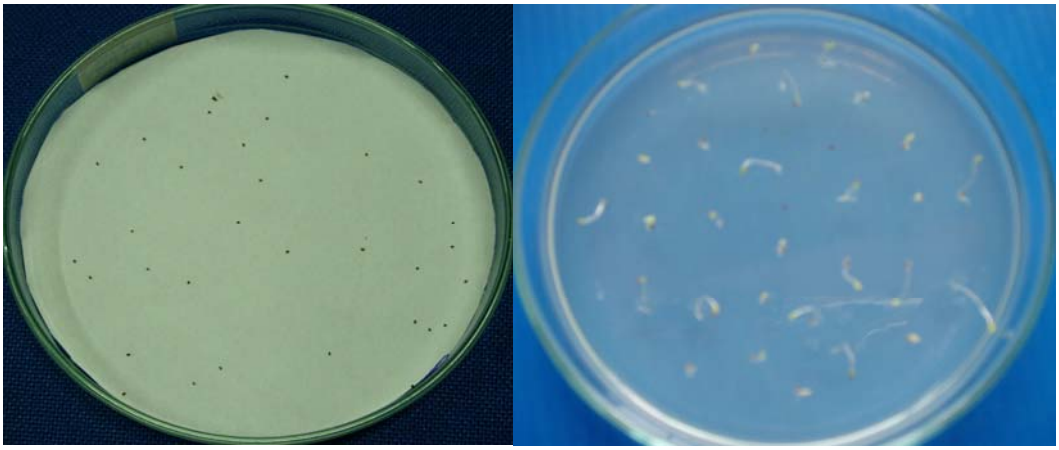
* T test

Control **Transgenic**

PLATE 9: Comparison of T_0 generations of transgenic and control tobacco plants grown in green house.

Table 7: Kanamycin assay for study of transgene expression in transgenic (T_1) and non transgenic tobacco seeds by germination on 100- ppm of kanamycin.

	NO. OF SEEDS SOWN	100- PPM OF KANAMYCIN	
		Germinated	Non-germinated
Non Transgenic	32	0	32
T1	32	23	9
T2	32	24	8
T3	32	25	7



Control **Transgenic**

Plate 10: Germination of seeds obtained from nontransgenic (control) and transgenic T_1 tobacco plants in presence of 100 ppm of kanamycin.

DISCUSSION

V DISCUSSION

Hepatitis B is a significant cause of morbidity and mortality. In India, there are 43 million people who are estimated to be *HBsAg* carriers, which constitutes to 4.7% of the total carriers, the second largest pool of HBV carriers in the world. Majority of these are vaccine preventable diseases. Hepatitis B is one among the most important infectious diseases coming under this group. There are about 350 million chronic carriers of HBV, which is about 5% of the total world population. It is estimated that 75-100 million of them will die of liver cirrhosis and/or hepatocellular carcinoma (Sunil Kumar *et al*., 2007).

Hepatitis is an inflammation of the liver, which can be caused by viruses, medications or toxic agents. It is usually characterized as viral hepatitis or non-viral hepatitis. Viral hepatitis can be considered "acute" (a condition that comes on rapidly with severe symptoms and a short course) or "chronic" (a condition that comes on slowly, may or may not have symptoms which has a long course).

Therapeutic vaccines against chronic infectious diseases aim at eliciting broad humoral and cellular immune responses against multiple target antigens. Importantly, the development of such vaccines will help to establish surrogate markers of protection in humans and thus will augment the subsequent development of efficient prophylactic vaccines. A combination of synthetic small-molecule drugs and immunotherapeutic is likely to represent a powerful means of controlling chronic infections in the future.

