EXPRESSION OF HEPATITIS C VIRAL NON-STRUCTURAL 3 ANTIGEN IN TRANSGENIC CHLOROPLASTS

by

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ABSTRACT

Hepatitis C viral infection is the major cause of acute hepatitis and chronic liver disease and remains the leading cause of liver transplants (NIH). An estimated 180 million people are infected globally (WHO). There is no vaccine available to prevent hepatitis C. The treatment with antiviral drugs is expensive, accompanied with various side effects and is limited only to those at risk of developing advanced liver disease. The treatment is also effective in only about 30% to 50% of treated patients and still a high percentage of patients are resistant to therapy. Therefore, there is an urgent need for the development of effective vaccine antigens and an efficacious HCV vaccine. The non-structural 3 protein of the hepatitis C virus is a multifunctional protein of the virus required for virus polyprotein processing and replication. Vaccine antigen production via chloroplast transformation system usually results in high expression levels and eliminates the possibility of contamination with vector sequences, human or animal pathogens. The HCV NS3 antigen was expressed in the chloroplast of *Nicotiana tabacum var. Petit havana* and *LAMD-609*. The 1.9kb NS3 gene was cloned into a chloroplast expression vector, pLD-AB-Ct containing the 16S rRNA promoter, aadA gene coding for the spectinomycin selectable marker, *psb*A 5' untranslated region to enhance translation in the light and 3' untranslated region for transcript stability and trnI & trnA homologous flanking sequences for site specific integration into the chloroplast genome. Chloroplast integration of the NS3 gene was first confirmed by PCR. Southern blot analysis further confirmed site-specific gene integration and homoplasmy. The NS3 protein was detected in transgenic chloroplasts by immunoblot analysis. The NS3 protein was further

quantified by ELISA. Maximum expression levels of NS3 up to 2% in the total soluble protein were observed even in old leaves, upon 3-day continuous illumination. These results demonstrate successful expression of the HCV non-structural 3 antigen in transgenic tobacco chloroplasts. Animal studies to test the immunogenecity of the chloroplast derived HCV NS3 will be performed using chloroplast derived NS3 antigen.

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LIST OF ACRONYMS/ABBREVIATIONS

NS3 - Non-Structural 3 protein

HCV- Hepatitis C Virus

INTRODUCTION

Hepatitis C: a silent epidemic

Hepatitis C is the major cause of acute hepatitis and chronic liver disease, including cirrhosis and liver cancer and remains the leading cause of liver transplants (NIH Consensus Conference, 2002). An estimated 170 million people are infected globally, with HCV and 3 to 4 million people newly infected each year (WHO, 1997). Over 30,000 new HCV infections still occur each year in the US and Canada. HCV infection is the most common chronic blood borne infection and is mainly transmitted through blood transfusions that are not screened for HCV infection, through the reuse of inadequately sterilized needles, syringes or other medical equipment, or through needle sharing among drug-users (Alter et al., 1995). The incidence of infection has changed over time as the risk of exposure has decreased by the introduction of blood screening measures in 1992, although infections continue to occur, primarily among high-risk groups such as injection drug users (IDUs). Hepatitis C has been described as an insidious disease and a 'silent epidemic' mainly because acute infection is asymptomatic in most patients. The most common symptoms are fatigue and jaundice. It is characterized by a high tendency to chronicity with about 70% to 80% of acutely infected patients progressing to develop chronic infection with 10% to 20% of this developing cirrhosis (Sharara et al., 1996). Most chronically infected patients experience a slow insidious progression of chronic liver disease, but have few, if any symptoms or physical signs of the disease for decades. An estimated 5% -10% of chronically infected persons eventually die from either cirrhosis or liver cancer (Mast, 1999).

There is no vaccine currently available to prevent hepatitis C. HCV is highly heterogeneous with six genotypes and multiple subtypes and the virus exists in chronically infected individuals as a quasi-species population that is, with multiple but related sequences. There is no in vitro replication system available to produce large quantities of the virus for studying viral replication and to assay immune responses. A transfection system producing subgenomic HCV RNA replicons and supporting high-level RNA replication has been recently established (Lohmann et al., 2001). Additionally, there is an incomplete understanding of the immunologic mechanisms that control the virus during natural infections. There is also no small animal model of HCV infection and the chimpanzee is the only naturally susceptible animal other than humans (Bukh et al., 2001). There is limited availability and high costs associated with utilizing the chimpanzee for studies (Bukh et al., 2001). The replication cycle of HCV is incompletely understood due to the lack of an efficient cell culture system and a small animal model permissive for HCV infection.

The treatment of chronic hepatitis C is currently based on antiviral drugs such as interferon α monotherapy or a combination therapy with pegylated interferon α and ribavirin (NIH Consensus Conference, 2002). Treatment with interferon α alone is effective in about 20% to 30% of treated patients (Camps et al., 1993). Recent trials combining IFN- α plus ribavirin administration have improved these results with the treatment effective in about 30% to 50% of patients but there is still a high percentage of patients resistant to therapy (Poynard et al., 1998). There are various side effects associated with the use of these antiviral drugs (McHutchison et al., 1999). The major types of side effects include fatigue, influenza-like symptoms, gastrointestinal

disturbances, neuropsychiatric symptoms, and hematologic abnormalities (Maddrey et al., 1999). The cost of treatment is also very high. The average cost of treatment using the marketed recombinant IFNα2b is $26,000 for a twelve-month course (Cowley et al., 1996). The high costs associated with the treatment, adverse side effects and the prolonged treatment limit the use of the therapy. Therefore, developing strategies for vaccination as well as for treatment of HCV infection is of great importance.

Hepatitis C virus (HCV) is one of the viruses (A, B, C, D, and E), which together account for the vast majority of cases of viral hepatitis. An estimated 1.5 million clinical cases of hepatitis A occur each year. The hepatitis B virus (HBV) is the only DNA-containing hepatitis virus. It is also a global health problem with more than two thousand million people infected and approximately 350 million chronically infected and at high risk of serious illness and death from cirrhosis of the liver and primary liver cancer (WHO, 1997). There are vaccines available to prevent hepatitis A and hepatitis B. The available inactivated whole virus HAV vaccine appears to be highly effective in decreasing the incidence of infection although vaccine costs and unfounded concerns about vaccine safety have been impediments (Averhoff et al., 2001). The current commercially available yeast derived recombinant hepatitis B vaccine which has been licensed since 1986, utilizes the HBV viral envelope, the hepatitis B surface antigen (HBSAg) as an immunogen (Mason et al., 1992). However, it is technology-intensive and therefore comparatively expensive as a health-care product for developing countries (Kong et al., 2001). As a result, efforts are being made to produce some of these in-demand vaccines in an edible form by plant-based oral delivery systems (Walmsley and Arntzen, 2000).

Although it was known in the 1970s that a certain fraction of post-transfusion hepatitis cases were not caused by the hepatitis A or hepatitis B virus, it was not until recently in 1989 that the causative agent of the disease (non-A, non-B hepatitis) was cloned (Choo et al., 1989). This initiated research efforts leading to the elucidation of the genomic organization and the definition of the functions of most viral proteins of the hepatitis C virus. Although information about the molecular mechanisms of HCV replication is still missing, it is often thought that it may follow a replication strategy similar to that of other plus strand RNA viruses. Hepatitis C virus is a small RNA virus that belongs to the flaviviridae family and is the sole member of the genus Hepacivirus (Houghton et al., 1996). Six major HCV genotypes and more than 100 subtypes have been identified throughout the world on the basis of molecular relatedness of conserved and non-conserved regions. HCV has a 9.6kb single stranded RNA genome that encodes a single, large polyprotein of about 3000 amino acids (Choo et al., 1989). The HCV-encoded polyprotein is cleaved post-translationally into multiple structural and nonstructural (NS) proteins. The polypeptides liberated from the amino terminus of the polyprotein are the structural proteins. These include the 21kDa nucleocapsid core protein and two envelope glycoproteins, 37kDa El and 61kDa E2. The non-structural domain encodes six proteins: NS2, NS3, NS4A, NS4B, NS5A and NS5B (Takamizawa et al., 1991). The cleavage of the polyprotein into mature proteins is dependent on host and viral encoded enzymes. The region of the polyprotein containing the non-structural proteins is processed by two HCV encoded enzymes. The NS2-NS3 junction is cleaved by a proteinase associated with NS2 and the N-terminus of NS3 (Grakoui et al., 1993). The C-terminal remainder of the HCV polyprotein is further processed by a virus-

encoded serine proteinase contained within the NS3 protein to generate NS3 (68 kDa), NS4A (6 kDa), NS4B (26 kDa), NS5A (58 kDa) and the 65 kDa NS5B (Tomei et al., 1993). The downstream proteolytic cleavages by NS3 protease are important to generate the functional mature viral proteins including the NS5B RNA – dependent RNA polymerase required for replication.

The 69 kDa non-structural 3 protein (NS3) of HCV is a multifunctional protein containing a proteinase domain that has been mapped to the N-terminal 180 amino acids and an RNA dependent NTPase/ helicase domain from amino acids 181-631 at the C-terminal (Han, 1995). Viral clearance after acute hepatitis or after IFN-α therapy is usually associated with strong CD4 and CD8 T cell responses (Botarelli et al., 1993, Ferrari et al., 1994, Missale et al., 1996, Hoffman et al., 1995, Lasarte et al., 1998, Lechman et al., 1996., Cooper et al., 1999, Gruener et al., 2000, Thimme et al., 2001). In particular, cellular T helper immune response against non-structural NS3 protein has been associated to viral clearance after acute infection, whereas absence of this T cell response leads to viral persistence and chronic hepatitis (Diepolder et al., 1995, Pape et al., 1999). It has been recently described that activation of a CTL response directed against NS3 after IFN –α therapy in HCV chronically infected patients, may have a role in the control and clearance of HCV infection (Vertuani et al., 2002). Moreover, several studies have identified cytotoxic T cell epitopes within NS3 protein in HCV infected patients (He et al., Cerny et al., 1995, Kurokohchi et al., 1996). Vaccination with an adenoviral vector encoding the NS3 protein protected against infection with a recombinant vaccinia virus expressing HCV polyprotein and induced anti-NS3 humoral, T helper and T cytotoxic responses (Arribillaga et al., 2002). Oral immunization with attenuated HCV NS3

transformed *Salmonella typhimurium* to deliver DNA directly to the gut-associated lymphoid tissue induced HCV specific CTL in mice challenged with recombinant HCV NS3 vaccinia virus (Wedemeyer et al., 2001). In one study, a strong NS3 specific CD4 T cell response that is associated with viral clearance in acute hepatitis C infection was dominated by the response to a single epitope aa 1248-1261. Since viral heterogeneity is thought to be important factor in establishing chronic infection, it was found out by searching databases for NS3 sequences that the epitope was completely conserved among the different genotypes (Diepolder et al., 1997). These data suggest that NS3 might be a good target for the induction of anti-HCV immune response. Despite substantial progress, however, HCV vaccine development remains at an early stage. There is an urgent need for the development of an efficacious vaccine to prevent HCV infection. Although the failure of candidate vaccines to uniformly induce sterilizing immunity is disappointing, the reduction in the risk of chronic infection suggested in these studies is exciting. Therapeutic vaccines could also be used for clearing the virus and/or to slow disease progression in infected people (Guha, 2003). Because nearly all the morbidity and mortality of HCV infection is a consequence of chronic infection, a vaccine that prevented chronic infection would be highly desirable. Recently, HCV vaccine antigens have been expressed in plants (Nemchinov et al., 2001, El Attar et al., 2004). A plant derived recombinant HCV vaccine would be a safe source and can potentially reduce expenses normally associated with production and delivery of conventional vaccines.