Immunization has/had a profound impact on global health, in rich countries as well as poor. Immunization is cheap, reliable and effective, and is reaching the majority of children in low-income countries. But capitalization on the full potential that immunization offers has not been possible. Many more lives in the developing world could be saved and improved with increased access to existing and new vaccines. First, there is need to improve the availability of existing vaccines. About 3 million people die every year of diseases that can be prevented with existing vaccines is evidence of a profound failure in the current system. This

failure can be remedied with more predictable financial resources, stronger political commitment to public health, greater investment in both immunization-specific and broader elements of health systems and better management at all levels, global to local. Second, there is need to accelerate the development of new vaccines targeted to and appropriate for the epidemiological conditions and health systems of developing countries. Another part of the solution is to ensure sufficient funding and the right incentives for innovation to develop new health technologies, both in the short and long term.

Plant biotechnology offers additional advantages in production scale, economy, product safety, and ways of delivery. At present, the main goal is to increase the overall expression, yield of functional plant-derived proteins, especially those of viral origin, which sometimes seriously impair transgenic plant growth and development.

There is a growing interest to develop oral vaccines for infectious diseases, as it is the most convenient and effective way to attain mucosal immunity. Progress in plant genetic engineering has enabled the transfer of useful genes for desirable traits. The recent trend is to use this technique to exploit plants as biofactories for the production of therapeutic proteins including vaccines (Sunil Kumar *et al*., 2007). The production of foreign proteins in plants has several advantages. In terms of required equipment and cost, mass production in plants is far easier to achieve than techniques involving animal cells. The biggest advantage of plant vaccine is that it does not need any refrigeration for its storage (Okada, 1997).

In the present investigation, we have selected tobacco as an expression system, since it serves as a model crop and also to study the stability pattern of the Hepatitis B recombinant protein accumulation in subsequent generation. Tobacco was transformed with hepatitis B surface antigen gene through *Agrobacterium* mediated transformation. This method of gene transfer is highly efficient one,

resulting in stable integration of the inserted gene. The integration of the gene in the resulting transgenics was tested through PCR analysis, SDS-PAGE and western blotting.

5.1. Confirmation of gene integration:

5.1.1. Polymerase Chain Reaction (PCR)

The genomic DNA was isolated from putative transformants and non transformed tobacco plants. The plasmid DNA of pHB118 carrying *HBsAg* gene was used as positive control. The genomic DNA from control plant was used as negative control. Integration of the gene was confirmed by using the *HBsAg* specific primers. In T_0 generation three putative transformed plants chosen for the analysis, all the three plants have shown the presence of single 900 bp amplified product, which is the size of the *HBsAg* gene. In T_1 generation out of eight putative transformed plants chosen for the analysis, all eight plants have shown the presence of single 900 bp amplified product. The results are in confirmation with Zhong *et al.* (2005), Hanumantappa (2006) and sandesh (2007). Cent percent PCR positive T_1 generation might also due to recombinant selection done on kanamycin media.

5.1.2. SDS-PAGE:

The protein was extracted from transformed and untransformed tobacco plants and subjected to SDS-PAGE to see the expression of *HBsAg* gene. The SDS-PAGE gel revealed the presence of the band around 24 kDa in three putative transformants in T_0 generation and seven putative transformants in T_1 generation of transgenic tobacco and the absence of same band in control plants indicating the over expression of the gene in the transgenic tobacco plants. The results are in confirmation with Zhong *et al.* (2005), Hanumantappa (2006) and sandesh (2007).

5.1.3 Western blotting analysis of the Hepatitis B surface antigen produced in transgenic tobacco plants:

The protein product of the Hepatitis B surface antigen gene was detected on western blot using monoclonal antibody specific for a hepatitis B virus. The successful expression of the hepatitis B surface antigen gene in transgenic tobacco plants shown positive results of the 24 kDa antigenic Hepatitis B surface antigen, which was not detected in the untransformed control plant samples. This result was consistent for all the T0 and T1 generation transgenic tobacco leaf protein samples tested. The results indicated that the transgenic tobacco plants positively contain the immunogenic hepatitis B surface antigen. The results are in confirmation with Zhong *et al.* (2005).