Plant expression system

The applications of plants as protein production systems are wide and varied. In

recent years, plants have been increasingly explored as renewable resources for the production of important vaccines and biopharmaceuticals. The production of recombinant vaccines in plants may overcome some of the major difficulties encountered when using traditional or subunit vaccines, especially in developing countries. In developing countries, difficulties include vaccine affordability, the need for "cold chains" from producer to the site of use of the vaccine and the dependence on injection. Plant based technology could contribute to global vaccine programs as plant systems can dramatically reduce costs associated with the production of important vaccines for mass vaccination. They have greater potential for scalability as compared to microbial or animal systems as they can also be stored as seeds and unlimited vaccine quantity can be produced from them in a limited time. Plant systems are more economical than industrial facilities using fermentation or bioreactor systems and the technology is already available for harvesting and processing plants and plant products on a large scale (Daniell, 2001). If the protein is delivered orally, then the expensive step of purifying the recombinant protein could be eliminated (Daniell, 2005). Proteins that require disulfide bonds or glycosylation are not well suited for expression in microorganisms (Glick et al., 1998). A recombinant protein can be toxic to the microorganism, form inclusion bodies, or be degraded by proteases (Kusnadi et al., 1997). Transgenic plants have the ability to carry out post-translational modifications and the recombinant proteins expressed in plant cells are naturally protected from degradation when taken orally through the protective effect of the plant cell wall. The plant cell wall consisting of cellulose provides protection against proteases in the stomach and gradual release of the antigen in the gut (Kong et al., 2001). Another aspect of vaccine production is that the vaccine should be safe and free from

contamination. Plant derived vaccines offer increased safety and are free of contaminants as there are no human and animal pathogens that affect plants (Streatfield *et al*. 2001). Plants may be engineered to accumulate the antigen in convenient intracellular compartments such as the chloroplast in an environmentally friendly approach.

Advantages of expressing vaccine antigen via chloroplast transformation system

Chloroplast transformation is a powerful tool in plant biotechnology and has several advantages over transgenic plants generated by transformation of the nuclear genome.

High levels of transgene expression: A remarkable feature of the plastid genome is its extremely high ploidy level. A single tobacco leaf cell may contain as many as 100 chloroplasts, each harboring approximately 100 identical copies of the plastid genome resulting in up to 10,000 plastid genomes per cell (Bogorad, 2000). Most plastid genomes have two inverted repeat regions and transgene integrated into the plastid genome can reach a copy number of approximately 20,000 copies per cell. Therefore, very high expression levels can be achieved as reported with the highest expression of a protein in transgenic plants of *Bacillus thuringiensis* (Bt) cry2Aa2 protein, which accumulated up to 46.1% tsp in transgenic tobacco chloroplasts (Decosa et al. 2001).

Transgene integration into the chloroplast genome occurs exclusively by homologous recombination of chloroplast DNA flanking sequences. This allows site-specific gene integration and eliminates concerns of position effect and gene silencing and introduction of vector sequences in chloroplast transgenic plants, which is a serious concern in nuclear transformants (Daniell, 2005).

A major advantage of chloroplast engineering is the expression of multiple transgenes as operons due to efficient translation of polycistronic messenger RNAs. Expression of polycistrons in transgenic chloroplasts is a unique feature, which facilitates the expression of entire pathways in single transformation event (De Cosa et al. 2001, Daniell and Dhingra 2002). Two bacterial enzymes that confer resistance to different forms of mercury- mercuric ion reductase (merA) and organomercurial lyase (merB) were expressed as an operon and conferred resistance to very high levels of mercury and organomercurial compounds (Ruiz, 2003).

The major concern with the genetic modification (GM) of plants is the possibility of the escape of foreign genes through pollen dispersal from transgenic plants to sexually compatible weedy relatives or to pathogenic microbes in the soil (Daniell 2002, Daniell 2004a). Such gene transfers could potentially result in the emergence of "superweeds" able to resist certain herbicides thereby undermining the benefits of GM crops. Most genes in the chloroplasts of higher plants are maternally inherited. Although pollen from plants shown to exhibit maternal plastid inheritance contains metabolically active plastids, the plastid DNA itself is lost during the process of pollen maturation and hence is not transmitted to the next generation (Daniell, 2002). Therefore, a foreign gene introduced by genetic engineering of the chloroplast genome cannot be transferred to the environment.

Chloroplasts have the ability to process eukaryotic proteins, including correct folding of subunits and formation of disulfide bridges (Daniell et al. 2001b). Functional assays showed that chloroplast-synthesized cholera toxin-B subunit binds to the intestinal membrane GM1-ganglioside receptor thereby confirming the correct folding and

disulfide bond formation (Staub et al., 2000; Daniell et al. 2001a, Molina et al. 2004). Chaperones present in chloroplasts facilitate correct folding and assembly of monoclonal antibody in transgenic chloroplasts (Daniell et al., 2001) and also result in fully functional human therapeutic proteins as seen in interferon alpha and gamma (Falconer 2002, Leelavathi and Reddy 2003).

The chloroplast could be a good place to accumulate certain proteins or their biosynthetic products that would be harmful if they were in the cytoplasm as trehalose, a pharmaceutical preservative, was toxic when accumulate in cytosol but was non-toxic when compartmentalized within the chloroplast (Lee, 2003). Cholera toxin B subunit (CTB), a candidate oral subunit vaccine for cholera was non-toxic when it was accumulated in large quantities within transgenic plastids; however, even very small quantities of CTB were toxic when expressed in the cytoplasm (Daniell, 2001). Transient plant transformation has been used for plant expression of vaccine antigens through integration of the gene of interest into a plant virus and subsequent infection of susceptible plants. For stable plant transformation, the vaccine antigen can be inserted into the chloroplast genome.

Chloroplast transformation

The Chloroplast genomes of higher plants contain multiple, circular double stranded DNA molecules ranging in size from 120-160kb and contains approximately 130 genes (Palmer, 1985). Chloroplast of higher plants has retained a largely prokaryotic system of gene organization and expression, with the eukaryotic nuclear genome exerting significant regulatory control (Hager and Bock, 2000). Chloroplast contain protein

synthesizing systems more similar to those of bacteria than to those of eukaryotes, consistent with the hypothesis that these organelles had endosymbiotic origins (Gillham, 1994) and are the endosymbiotic remnants of once free living cyanobacterial progenitor (Moreira, 2000). During the gradual integration of the acquired endosymbionts into the host cell's metabolism, the organellar genomes underwent a dramatic size reduction due to both massive gene loss and gene transfer to the nuclear genome (Martin, 1998). Chloroplast transformation generally results from homologous recombination and the large inverted repeat segments of chloroplast genome attracted attention as regions for intramolecular recombination and copy correction since the time of their initial discovery by restriction mapping (Bedbrook and Bogorad, 1976) and electron microscopy (Kolodner and Tewari, 1979).

The concept of chloroplast genetic engineering was first conceived in the mid 80's with the introduction of isolated intact chloroplasts into protoplasts (Daniell and Dhingra 2002). Focus was later laid on the development of chloroplast system capable of efficient, prolonged protein synthesis and the expression of foreign genes (Daniell and McFadden 1987). In 1988, successful chloroplast transformation was reported by the Boynton and Gillham laboratories for Chlamydomonas reinhardtii, unicellular green algae with a single large chloroplast occupying approximately 60% of the cell volume. Employing photosynthetically incompetent mutants carrying defective alleles of the chloroplast atpB gene (and thus lacking chloroplast ATP synthase activity), the wild-type atpB gene was used in this study to complement the mutant phenotype under selection for restored photoautotrophic growth. The DNA was deposited on tungsten microprojectiles ~ 1um in size and propelled into cells spread on agar plate using a gunpowder charge

(Klein, 1987). Stable chloroplast transformants were obtained in which the mutant atpB allele had been replaced by the wild type gene. Later, compressed gases were used for biolistic transformations and the discovery of the gene gun as a transformation device opened the possibility of direct plastid transformation without the need to isolate them (Sanford, 1991). It was also shown that foreign DNA sequence is incorporated and stably maintained in the Chlamydomonas chloroplast chromosome (Blowers, 1989). The first transient expression of foreign genes in cultured tobacco cells used autonomously replicating chloroplast vectors (Daniell et al., 1990). This work was repeated in wheat leaves, calli and somatic embryos (Daniell et al., 1991). Stable integration of the aadA selectable marker into the tobacco chloroplast genome was accomplished using the gene gun (Svab and Maliga, 1993). Initially, when the transgenes were introduced via the chloroplast genome, it was believed that foreign genes could be inserted only into transcriptionally silent spacer regions within the chloroplast genome (Zoubenko et al, 1994). The approach of inserting transgenes into functional operons and transcriptionally active spacer regions facilitated the insertion of multiple genes under the control of a single promoter, enabling the coordinated expression of transgenes (Daniell and Dhingra, 2002).

Plants have also been genetically engineered via the chloroplast genome to confer agronomic traits. Insect resistance was achieved by high levels of foreign gene expression of the Bt cry2Aa2 operon (up to 46% tsp) in chloroplasts and as a result the protein accumulated as cuboidal crystals (DeCosa, 2001). This study also demonstrated the ability of transgenic chloroplasts to express bacterial operons, opening the door for multigene engineering via the chloroplast genome. Herbicide resistance was also shown

against glyphosate, a broad-spectrum herbicide that is highly effective against a majority of grasses and broad leaf weeds and works by competitive inhibition of the enzyme 5-enol-pyruvyl shikimate- 3-phosphate synthase (EPSPS) of the aromatic amino acid biosynthetic pathway of plants and microorganisms. Tobacco plants engineered with EPSPS gene developed resistance to glyphosate over the wild type plants (Daniell et al., 1998). Plants have also been genetically engineered via the chloroplast genome to confer bacterial and fungal disease resistance by expressing antimicrobial peptides within transgenic chloroplasts (De Gray, 2001) and drought tolerance by engineering the yeast trehalose-6-phosphate synthase gene (Lee, 2002). An antimicrobial peptide, MSI-99, a magainin analogue was expressed in transgenic chloroplasts and expression levels of approximately 21.5% of the total soluble protein were achieved and it was shown to inhibit the growth of several plant pathogens including *Pseudomonas syringae*, *Aspergillus flavus*, and the multidrug-resistant human pathogen *Pseuodomonas aeruginosa* (De Gray et al., 2001).

Other useful proteins that have been expressed in chloroplasts include GVGVP, a protein based polymer with varied medical applications as prevention of post-surgical adhesions and scars and wound coverings (Guda et al. 2000), Guy's 13 monoclonal antibody (IgA-G) which targets the surface antigen of the bacterium *Streptococcus* mutants, the causative agent of tooth decay was successfully synthesized in transgenic tobacco chloroplasts (Daniell et al., 2001a), human serum albumin (Fernandez et al; 2003), interferon and insulin- like growth factor (Daniell, 2004). The spinach betaine aldehyde dehydrogenase (BADH) gene was developed as a selectable marker to transform the chloroplast genome (Daniell et al. 2001b). The toxic betaine aldehyde (BA)

is converted to non-toxic glycine betaine by the chloroplast BADH enzyme and eliminates concerns of antibiotic resistance genes in transgenic plants. This glycine betaine also serves as an osmoprotectant and confers salt tolerance (Kumar et al. 2004a). Expression of Chorismate Pyruvate Lyase (CPL), an enzyme encoded by the *ubiC* gene in *E.coli* which catalyzes the conversion of chorismate to pyruvate and p-hydroxybenzoic acid (pHBA). pHBA is the principle monomer found in all commercial thermotropic liquid crystal polymer's (LCP's). Chorismate the substrate for CPL is an intermediate of the shikimate pathway, which is located in chloroplasts. CPL is not normally present in plants instead the entire reaction leading from chorismate to pHBA involves up to 10 successive enzymatic reactions. Therefore, pHBA was expressed in plants and it accumulated to about 30% tsp (Viitanen *et al*. 2004).