5.1.4 Testing the immunogenic nature of the transgenic tobacco plant protein samples by ELISA:

ELISA is a rapid and sensitive technique by which the immunogenic nature of a large number of samples can be detected. The crude protein samples of T0 and T1 generation transgenic tobacco leaves were coated at the concentration of 1 μg per well as an antigen. Enriched mouse anti Hepatitis B surface antigen was used as the primary antibody. The immune complex formation was detected by using goat anti-mouse IgG horse radish peroxidase conjugate and the chromogen orthophenylene diamine with hydrogen peroxide as substrate. The negative control used was an untransformed tobacco plant protein sample whereas the positive control was the Hepatitis B commercial vaccine. The highest OD was recorded in one of the transgenic sample (1.10) which was significantly higher than the negative control which gave an OD of 0.59. The commercial vaccine gave an OD of 1.21. A similar trend was seen in case of the T1 generation transgenic tobacco plants. All the T1 generation transgenic tobacco leaf samples gave a significantly higher OD of around 0.16 which was well over that of the negative control which gave an OD of 0.09. The higher OD values of the transgenic plant samples

indicate the immunogenic nature of the sample which is a confirmation of the presence of the recombinant Hepatitis B surface antigen. This also indicates that the Hepatitis B surface antigen is expressing the immunologically important epitopes. This forms the validation for the earlier SDS-PAGE and western blot confirmation**.**

5.2 Observation on growth parameters of T_0 and T_1 generation transgenic and control Tobacco plants:

The T_0 generation transgenic tobacco plants were raised in the green house along with the control evaluated for their growth characters besides checking for the integration of the gene and accumulation of the Hepatitis B surface antigen. There was no inhibition of growth in the transgenic plants. The average height of the transgenic plants was 70.29 cm which was higher than the average height of the control plants which were 67.85 cm. All the transgenic plants showed better growth when compared to the control plants. The average number of leaves per plant in case of transgenic (18.68) was higher than in the control plants (18.32).

The seeds of the T_0 generation transgenic tobacco were sown and 8 T_1 generation plants were raised in the green house along with 2 controls. The average height of the transgenic plants was 3.67 cm which was higher than the average height of the control plants which was 3.63 cm. The average number of leaves per plant in case of transgenic (4.69) was higher than in the control plants (4.54).The increase in plant height in case of transgenic may be due to knocking out of a gene during the random integration of the Hepatitis B surface antigen gene during *Agrobacterium* mediated transformation and also may be due to variation in the fertility levels or may be due to segregation of the plants.

5.3 Studies on inheritance of Hepatitis B surface antigen gene in the seeds obtained from transgenic tobacco:

In this study, the seeds obtained from the transformed tobacco plants with Hepatitis B surface antigen gene were tested for their germination in presence of kanamycin. The seeds obtained from the transgenic tobacco plants show the hepatitis B surface antigen segression ratio of 3:1 as expected confirming to Mendelian inheritance, when they were germinated in presence of 100 ppm of kanamycin. This results in confirmation with the Vasil *et al*., (1993), where he transferred gene coding for phosphinothricin acetyl transferase (PAT) in wheat plants, PAT activity was detected in a 3:1 ratio in R1 generation plants following cross or self pollination. Both male and female transmission of the PAT gene and its segregation as a dominant Mendelian ratio in R1 and R2 plants. And chowda reddy, (1999) showed that the seeds obtained from the transgenic tobacco plants show the glucane-chitinase gene segregation in the ratio of 3:1 in R_1 generation. But contradictory results showed by Becker *et al*., (1994).He reported that the histochemically stained pollen grains of a GUS gene transformed wheat plants showed a 1:1 segregation of the uid A gene in all the plants tested. A 3:1 segregation of the introduced gene was demonstrated by enzyme sensitivity test and southern blot analysis of R_1 generation plants.