Chloroplast genetic engineering technology is currently applied to other useful crops. Difficulties include identification of regulatory sequences that function in non-green plastids, regeneration of transgenic plants through somatic embryogenesis and achieving homoplasmy as subsequent rounds of regeneration are not possible as opposed to organogenesis. Recently, the first successful demonstration of transformation of non-green plastids and regeneration of transgenic plants through somatic embryogenesis in carrot and cotton has been reported (Kumar et al. 2004a**,** Kumar et al. 2004b). Carrot cultures were transformed with two separate vectors aadA/GFP and aadA/BADH vectors and targeted to 16S/trnI-trnA/23S plastid genome invert repeat region. Expression of BADH conferred high levels of salt tolerance (up to 500mM) and also transgenic green cells could be visually selected from non-transformed yellow cells (Kumar et al. 2004a). Cotton cultures were transformed with a plastid transformation vector that harbors two

selectable marker genes aphA-6 and nptII driven by regulatory elements that function in green and non- green plastids. Chloroplast transgenic lines were fertile, flowered and set seeds similar to untransformed plants. Transgenes stably integrated into the cotton chloroplast genome were also shown to be maternally inherited (Kumar et al. 2004b).

Chloroplast derived vaccine antigens

There are several advantages associated with plant-based vaccines that include antigen protection through bioencapsulation, generation of systemic and mucosal immunity, the reduced need for medical personnel and sterile injection conditions, heat stability, and improved safety via the use of a subunit vaccine (Daniell et al. 2004b). Vaccine antigens expressed in plants have shown to be protected in the stomach through bioencapsulation. Also, they have been proven immunogenic when administered orally in clinical trials (Tacket et al., 1998, Tacket et al., 2000, Castanon et al., 2000, Tuboly et al., 2000, Walmsley and Arntzen, 2000, Tacket et al., 2003). Using plastid transformation technology, large quantities of vaccine antigen can be produced.

Cholera toxin B sub-unit (CTB) of *Vibrio cholerae* has been expressed in chloroplasts and this resulted in accumulation of up to 31.1% of total soluble protein and also, CTB synthesized from transgenic chloroplasts assembled into as functional oligomers and were antigenically identical to purified native CTB (Daniell et al., 2001b). *Bacillus anthracis* protective antigen (PA) was expressed in transgenic tobacco chloroplasts as the present vaccine for anthrax (Protective Antigen) produced by cell-free filtrate of toxigenic, nonencapsulated strain of *Bacillus anthracis* contains trace amounts of Lethal factor (LF) and Edema factor (EF) which are toxic and cause side-effects (Watson, 1994).

The gram-negative bacterium *Yersinia pestis* is the causative agent of plague. *Yersinia pestis* F1~V fusion antigen was expressed in transgenic chloroplasts. The expression levels obtained were as high as 14.8% of the total soluble protein (Singleton, 1994). The 2L21 peptide, which confers protection to dogs against virulent canine parvovirus (CPV), was expressed in tobacco chloroplasts as a fusion protein with cholera toxin B (CTB) and with green fluorescent protein (GFP). Very high levels of expression of 31.1 % of total soluble protein were achieved with CTB-2L21 and 22.6 % of total soluble protein with GFP-2L21 (Molina, 2003).

The use of plants to produce vaccine antigens for human diseases is a novel and promising system with several practical advantages as discussed above such as delivery of edible vaccines by the oral route, the ease of agricultural scale production, safety from contaminating human and animal pathogens, and the possibility of growing and manufacturing vaccine materials locally for specific demands. HCV is an important pathogen affecting more than 180 million people worldwide including nearly 4 million in the United States (Purcell, 1997). A plant derived recombinant HCV vaccine can potentially reduce expenses normally associated with the production and delivery of conventional vaccines. The plant system has been explored as a safe and inexpensive source for the production of HCV vaccine antigen.

MATERIALS AND METHODS

NS3-pcDNA3.1 Vector

The plasmid encoding HCV NS3 protein (initial concentration of the plasmid-280ng/ul in H2O) was sent cloned in the commercial plasmid pcDNA3.1/V5/His –TOPO (Invitrogen). A PCR product encoding NS3 had been inserted in this plasmid (in the cloning box) as described by the protocol supplied by the manufacturer. The plasmid encoded NS3 with a ATG and a Kozak sequence (5' end) and a TGA (3'end). The plasmid was transformed into Ultra competent XL1 Blue MRF' Tetracycline (tet) *E. coli* cells (Stratagene) that were endonuclease negative.

Preparation of Ultra competent cells (Rubidium chloride method)

The ultra competent cells were prepared using rubidium chloride method (http://www.nwfsc.noaa.gov/protocols-/rbcl.html). XL1 Blue MRF' (tet) *E. coli* cells (Stratagene) were made competent by the rubidium chloride method. The E.coli glycerol stock was streaked on the LB agar plate (1liter LB broth, 15 grams agar), containing 12.5 ug/ml Tetracycline and incubated at 37°C overnight. An isolated colony was picked and it was grown in 5 ml of Psi broth (per liter- 5g Bacto yeast extract, 20g Bacto Tryptone, 5 g magnesium sulfate, pH 7.6) with 5ug/ml Tetracycline and incubated at 37°C for 12-16 hrs in a shaker at 225 rpm. From the overnight culture, 1 ml was taken and inoculated in 100 ml of Psi broth and was incubated at 37°C for about 2 hours in a shaker at 225

rpm. After 2 hours, the O.D was checked at 550 nm and rechecked again after intervals, until the O.D reached 0.48. The culture was then kept on ice for 15 minutes and then the cells were centrifuged at 3000g/5000 rpm for 5 minutes in a sorvall centrifuge. The supernatant was discarded and the pellet was resuspended in 40 ml cold TFB-I solution (per 200ml- 0.558g Potassium acetate, 2.42 g rubidium chloride, 0.294g calcium chloride, 2.0g manganese chloride, 30 ml glycerol, pH 5.8). The cells were centrifuged at 3000g/5000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 4 ml of TFB-II solution (per 100ml- 0.21g MOPS, 1.1g calcium chloride, 0.121 g rubidium chloride, 15ml glycerol, pH 6.5) and then kept on ice for 15 minutes. The suspension was aliquoted (100ul) and quick freezed in dry ice/liquid nitrogen and the aliquots were stored at - 80°C.

Transformation of pcDNA3.1 plasmid into Competent XL1 Blue MRF' (tet) *E. coli* Cells

The competent cells were removed out of –80°C and thawed on ice. 100µl of competent cells were taken and 1ul (100 ng) of plasmid pcDNA3.1 DNA was added and mixed by gently tapping. The cells were left on ice for 30 minutes, and the tube was gently tapped every 15 minutes. The cells were heat shocked at 42°C for 90-120 seconds and then left on ice for 2 minutes, and then 900 µl LB broth was added to the cells and the cells were incubated at 37°C at 225 rpm in a shaker for 45 minutes. The cells were pelleted by centrifugation at 13,000 rpm for 30 seconds and the supernatant was discarded. Almost 800ul of supernatant was discarded and only approximately 100ul was left .The remaining 100ul of the cells were mixed well with the pellet. About 50ul and 100ul of the transformed cells and untransformed (control) were plated onto LB/amp agar

plates (1liter LB broth, 15gr agar, 100μg/ml ampicillin, pH 7) under the hood. Plates were covered and incubated O/N at 37°C (http://www.nwfsc.noaa.-gov/protocols-/rbcl.html).

Rapid Colony Screening by Cracking Method

To check the colonies for the presence of plasmids, the Rapid Screen procedure by Promega was used. Sterile toothpicks were used for picking 8 colonies from the incubated LB agar plates to the bottom of an individual sterile microcentrifuge tube. 25 μl of 10mM ethylene diamine tetra-acetic acid (EDTA), pH 8 was added to the tubes and vortexed to mix. Then 25 μl of fresh 2X cracking buffer (2N NaOH, 10% sodium dodecyl sulfate, 1M sucrose) was added to each colony and vortexed. The tubes were then incubated at 65°C for 10 minutes and were cooled at room temperature. 1.5 μl of 4M KCl and 3.5 μl of 6X bromophenol blue (.25% Bromophenol blue, 40% sucrose) was added to the tubes. The tubes were placed on ice for 5 minutes and centrifuged at 12,000 rpm for 3 minutes at room temperature. 20 μl of the supernatant form each tube was run on 0.8% agarose gel to visualize which of the selected colonies contained plasmids. Positive colonies were inoculated into 5 ml of fresh LB broth with 100 μg/ml amp and incubated overnight at 37°C on shaker.

Midi- prep of pcDNA3.1

Inoculated a colony obtained from the plate in 50 ml of liquid LB broth, to which 50 μl of ampicillin (stock concentration; 100 mg/ml) was added and incubated at 37°C

for 12 hours in a shaker. 40 ml of the overnight culture was transferred to a clean 50 ml round bottom sorvall centrifuge tube. The cells were centrifuged for 10 minutes at 8000 rpm at 40 C. The supernatant was discarded. The pellet was resuspended by vortexing in 5 ml of Solution 1 (50 mM Glucose, 10mM EDTA, 25 mM Tris, pH- 8) with 5ul of 100mg/ml of RNAse freshly added to it. Solution II (500ul of 2N NaOH, 100ul of 10% SDS, 4400ul of sterile water) which is a cell lysis solution was prepared freshly, added, and mixed by gently inverting the tube 6-8 times and the solution turned from milky to clear. Then 5 ml of Sol III (60 ml of 5M Potassium Acetate, 11.5 M glacial acetic acid, and 28.5 ml sterile dH2O) which is neutralizing solution was added to the clear solution and mixed well by inverting the tube 6-8 times and the solution precipitated. The solution was centrifuged for 15 minutes at 12,500 rpm at 40 C. The clear supernatant was poured into a new 50 ml Sorvall centrifuge tube. Cold absolute ethanol (24 ml) was added to the supernatant and mixed well by inverting the tube 6-8 times. The tube was then centrifuged for 10 minutes at 10,000 rpm at 40 C to pellet the plasmids. The supernatant containing contaminants was discarded .The pellet was washed with 12 ml of 70% ethanol and resuspended by shaking. The solution was centrifuged for 5 minutes at 10,000 rpm at 40 C. The supernatant was discarded and the pellet was dried in a speed vacuum or air-dried before resuspending the DNA pellet in 500 ul of TE (TE: 1M Tris, pH 8.0, 0.5M EDTA). The DNA sample was loaded in a 0.8% agarose gel and run at 60 volts for 30 minutes to check for plasmid isolation (Sambrook et al., 1989).

Phenol: Chloroform Extraction

Plasmid DNA (500ul) was taken and 250ul Phenol and 250ul Chloroform was added (1:1) and mixed well. The tube was then centrifuged at 14,000 rpm at 40 C for about 10 minutes. The supernatant was transferred to a new tube and 500ul of Chloroform: IAA (Isoamyl alcohol) was added and centrifuged at 14,000 rpm at 40 C for 10 minutes. The supernatant was transferred to a new tube and 0.1 volume of 3M sodium acetate (pH: 5.2) was added. Absolute ethanol (900ul) was added and mixed well by inverting several times and then centrifuged at 14,000 rpm, 40 C for 10 minutes. The supernatant was discarded and the pellet was rinsed with 70% ethanol (400ul) and centrifuged for 10 minutes at 14,000 rpm at 40 C. The supernatant was discarded again and the pellet was dried in a speed vacuum or air-dried before resuspending the DNA pellet in Elution buffer,10mM Tris Cl , (Sambrook et al., 1989).

PCR amplification of NS3 gene

The NS3 gene (first 134bp) were amplified to introduce the Sac1 and SnaB1 restriction sites at the 5' terminal end and Not1 at the 3'end of the 134bp of the NS3 gene for further subcloning. This was done to clone the 134 bp of the NS3 gene first into p-bluescript between Not1 and Sac1 sites. The primers used for amplification were the NS3-F primer (5'CAGTGTGGAGCTCTTGTACGTACCACCATGGCG3') and the NS3-R primer (5'TGGAGAGCACCTGCGGCCGCCCATCGACCTGG3'). Primers

(Invitrogen) were diluted with EB to give a 100 µM stock that was stored at –20°C. The PCR reaction was set up with 0.5 ul plasmid DNA (60ng), 10X PCR buffer, 5.0 µl of 10mM dNTP's, 1 µl of forward primer (NS3-F), 1 µl of reverse primer (NS3-R), 0.5 µl of Pfu polymerase and 37.0 µl of distilled, autoclaved H20 to a total volume of 50 µl.). Samples were carried through 35 cycles using the following temperatures and times: 94°C for 5 minutes, 94°C for 45 seconds, 56°C for 45 seconds, 72°C for 45 seconds, and followed by a 10-minute extension time at 72°C. The final PCR product (0.1 ul) was run on a 0.8 % agarose gel to analyze the PCR products. The PCR product was purified using the PCR purification kit (Qiagen).

Ligation of the PCR Product (Sac1/SnaBI/NS3/Not1) into p-Bluescript vector

The PCR product was ligated into p-Bluescript cloning vector (Invitrogen) between NotI and SacI restriction sites. The ligation mixture consisted of 4 µl of PCR product after PCR purification, 16 ul of p-Bluescript, 0.5 ul of T4 DNA ligase, 6.0ul of Ligase buffer, and 3.5 ul distilled, autoclaved H20 to a total of 30ul total volume. The solution was gently mixed and incubated overnight at 12°C. Competent E.coli cells were taken from –80°C freezer and thawed on ice and transformation was started immediately after cells thawed. 15ul of the ligation mixture was mixed into a vial containing the 100ul of E.coli competent cells and transformation was done as previously described (Sambrook et al., 1989).

Selection of Transformants

The p-Bluescript cloning vector has the β-galactosidase gene (lacZ). Within this coding region is a multicloning site. Insertion of a fragment of foreign DNA into the multicloning site of p-Bluescript almost invariably results in production of an amino-terminal fragment that is not capable of α-complementation. Selective plates were made with LB agar with 100 μg/ml ampicillin and 12.5μg/ml tetracycline. About 1 hour before transformation was complete, 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was spread onto the top of the plates while under the hood. X-gal is a lactose analog that turns dark blue when it is hydrolyzed by β-galactosidase. After the X-gal dried (about 15 minutes), 40 μl of 100 mM of isopropyl-β-D-thiogalactoside (IPTG) was spread onto the plates. IPTG, another lactose analog, is a strong inducer of lacZ transcription but is not hydrolyzed by β-galactosidase. The plates were warmed 37°C for 30 minutes and then the plates were streaked with 100 μl of the transformed bacterial cells were spread over the top of the agar. Allowed the plates to dry for 5 minutes, and then incubated the plates in an inverted position at 37°C overnight. Colonies without an interrupting insert were blue because they had an active β- galactosidase. Colonies with an insert were white, so these were picked to culture, and midi-prep was done with the Midi-prep kit (Qiagen).

Sequencing of NS3 in p-Bluescript

The PCR product in the plasmid (NS3-p-Bluescript) was sequenced using M13 forward (5'-TGACCGGCAGCAAAATG-3') and M13 reverse (5'GGAAACAGCTATGACC-ATG-3') primers. Sequencing results confirmed that the fragment in the p-Bluescript vector was the NS3 gene.

Construction for pLD-AB-NS3 vector for transformation of tobacco chloroplasts

The original vector pcDNA3.1 was digested with BstXI and EcoRV and the NS3 gene (remaining 1760bp) was ligated between BstXI and EcoRV in p-Bluescript. The entire NS3 gene was digested from p-bluescript with SnaBI and HindIII and ligated in pCR2.1 vector downstream of the psbA 5'UTR. Finally, the pCR 2.1 vector containing the 5'UTR and the NS3 gene was digested with EcoRI and EcoRV (fragment size 2.1) and was cloned between the same sites in the universal vector pLD-AB-Ct.

Extraction of NS3 Protein from Transformed E.coli Cells

5 ml of Terrific Broth (TB) containing 5ul ampicillin (100µg/µl) and tetracycline (50µg/µl) was inoculated with the scrapping from the glycerol stock of E.coli transformed with pLD-AB-NS3 and incubated in a shaker at 37°C for 10-12 hours. 5 ml of Terrific Broth (TB) with untransformed E.coli cells was used as a negative control. The buffers and gels used in this study were made from protocols in SDS-PAGE Buffer

System (Laemmli, 1970). 800 µl of cultured cells were taken and centrifuged for 2 minutes at 12,000 rpm. The supernatant was discarded from pelleted E.coli cells and then washed with 1ml of 1x Phosphate-Buffered Saline (PBS: 140mM NaCl, 2.7Mm KCl, 4mM Na2HPO4, 1.8mM KH2PO4, pH 7.2). The pellet was resuspended and then centrifuged for 1 minute at 13,000 rpm. The supernatant was then discarded. 50 µl of 1x PBS was added and mixed well. 50 µl of 2x loading buffer, also called Sample Buffer or SDS Reducing Buffer (1.25 ml of 0.5 M Tris-HCl, pH 6.8, 2.0 ml of 10% (w/v) SDS, 0.2ml of 0.5% (w/v) bromophenol blue, 2.5 ml of glycerol, dH20 to a total volume of 9.5ml, then add 50 µl of β-mercaptoethanol to the 9.5 ml) was added. The sample extracts were boiled for 4 minutes and then immediately loaded onto gels (Sambrook et al., 1989).

SDS-PAGE

The buffers and gels used in this study were made from protocols in SDS-PAGE Buffer System below (Laemmli 1970). To detect the protein extracted from E.coli cells containing pLD-AB-NS3, SDS-PAGE gels were made in duplicate utilizing the following solutions: 1.) Bio-Rad (cat#161-0158), which is a 30% Acrylamide/Bis solution according to the ratio 37:5:1. 2.) The resolving buffer, which was used to make the lower portion of the gel: 1.5M Tris-HCl, pH 8.8. The pH was adjusted with 6N HCl and brought to a total volume of 150ml with dH20. 3.) The stacking buffer that was used to make the stacking gel layered over the resolving gel and concentrated the samples at top of the resolving gel to improve resolution: 0.5M Tris-HCl, pH 6.8. 4.) Electrode buffer (1x) which was the gel running buffer. For 10x Electrode buffer: Dissolved 30.3g

Tris base, 144.0g glycine and 10.0g SDS into 1000 ml dH20. 5.) 2x loading buffer also called the Sample buffer and the SDS Reducing Buffer. 6.) 10% (w/v) Sodium Dodecyl Sulfate (SDS): 7.) N,N,N,N'-Tetra-methyl-ethylene diamine (TEMED) from BIO-RAD (cat# 161-0800). 8.) 20% Ammonium Persulfate (APS): Dissolved 20 mg of APS into 1ml dH20 and this solution can be stored at 4ºC for about a month. To make the 10% resolving gel, in 4.1 ml dH20, 3.3 ml of 30% Acrylamide/Bis, 2.5ml of resolving buffer and 100 μl of 10% SDS was added. 40 μl of 20% APS and then 10 μl of TEMED was added to the gel mixture. The gel mixture was poured between the two, vertical, glass plates leaving about 1.5 cm at the top of glass plates for the stacking gel. The gel was allowed to polymerize for 20 minutes. To make the 4% stacking gel, in 6.1 ml dH20, 1.3 ml of 30% Acrylamide/Bis, 2.5 ml of the stacking buffer and 100 μl of 10% SDS was added. 40 μl of 20% APS and then 10 μl of TEMED was added to the gel mixture. The 4% gel mixture was layered on top of resolving gel and then the comb is inserted for the formation of wells. After polymerization for about 20 minutes, the comb is removed and put vertically into PAGE apparatus containing 1x Electrode (running) buffer. 20 μl of protein extract from pLD-AB-NS3 transformed and untransformed E.coli cells was loaded along with 10 ul protein marker. Gel was ran at 50V until samples stacked onto the top of the resolving gel, then ran gel at 80V for 2-3 hours so that protein marker bands could spread out sufficiently (Sambrook *et al*. 1989).

Transfer to Membranes and Immunoblot Analysis

The separated proteins were transferred onto a 0.2 μm Trans-Blot nitrocellulose membrane (Bio-Rad) by electroblotting in Mini-Transfer Blot Module at 80V for 45

minutes in Transfer buffer (360 ml of 10x Electrode buffer, 360 ml of methanol, 0.18 grams of SDS, 1080 ml distilled H20). The membranes were taken out and rinsed with water and placed in blocking solution (100 ml 1x PBS, 100µl of Tween 20, 5g of non-fat, Carnation powdered milk) and incubated for an hour at room temperature in a shaker. The P-T-M was poured off and the Hepatitis C Virus (NS3)-specific primary mouse monoclonal antibody (HCV NS3 Ab-1, Clone MMM33, from Neomarkers) was added in the ratio of antibody: PTM as 1:1000 and incubated for 2 hours at room temperature in a shaker. Membranes were then washed with distilled water and transferred to P-T-M containing goat derived anti-mouse IgG antibody conjugated with Horseradish peroxidase (Sigma, St. Louis, MO), in the ratio of antibody: PTM as 1:10,000 and incubated for 1.5 hours at room temperature in shaker. Blots were washed three times with PBST for 15 minutes each time and then washed with only PBS for 10 minutes. Then 750 µl of 2x Stable Peroxidase Solution and 750 µl of 2x Luminol /Enhancer Solution (Pierce) was poured over the membrane and a film was developed in the to visualize the bands (Sambrook *et al*. 1989).

Sterilization of Seeds for Wild-type and T1

For generating wild-type (untransformed) tobacco plants to use for bombardment, pods were picked from both varieties of tobacco when the pods were dry. The pods were broken under hood and then poured into labeled eppendorf about until about 1/3 full. To germinate seeds, fresh MSO (Murashige and Skoog, 1962) plates with no antibiotic were made. The sterilization solution consisted of 1.5 % bleach (4 ml of 5.25% Chlorox bleach), 16 ml d/aH20, 0.05 % Tween 20 (20 µl of Tween 20). 1.2 ml of the sterilization

solution was added to each eppendorf and then vortexed for 20 minutes and then rinsed 7 times with sterile H20. Then the seeds were dried and then spread onto the surface of the MSO plates, covered and wrapped in parafilm. Put plates at 26°C with a 16 hour photoperiod. For germination of T1, 1.2 ml of sterilization solution was added and sterilized as above, except the dry seeds were spread onto MSO plated with 500 μg/ml spectinomycin (Petit Havana) and 350 μg/ml to select for transformants (Kumar and Daniell, 2004).

Preparation of tobacco tissue culture media (RMOP and MSO media)

The shoot-inducing RMOP media was made by adding one packet MS salts mixture, 30 gm sucrose, 1 ml benzylaminopurine, BAP (1mg/ml stock); 100 ul napthalene acetic acid, NAA (1mg/ml stock), 1ml thiamine hydrochloride (1mg/ml stock) to 1L dH2O. The pH was adjusted to 5.8 with 1N KOH and 7.0 g/L phytagar was added to the mixture which was autoclaved, cooled and plated out under the hood (Kumar and Daniell, 2004). The root-inducing MSO media was prepared by adding 30 g sucrose and one packet (4.3 g) of Murashige & Skoog (MSO) salt mixture (Gibco BRL) to 1L dH2O. The pH was adjusted to 5.8 with 1N KOH, then 7 g/L phytagar was added and the mixture was autoclaved (Kumar and Daniell, 2004).