SUMMARY

VI SUMMARY

Despite medical advances that have produced hundreds of drugs that are safe and effective against bacteria, viruses, fungi and parasites, infectious diseases are still a major cause of death, disability and social and economic upheaval for millions around the world. More than 90 percent of the deaths from infectious diseases worldwide are caused by only a handful of disease. Each year, infectious diseases kill 17 million people worldwide and are a major public health concern. Of these, 6.3 million belong to developing countries. Hepatitis B is one among the most important infectious diseases coming under this group.

Hepatitis B is caused by Hepatitis B virus (HBV) that affects more than 2 billion people worldwide. It is a major global health problem, 360 million people have chronic infection and 6.0 lakh people die each year from HBV-related liver disease or hepatocellular carcinoma. The estimated number of annual new cases due to hepatitis B disease is at closer to 2.0 lakh. In India, there are 43 million people who are estimated to be *HBsAg* carriers which constitutes to 4.7% of the total carriers, the second largest pool of HBV carriers in the world. It has been estimated that presently hepatitis B kills more people every day than AIDS kills in a year world-wide.

HBV causes acute and chronic hepatitis. The chances of becoming chronically infected depend upon age. About 90% of infected neonates and 50% of infected young children will become chronically infected. In some individuals who become chronically infected, especially neonates and children, the acute infection will not be clinically apparent. The treatment for acute hepatitis B patient is rest, combined with a high protein/high carbohydrate diet to repair damaged liver cells and protect the liver. If hepatitis B persists, the doctor may recommend an antiviral agent called interferon.

Immunization with Hepatitis B vaccine is the most effective means of preventing HBV infection and its consequences. Vaccination is given three times spaced over 6 periods. The first dose of hepatitis B vaccine should be administered immediately and the remaining doses one and six months later. As the available recombinant vaccine is expensive, there is still need for a less expensive vaccine source in most developing countries.

New and improved vaccines are urgently needed to address the global scourge of infectious diseases. With over two billion people worldwide infected with HBV there is great need for an inexpensive vaccine that would allow vaccination of large segments of the population. Plants are a potential source of *HBsAg* that is not dependent upon process technology to ensure protein folding and particle assembly. In addition, a plant based *HBsAg* expression system makes possible the testing of an oral immunization strategy by simply feeding the plant samples.

The primary means of transformation is the *Agrobacterium* mediated gene transfer which has provided a reliable means of creating transformants in a wide variety of species and also can express a wide variety of pharmaceutically important products including recombinant vaccines.

The present investigation lays emphasis on study of integration and stability of the recombinant protein expressed in Tobacco plants. The highlight of this investigation is summarized below:

1. Restriction digestion analysis of the gene construct pHB118 with restriction enzymes *Eco*RI and *Bam*HI yielded two separate bands of 9.7kb and 3.6kb size the size of uncut plasmid DNA was 13.3kb when run on agarose gel along with ladder.
2. The leaf explants of tobacco were transformed with Hepatitis B surface antigen gene along with *npt-II* as an antibiotic selection marker gene. The

presence of *HBsAg* gene in putative transformants were confirmed by PCR analysis. All the putative transformed tobacco plants showed the presence of 900 bp band in the PCR analysis.

3. The crude protein obtained from the transformed tobacco plants were tested by SDS-PAGE, western-blot and ELISA, which gave positive results indicating that there was no loss in confirmation and immunogenic nature of the Hepatitis B surface antigen.
4. The presence and expression of hepatitis B surface antigen small protein in the transformed tobacco plants were confirmed by the presence of a 24 kDa size foreign protein that was expressing only in the transgenic tobacco plants.
5. Three putative transformants from T_0 and seven putative transformants from the T_1 generation tobacco were subjected to western blotting have indicated expression of foreign gene.
6. The T_0 generation seeds obtained from the transgenic tobacco plants were tested for the germination in presence of kanamycin. It was observed that the segregation ratio was 3:1 indicating Mendelian inheritance.
7. The growth parameters of T_0 and T_1 generation transgenic and control tobacco plants were recorded. There was no major variation in growth between transgenic and control plants.