Biolistic transformation of tobacco leaf chloroplast

About 4 weeks prior to the planned bombardment, wild-type (untransformed) tobacco plants were micropropagated from seeds using sterile techniques. Two varieties of tobacco were generated for the bombardment: Petit Havana (model) and LAMD-609

(low nicotine hybrid produced by backcrossing a Maryland type variety, MD-609, to a low-nicotine producing burley variety, LA Burley 21 (Collins et al., 1974).

Preparation of the gold particles and DNA/particle suspension

Fifty mg of gold particles (0.6 μm) were placed in a micro centrifuge tube and 1 ml of freshly prepared 70% ethanol was added. The mixture was vortexed for 3-5 minutes and incubated at room temperature for 15 minutes. The gold particles were pelleted by spinning for 5 seconds and then the supernatant was discarded. 1 ml of H2O was added to the particles and vortexed for a minute. Particles were allowed to sit for 1 minute and pulse centrifuged for 3 seconds. The supernatant was discarded and this was repeated three times. After the last spin, 50% glycerol was added to a concentration of 60 mg/ml. The gold particles were stored at –20°C (Kumar and Daniell, 2004).

Coating DNA onto macrocarriers

The gold particles prepared in 50% glycerol (60mg/ml) were vortexed for 5 minutes to resuspended the particles. Fifty ul of gold particles was removed and placed in a micro centrifuge tube. 10 ul (1μg/μl) of the pLD-AB-NS3 vector DNA was added and quickly vortexed. Then, 50 ul of freshly prepared 2.5M CaCl2 (367.5 mg of CaCl2 into 1 ml of d/aH2O) was added and vortexed. Finally, 0.1M spermidine-free base (20 ul) was added and the tube was vortexed for 20 minutes at 4°C. 200 ul of absolute ethanol added to each tube and centrifuged for 2 seconds, then the ethanol was discarded. The wash was repeated 4 times. After the washes, the particles were resuspended in 40 ul of absolute ethanol and kept on ice (Kumar and Daniell, 2004).

Preparing the Biolistic Gun and Consumables

Stopping screens, rupture disk holders, macrocarrier holders were autoclaved to ensure that they were sterile. Rupture disks and macrocarriers were washed in 50 ml of autoclaved H2O and 70% ethanol. The Bio-Rad PDS-1000/He (gene gun) shelves, macrocarrier holder, rupture disk holder were washed with 70% ethanol. After the pump under the hood was turned on, the main valve on the helium tank was opened and the valve controlling pressure to the gene gun was set to 13500 psi (Kumar and Daniell, 2004).

Bombardment

The bombardment was performed as described previously (Daniell, 1997). Stopping screens were placed in macrocarrier holders. 6 ul of particle mixture was spread evenly onto the macrocarrier. The gold suspension was allowed to dry. One rupture disk was placed in the holder ring and screwed in place at the top of the vacuum chamber. The stopping screen and macrocarrier with the gold/DNA (in holder) were placed into the retaining assembly. The assembly was placed into the vacuum chamber. A piece of sterile whatman #1 filter paper was placed on solidified RMOP media in a petri dish. One leaf at a time was placed on the whatman paper abaxial side upwards. The petri dish with leaf was placed on a plastic holder and placed in the next to last slot in the vacuum chamber. The chamber door was closed and secured. The power switch for the gene gun was turned on. A vacuum was allowed to build to 28 psi in the

bombardment chamber. When 28 psi was reached, the fire switch was pressed until the rupture disk ruptured (u1100psi). After delivery of the gold particles with vector DNA, the vacuum was released and the Petri dish taken out and covered. The petri dishes were wrapped in aluminum foil and kept in the dark for 48 hours at room temperature to recover from the shock of bombardment (Kumar and Daniell, 2004).

Selection and Regeneration of Transgenic Lines

After recovering in the dark for 48 hours from bombardment, leaves were cut into 5mm2 squares and placed on a petri dish containing RMOP media containing spectinomycin. For Petite Havana, 500 ug/ml of spectinomycin was used and for LAMD-609, 350ug/ml of spectinomycin was used for the first round of selection (with the abaxial side down). Four to six weeks later when the shoots appeared, they were cut into 2mm2 pieces and transferred to fresh RMOP media with spectinomycin for the second round of selection (500 ug/ml for Petite Havana and 350ug/ml for LAMD-609). During the second selection, the shoots that appeared and tested positive for cassette integration into the chloroplast by PCR analysis were grown in sterile glass jars containing fresh media with spectinomycin until the shoots grew to fill the jar. Then the plants were transferred to pots with soil containing no antibiotic. Potted plants were grown in a 16 hour light/ 8 hour dark photoperiod in the growth chamber at 26°C (Kumar and Daniell, 2004).

Isolation of total plant genomic DNA from Tobacco Leaf

The QIAGEN's DNeasy® Plant Mini Kit was used for isolating the total DNA from plant tissue as described in the Qiagen manual. 100mg of the tissue was grounded in liquid nitrogen to a fine powder and was transferred to a cooled eppendorf and 400 ul of Buffer AP1 and 4 ul of RNase A stock solution (100 mg/ml) was added and vortexed. The mixture was incubated for 10 minutes at 65°C and mixed about 2-3 times during incubation by inverting the tube. 130 ul of Buffer AP2 was added to the lysate, mixed, and then incubated on ice for 5 minutes. The lysate was applied to the QIAshredder spin column (lilac) sitting in a 2 ml collection tube and then centrifuged for 2 minutes. The flow-through was transferred to a new tube and 1.5 volumes of buffer AP3/E were added to the lysate and mixed immediately. 650 ul of the mixture was applied to the DNeasy mini spin column sitting in a 2ml collection tube and then centrifuged for 1 minute at 8000 rpm. The DNeasy column was placed in a new 2 ml collection tube and 500 ul Buffer AW was added to the DNeasy column and centrifuged for 1 minute at 8000 rpm. The flow-through was discarded and collection tube was reused in the next step. 500 ul Buffer AW was added to the DNeasy column and centrifuged for 2 minutes at maximum speed to dry the membrane. The DNeasy column was transferred to a 2 ml microcentrifuge tube and 100 ul of preheated (65°C) Buffer AE was directly added onto the DNeasy membrane. The membrane was incubated for 5 minutes at room temperature and then centrifuged at 8,000 rpm for 1 minute to elute the DNA. The DNA was kept at -20°C for use in PCR and Southern analysis.

PCR Analysis of Integration into the Chloroplast Genome

To confirm the transgene cassette integration into the chloroplast genome, two primers sets were designed and assigned numbers with the plus (P) being for the forward primer and minus (M) being for the reverse primer. The 3P/3M (3P: 5'-AAAACCCGTCCTCCGTTCGGAT-TGC-3') primer annealed to anneal to a unique portion of the chloroplast genome and 3M (5'-CCGCGTTGTTTCATCAAGCCTTACG-3') annealed to the integrated aadA gene (Daniell et al, 2001b). For the PCR reaction, 200ng of plant DNA, 5 µl of 10X buffer, 4µl of 2.5 mM dNTP, 2µl of each primer from the stock, 0.5µl Taq DNA polymerase and H2O to make up the total volume to 50ul. The amplification was carried for 25 cycles of the following reaction : 94°C for 5 mins, 94°C for 45 sec, and 65°C for 45 sec, 68°C for 1.5 min, 68°C for 7 mins. To confirm the integration of gene of interest, PCR was performed using primer pairs 5P (5'-CTGTAGAAGTCACCATTGTTGTGC-3' and 2M (5'-TGACTGCCCACCTGAGAGCGGACA-3'). The amplification was carried during 25 cycles of the following reaction : 95°C for 5 mins, 95°C for 1 min, and 68°C for 1 min, 72°C for 3 min, 72°C for 10mins. 5 ul of each PCR products including the controls were loaded into a 0.8% agarose gel to confirm the results. pLD- NS3 was used as the positive control and wild type petite Havana was used as a negative control.

Southern Blot Analysis

These steps were performed as described in (Daniell *et al.*, 2004a). The total DNA isolated from T0 plants as well as from untransformed tobacco plants with QIAGEN's

DNeasy® Plant Mini Kit was digested as follows: 10ul (2ug) DNA from DNeasy, 3 µl of 10x buffer 3 , 2 µl BglII enzyme (NEB), 14.7 µl sterile H2O, to a total volume of 30 µl. The digest was incubated O/N at 37°C. The digestion was separated on a 0.8% agarose at 50V for 3.5 hours. The gel was observed under UV light to verify the complete digestion of the plant DNA. The gel was soaked in 0.25N HCl (depurination solution) for 15 minutes in a continuous agitation. The depurination solution was discarded, and the gel was rinsed 2 times with sterile H2O for 5 minutes. The gel was then soaked in transfer buffer on a rotary shaker for 20 minutes. The transfer apparatus was assembled for the transfer of the DNA to Duralon-UV nylon membrane. Four pieces of the Whatman paper were cut slightly larger than the gel and the membrane. Two pieces of Whatman paper were dipped into the transfer solution and placed on three sponges placed in a large pyrex dish partially filled with transfer buffer. The gel was removed from the transfer buffer and inverted on the Whatman paper. The nylon membrane was soaked in water and then placed on the gel. Removed air bubble gently and arranged parafilm along all the side to prevent horizontal DNA transfer. A stack of ordinary paper towels onto the top of Whatman filter paper and then added a 500g weight to encourage transfer. From the bottom of the pyrex dish the transfer was in the following order: sponges, 2 filter paper, gel, parafilm at edges, nylon membrane, 2 filter paper, paper towels and weight. The set up was left for transfer over night and the next day the membrane was washed on 2X SSC (3M NaCl, 0.3M Na citrate, H2O, the pH was adjusted with 1N HCl to 7 and water was added to 1L) for 5 minutes. The membrane was air-dried and then cross-linked using the GS Gene Linker UV Chamber (BIO-RAD) at the C3 setting.

Generating and Labeling Probes

The probes were prepared by the random primed ^{32}P-labeling (Ready-to-go DNA labeling beads, Amersham Pharmacia).A pUC universal vector containing the chloroplast flanking sequences was used to generate the flanking probe. The restriction digest was set-up as follows: 20 µl of pUC-ct, 1 µl 10x buffer 3, 1 µl BamHI (NEB), 1 µl BglII (NEB), 0.3µl of BSA, 6.7 µl of sterile H2O to a total volume of 30 µl. The reaction was incubated overnight at 37°C. The restriction digest for the gene specific probe was as follows: 20 µl of pLD-AB-NS3, 1 µl of EcoRI (NEB), 1 µl of EcoRV (NEB), 3 µl of 10x buffer #3 (NEB), 0.3ul of BSA, 4.7 µl sterile H2O to a total volume of 30 µl. The reaction was incubated O/N at 37°C. 45 µl of each probe was denatured at 94°C for 5 minutes and then placed on ice for 3 minutes. The probes were added to the ready mix tube (Quantum G-50 Micro columns, Amersham) and gently mixed by flicking. 5 µl of α32P was added to the ready mix tube and then it was incubated at 37°C for 1 hour. The resin in the G50 column was resuspended by vortexing. The cap was loosened and the bottom plug broken off. Then the column was placed in a microcentrifuge tube with the top cut off and centrifuged for 1 minute at 3000 rpm. The collection tube with the supernatant was discarded and the column was transferred to a new tube. The probes were added to the center of the resin and centrifuged for 2 minutes at 3000 rpm and then the column was discarded. The amount of labeled DNA probe to be used was determined.

Prehybridization, Hybridization and Washing of the membrane

For prehybridization, the membrane was washed with sterile water. The Quick Hyb solution was gently mixed by inverting and warmed. The membrane was placed in a bottle with the top facing in towards the solution and 5ml of the pre-Hyb solution was added and incubated for 60 mins at 68°C. 100 µl of salmon sperm (10 mg/ml) was added to the labeled probes and the mixture was heated at 94°C for 5 minutes. The probes were added to the pre-Hyb solution and the blot was incubated for 1 hour at 68°C. After hybridization, the membrane was removed from the bottle and washed twice in 50 ml of 2X SSC and 0.1% SDS for 15 minutes at room temperature. Then, the membrane was washed twice in 50 ml of pre-heated 0.1X SSC and 0.1% SDS for 15 minutes at 60°C. The membrane was then placed on top of Whatman filter paper for 30 minutes to dry and then wrapped in saran wrap. The membrane was exposed to film overnight, stored at -80°C and then developed.

Plant Expression of NS3 and Immunoblot Analysis

Petit Havana and LAMD-609 leaf sections were cut and 100 mg plant leaf tissue was weighed and grounded with liquid nitrogen in cold mortar and pestles and transferred to a microcentrifuge tube. Fresh plant extraction buffer (PEB: 60 ul of 5M NaCl, 60 ul of 0.5M EDTA (pH 8), 600 ul of 1M Tris-HCl (pH 8), 2 ul of Tween-20, 30 ul of 10% SDS, 3 ul of 14mM β-mercaptoethanol (BME), 1.2 ml of 1M sucrose, 1ml sterile H2O and 120 ul of 100 mM PMSF) was made and kept on ice. To make 100 mM of PMSF, 17.4 mg of powdered PMSF (Sigma) was weighed out, put into 1 ml of methanol and vortexed, and

stored at up to 1 month at –20°C. 200 ul of PEB was added to each plant sample on ice and then samples were mixed for 3 minutes with a micropestle. The samples were centrifuged at 13,000 rpm for 10mins to obtain the supernatant containing the soluble proteins. 20 µl of these extracts were mixed with 20 µl of sample loading buffer containing BME. Samples were then boiled for 5 minutes and loaded into SDS-PAGE gel. The procedure for the rest was identical to the protocol for E.coli-expressed NS3 and Immunoblot Analysis (see above sections).

Enzyme Linked Immuno Sorbant assay (ELISA)

The levels of NS3 in transgenic LAMD-609 were calculated as a percentage of the total soluble protein of leaf extracts. The quantification of NS3 in the plant crude extract was done using the enzyme linked immunosorbant assay (ELISA). 100mg of transgenic leaf samples (young, mature, old) and the wild type leaf samples (young, mature, old) were collected. The leaf samples were collected from plants exposed to regular lighting pattern (16 h light and 8 h dark), 3 day continuous light, and 5 day continuous light. The leaf samples were finely grounded in liquid nitrogen and the leaf powder was transferred into an eppendorf tube. To extract the protein, plant protein extraction buffer (15mM Na2CO3, 35mM NaHCO3, 3mM NaN3, pH: 9.6, 0.1% Tween, 5mM PMSF) was used to resuspended the leaf powder. In order to check the protein concentration, the standards, test samples and antibody were diluted in coating buffer (15mM Na2CO3, 35mM NaHCO3, 3mM NaN3; pH: 9.6). The standards ranging from 50 to 500ng/ml (500ng/ml, 400ng/ml, 300ng/ml, 200ng/ml, 100ng/ml and 50ng/ml) were made by diluting purified NS3 in coating buffer (stock: 1000ng/ml). The standards and protein samples (100 µl)

were coated to 96-well polyvinyl chloride microtiter plate (Cellstar) for 1 h at 37 C followed by 3 washes with PBST and 2 washes with water. Blocking was done with 3% fat-free milk in PBS and 0.1% Tween and incubated for 1h followed by washing. The primary anti-NS3 antibody (Neomarkers) diluted (1:500) in PBST containing milk powder was loaded into wells and incubated for 1h followed by washing steps and then again incubated with 100 µl of anti-mouse goat-HRP conjugated antibody (American Qualex, 1: 5000) diluted in PBST containing milk powder. The plate was then incubated for 1h at 37 °C. After the incubation, the plate was washed thrice with PBST and twice with water. The wells were then loaded with 100 µl of 3,3,5,5-tetramethyl benzidine (TMB from American Qualex) substrate and incubated for 10–15 min at room temperature. The reaction was terminated by adding 50 µl of 2N sulfuric acid per well and the plate was read with a plate reader (Dynex Technologies) at 450 nm (Modified form of protocol from Ausubel *et a*l*.,* 4 th edition).

Bradford assay for protein quantification (Bio-rad manual)

The Bradford assay was used to determine the total protein from the plant extracts prepared as described above. This was used to determine the percent of NS3 antigen in the total soluble protein extract (or %TSP). An aliquot of plant extract as prepared above was thawed on ice. Extraction buffer (15 mM Na2CO3, 35 mM NaHCO3, 0.2 g NaN3, 0.1% Tween 20, and 5mM PMSF adjusted to pH 9.6) was used to make Bovine Serum Albumin (BSA) standards ranging from 0.05 to 0.5 µg/µl. Plant extracts were diluted 1:20 and 1:30 with extraction buffer. 10 µl of each standard and 10 µl of each plant dilution were added to the wells of a 96 well microtiter plate (Costar) in duplicates.

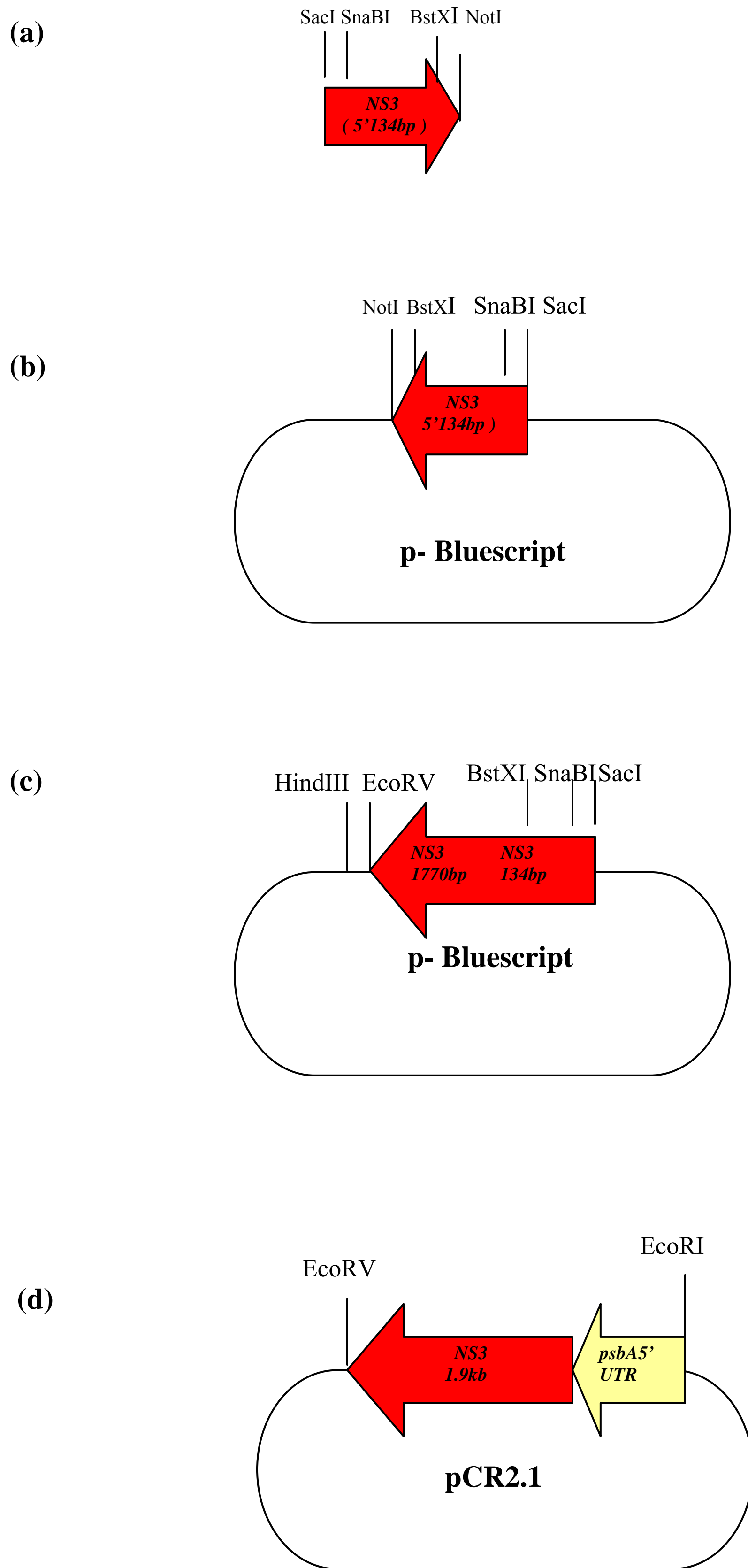
Bradford reagent (Biorad protein assay) was diluted 1:4 with distilled water as specified and 200 µl was added to each well. Absorbance was read. The comparison of the absorbance to known amounts of BSA to that of the samples was used to estimate the amount of total protein.

RESULTS

Construction of pLD-5'UTR/NS3 Vector for tobacco chloroplast transformation

The NS3 gene (starting 134bp) in pcDNA3.1D/V5-His-TOPO was PCR amplified and the restriction sites, SacI and SnaBI at the 5' end and NotI at the 3' end of the 134bp of the NS3 gene were created for further subcloning. A PCR product of 134bp in size was obtained by amplification. The PCR product was then digested with SacI and NotI and was ligated between the same sites in p-Bluescript II KS vector. The transformed colonies were selected as the pBluescript vector contains the LacZ gene for α complementation and blue/white selection. The ligated plasmid pBS-NS3 was isolated using midi-prep and the PCR product was sequenced. The sequence was compared with the original NS3 sequence sent by Dr. Lasarte. After confirming that the 5'of the NS3 gene (beginning 134bp) was successfully cloned into pBluescript, the remaining NS3 gene (1770bp) was digested from the original pcDNA3.1D/V5-His-TOPO vector with BstXI and EcoRV and ligated between the same sites in pBluescript vector. Therefore, the entire NS3 gene (1.9kb) was cloned into p-Bluescript vector. The entire NS3 gene was digested with SnaBI and HindIII and cloned downstream of psbA 5'UTR in pCR2.1. Finally, the psbA 5'UTR and the NS3 gene were digested with EcoRV and EcoRI (fragment size 2.1kb) from pCR2.1 and ligated into the final universal vector, pLD-AB-Ct. The 5.9 kb expression vector was developed with unique features facilitating the genetic engineering of plant chloroplasts (Fig.2). The integration of cloned chloroplast DNA into the plastid genome occurs exclusively through site-specific homologous

recombination and excludes the foreign vector DNA (Kavanagh et al., 1999). The pLD-AB-Ct uses trnA and trnI genes (chloroplast transfer RNAs coding for alanine and isoleucine) from the inverted repeat region of the tobacco chloroplast genome as flanking sequences for homologous recombination (Daniell, 1999). This chloroplast expression vector is considered universal because it can be used to transform the chloroplast genomes of not just tobacco, but several other plant species as well (Daniell, 1999). Therefore, this pLD-AB-Ct was successfully used as the backbone for the 5'UTR/NS3 cassette (Fig.1).