In conclusion, our study has shown that it is possible to transform and express tobacco with *HBsAg* gene using *Agrobacterium* mediated transformation. The integration of recombinant *HBsAg* gene in transgenic tobacco plants have shown the presence of 900 bp band with *HBsAg* gene specific primers. The expression of 24 kDa Hepatitis B surface antigen in transgenic tobacco plants were shown by SDS-PAGE analysis. The specificity of the crude protein from transgenic tobacco plants were also analyzed by western blotting and dot blot. The

stable integration and expression of the gene in T_0 and T_1 generations were confirmed by PCR, SDS-PAGE, western blot, ELISA and Kanamycin assay.

Future line of work:

1. Standardization of the purification method for *HBsAg* S-protein from the transgenic plants.
2. Immunological studies of the plant extracted *HBsAg* S-protein on experimental animals.

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VII REFERENCES

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* Original not seen.

APPENDIX

APPENDIX

REAGENTS AND BUFFERS

Phenol: Chloroform: Isoamylalcohol (25:24:1)

A mixture consisting of equal parts of equilibrated phenol and chloroform:isoamylalcohol (25:24:1) was used to remove proteins and lipids from preparations of nucleic acids. Isoamylalcohol was used to reduce foaming during extraction.

Neither chloroform nor isoamylalcohol required treatment before use. The phenol:chloroform: isoamylalcohol mixture was stored in a brown bottle at 4^0C.

Preparation of commonly used stock solution:

1. 0.5 M EDTA (pH 8.0): 186.11g of disodium Ethylenediaminotetra acetate was added to 800mL of H_2O. Stirred vigorously on magnetic stirrer. The pH was adjusted to 8.0 with NaOH (20g of NaOH pellets) and dispensed into aliquots and sterilized by autoclaving.

2. Ethidium Bromide: 1.0g of ethidium bromide was added to 100 mL of H_2O. Stirred on a magnetic stirrer for several hours to ensure that the dye had dissolved. The container was wrapped with aluminium foil or the solution was stored in a brown bottle at room temperature.

3. 3M Sodium Acetate: 408.1g of sodium acetate $3H_2O$ was dissolved in 800 mL of H_2O, the pH was adjusted to 5.2 with glacial acetic acid or the pH was adjusted to 7.0 with dilute acetic acid. The volume was adjusted to 1 litre with water, dispensed into aliquots and sterilized by autoclaving.

4. 5M NaCl: 292.7g of NaCl was dissolved in 800mL of water, adjusted the volume to 1 litre with water, dispensed in aliquots and sterilized by autoclaving.

5. 10% SDS: 100g of electrophoresis grade SDS was dissolved in 900mL of H_2O heated to 68^o C to assist dissolution. Adjusted pH to 7.2 by

adding few drops of concentrated HCl the volume was adjusted to 1 litre.

6. 1M Tris: 121.1g of Tris-base was dissolved in 800mL of H_2O, adjusted the pH to the desired value by adding concentrated HCl. Allowed the solution to cool to room temperature before making final adjustment in pH, adjusted the volume to 1 litre, sterilized by autoclaving.

ELECTROPHORETIC BUFFER

Tris-borate (TBE)	0.5X: 0.045M Tris borate 0.001M EDTA	5X: 54g Tris base 27.5g boric acid 20mL of 0.5M EDTA pH 8.0

GEL LOADING BUFFER

Buffer Type	**6X**	**Storage Temperature**
I	0.25% Bromophenol blue 0.25% Xylene cyanol FF 30% (w/v) Glycerol in water	4^oC

CALCULATIONS:

A) DNA concentration: The concentration of DNA in the dilution was calculated by assuming that DNA at a concentration of 50µg/mL has an OD of 1.0 at 260nm.

DNA concentration = (OD) X (dilution factor) X (50µg DNA/1.0 A_{26o} unit)

B) Calculation of the total yield:

µg DNA recovered = (DNA concentration in µg/mL) X (total volume in mL)