(a)
SacI SnaBI BstXI NotI
NS3
(5'134bp)
(b)
NotI BstXI SnaBI SacI
NS3
5'134bp)
p- Bluescript
(c)
HindIII EcoRV BstXI SnaBI SacI
NS3
1770bp
NS3
134bp
p- Bluescript
(d)
EcoRV
EcoRI
NS3
1.9kb
psbA5'
UTR
pCR2.1

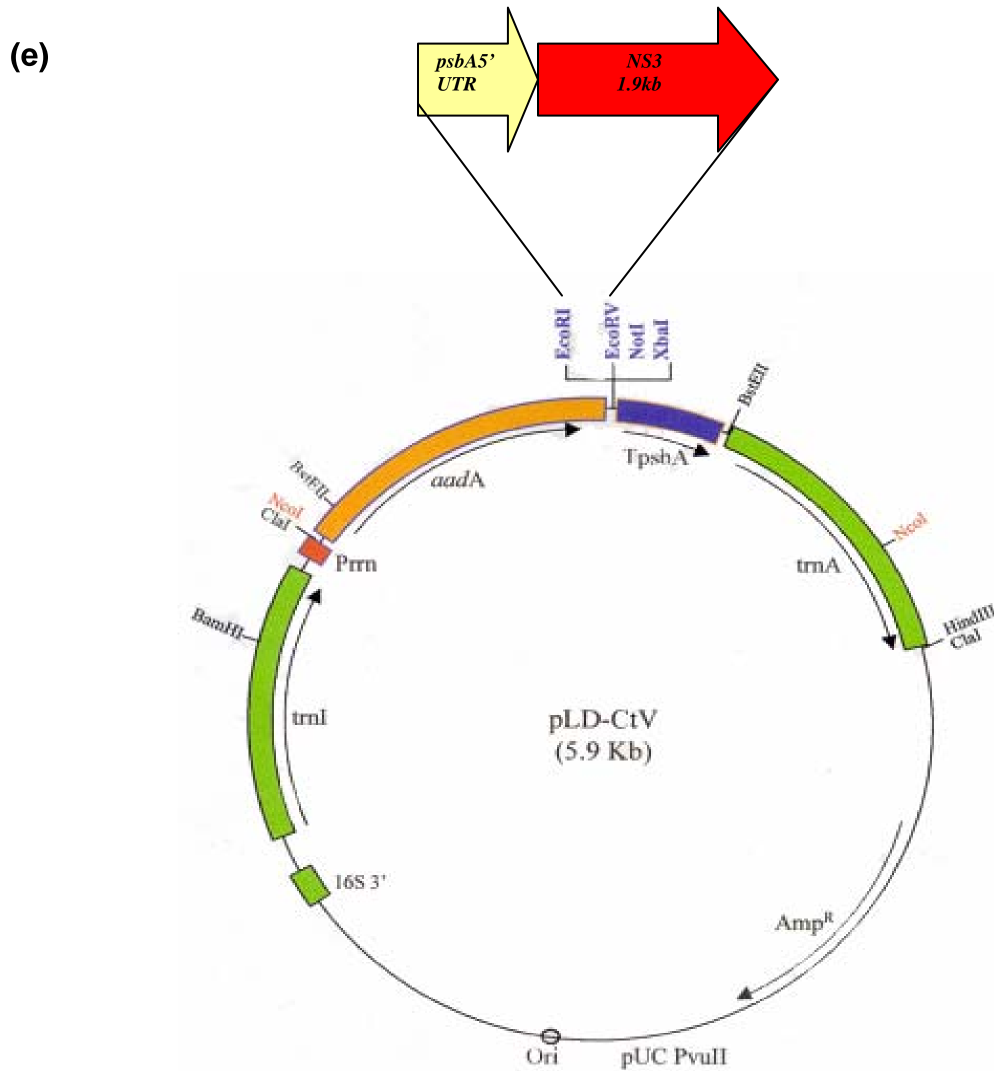


Figure 1: Schematic steps to clone pLD-AB-NS3

(a) Amplification of 5' terminal 134 bp of NS3 gene using PCR. SacI and SnaBI and NotI are introduced for further subcloning. (b) Cloning of PCR product in p-Bluescript between SacI and NotI. (c) pcDNA3.1-NS3 vector digested with BstXI and EcoRV and cloned between same sites in p-Bluescript. NS3 gene cloned in p- Bluescript between SacI and EcoRV. (d) NS3 gene in p- Bluescript digested with SnaBI and HindIII and cloned in pCR2.1 between the same sites and upstream of 5'UTR. (e) NS3 gene and 5'UTR (2.1kb) digested from pCR2.1 with EcoRI and EcoRV and cloned in between same sites in pLD-AB-Ct vector.

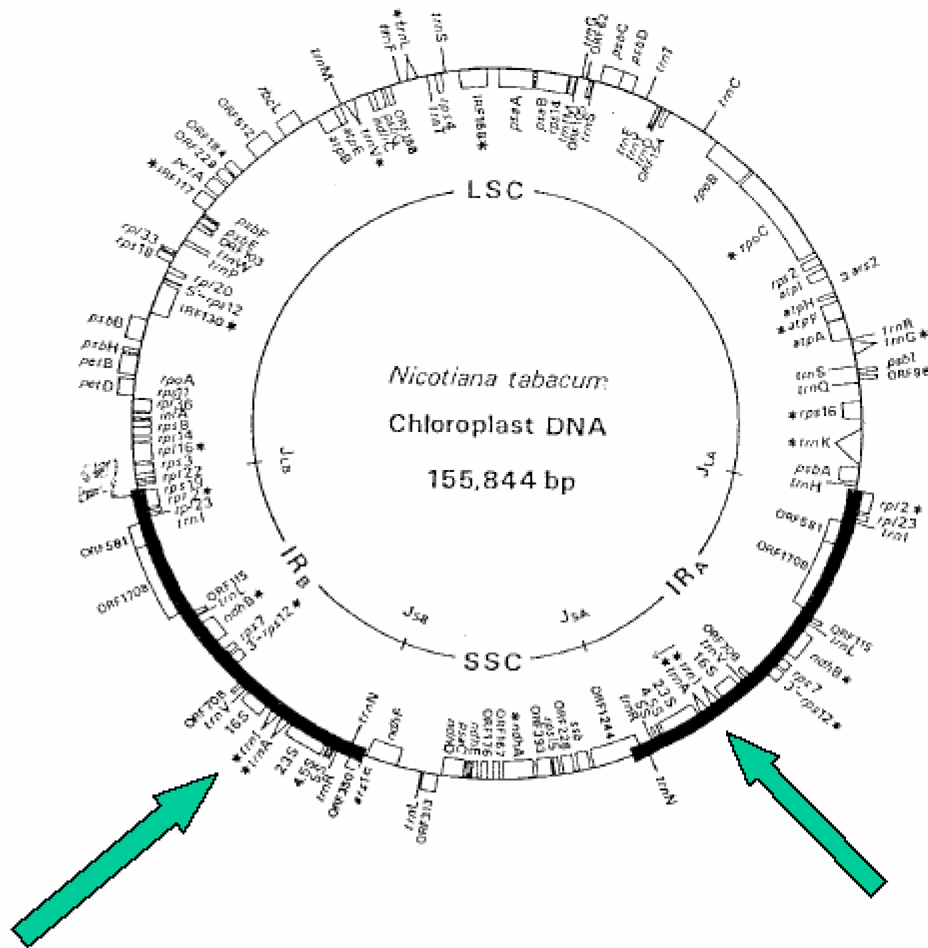


Figure 2: Nicotiana tabacum chloroplast genome

The pLD contains the chloroplast transfer RNAs coding for Isoleucine and Alanine *(trn*I and *trn*A*)*. These homologous flanking DNA sequences direct the insertion of the *Prrn/ aad*A/ *5'UTR/ NS3'UTR* genes into the chloroplast genome by two homologous recombination events.

E.coli expression of NS3and Immunoblot Analysis

Competent E.coli cells were transformed with pLD-AB-NS3. Western blot analysis was performed on the cell lysates. Total E.coli protein was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The NS3 protein was detected by chemiluminescent detection method which utilized the anti-NS3 antibody shows the presence of 69 kDa NS3 protein (Fig.3).

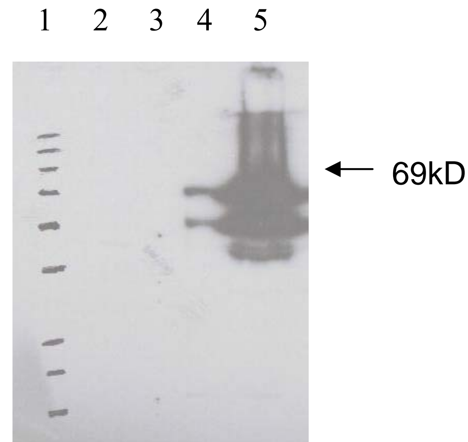


Figure 3: Chemiluminescent Detection of *E.coli*-expressed NS3

Total *E.coli* proteins were separated on SDS-PAGE and detected with monoclonal anti-NS3 as the primary antibody. The secondary antibody was goat anti-mouse IgG conjugated to horseradish peroxidase. Samples: Protein marker (lane1); Extracts of untransformed *E.coli* cells (lane 2 and 3); Protein extracts from lysates of *E.coli* transformed with pLD-AB-NS3 (lane5 and spillover in lane 4).

Selection and Regeneration of Transgenic Lines

After recovering in the dark for 48 hours from bombardment, leaves were cut into 5mm2 pieces and placed on RMOP (Daniell, 1993) plates containing 500 μg/ml spectinomycin for Petite Havana and 350 μg/ml for LAMD-650, for the first round of selection as described in Daniell (1997). From 10 bombarded Petit Havana leaves, 15 green shoots appeared after 4 weeks. From 10 bombarded LAMD leaves, 3 green shoots appeared within 7 weeks, so the shoots from the low-nicotine tobacco took longer to sprout and were less numerous. Untransformed cells appeared bleached on the antibiotic because they did not contain the aadA gene (Fig.4). For second selection the shoots were cut into $2mm^2$ pieces and then transferred to fresh RMOP plates with 500 μg/ml and 350 μg/ml spectinomycin for Petite Havana and LAMD spectinomycin respectively (Fig.5).

During the second round of selection, the shoots that appeared and tested positive for cassette integration into the chloroplast genome by PCR analysis were grown in sterile jars containing fresh plant media with spectinomycin until the shoots grew to fill the jars (Fig. 6A).Then the plants were transferred to pots with soil containing no antibiotic (Fig. 6B). Potted plants were grown in a 16 hour light/ 8 hour dark photoperiod in the growth chamber at 26°C.

A.

B.

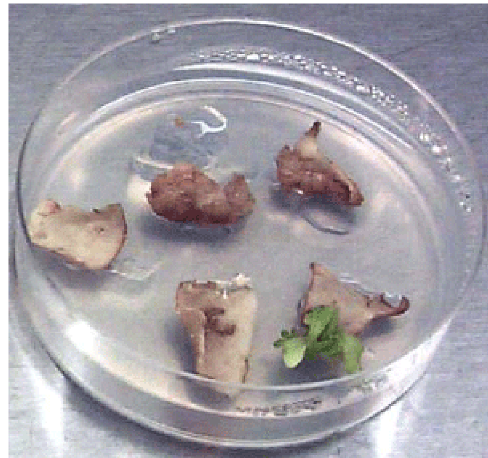
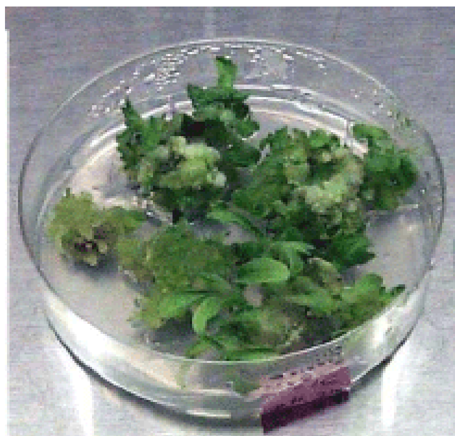


Figure 4: First Round of Selection

A. Shoots from bombardment of Petit Havana leaves appeared within 4 weeks

B. Shoots from bombardment of LAMD-609 leaves appeared within 7 weeks

A.



B.

Figure 5: Second Round of Selection

A. Pettit Havana shoots from first selection on 500 μg/ml spectinomycin
B. LAMD-609 shoots from first selection on 350 μg/ml spectinomycin

A. B.

Figure 6: Propagation of Petit Havana Transgenic Lines

A. Petit Havana transgenic lines in jars containing MSO 500ug/ml spectinomycin.
B. Petit Havana transgenic plant in pots with no added antibiotic.

PCR Analysis of Transgenic Lines

Two primer sets were used to identify transgenic lines. The 3P/3M set, the 3P primer annealed to the chloroplast genome outside of the inserted cassette and the 3M primer annealed to the chimeric aadA gene (Fig.7A). When both of the primers annealed, a 1.65 kb PCR product was observed, however, there was no PCR product in the untransformed (-) Petit Havana and LAMD line (Fig.7B). In addition, no PCR product should be observed if the foreign gene cassette was integrated into the nuclear genome or if the plants were mutants lacking the aadA gene. Out of the 7 putative transgenic lines shown, all 7 were positive for insertion of the foreign gene cassette (Fig.7B).

For the 5P/2M set, the 5P primer annealed to the chimeric aadA gene and the 2P primer annealed to trnA gene within the cassette (Fig.8A). When both of the primers annealed, a 3.7 kb PCR product was observed, however, there was no PCR product in the untransformed (-) petit Havana or LAMD line (Fig.8B). The correct size of PCR product (3.7kb) indicated that the entire foreign gene cassette and not just the aadA gene had been integrated into the chloroplast genome (Fig. 8A).

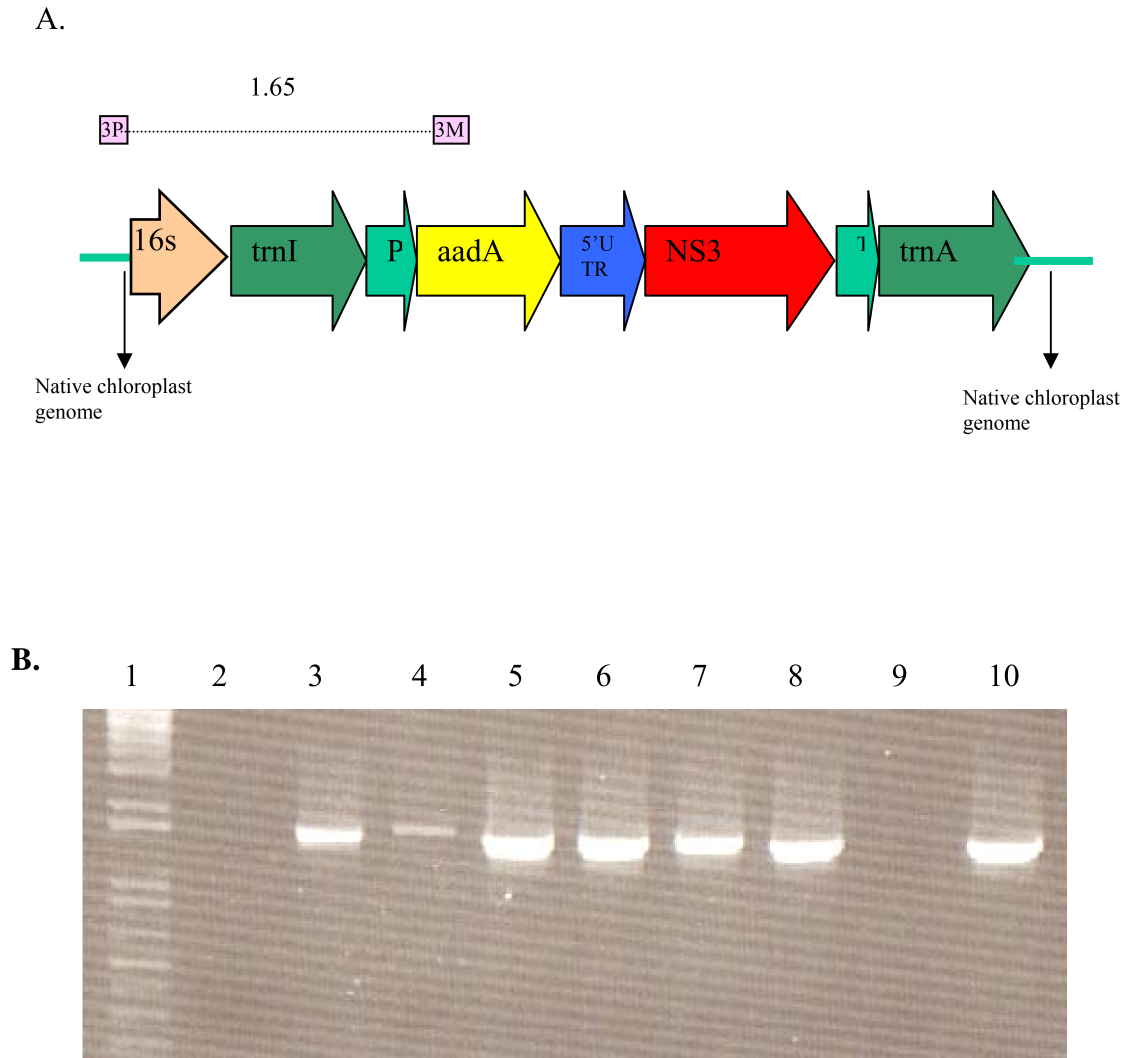
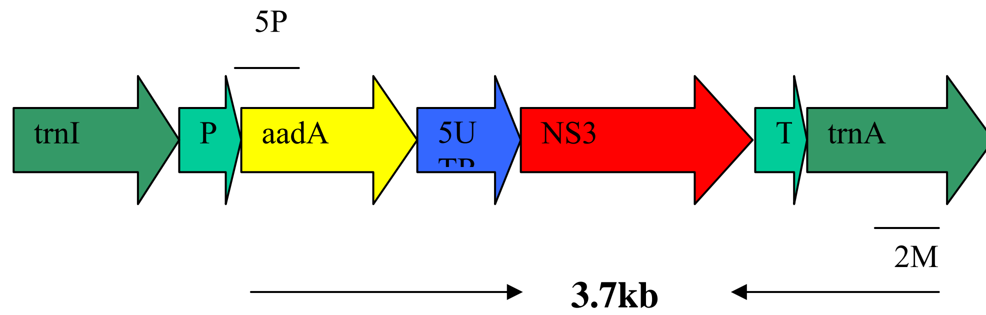


Figure 7: 3P/3M PCR Analysis of Putative Petit Havana and LAMD Transgenic Lines

A. 3P/3M primers annealing to sequences in the chloroplast genome of Petit Havana and LAMD.
B. A 1.65kb PCR product with 3P/3M primers: 1kb DNA ladder (lane1); untransformed (-) petit Havana (lane 2); transgenic PH lines (lanes 3-8); untransformed LAMD (lane 9, control); LAMD transgenic line (lane 10).

A.



B.

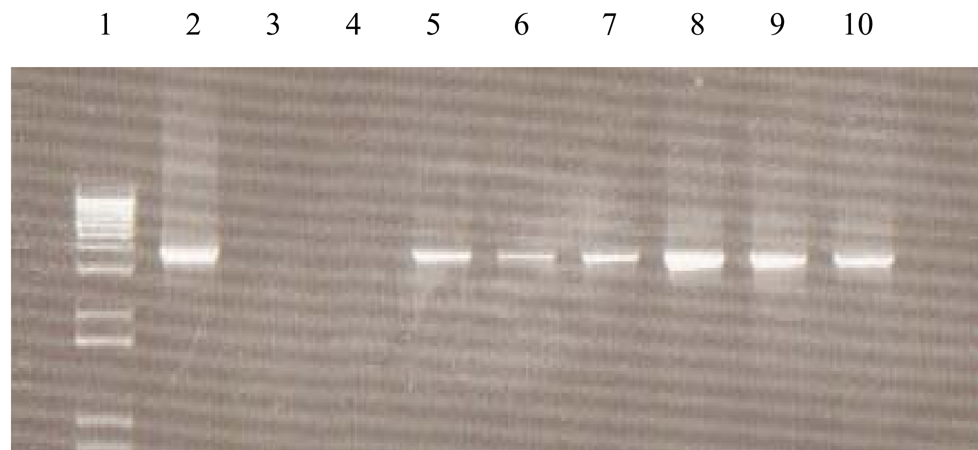


Figure 8: 5P/2M PCR of Putative Petit Havana and LAMD Transgenic Lines

A. 5P/2M primers annealing to sequences in the chloroplast genome of Petit Havana and LAMD.

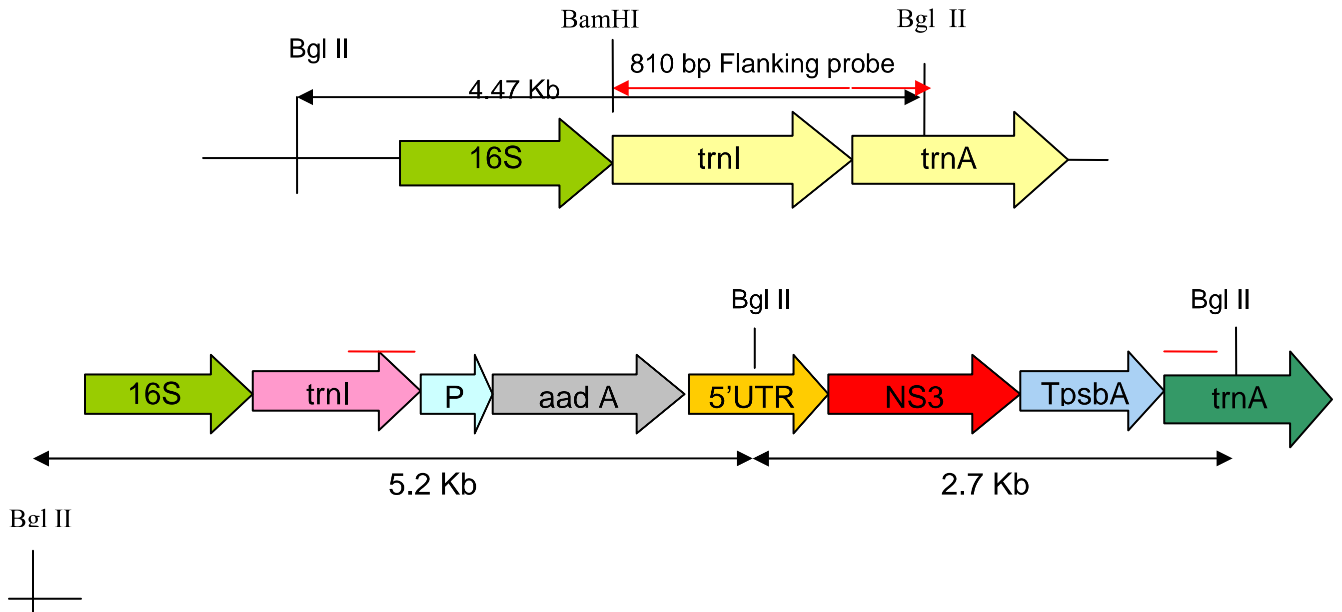
B. 0.8% agarose gel shows 3.7 kb PCR product utilizing 5P/2M primers**;** 1kb DNA ladder (lane1); 1µg of pLD-AB-NS3 as the positive control (lane 2); untransformed (-) Petit Havana (lane 3); untransformed (-)LAMD (lane 4); transgenic petite havana lines (lanes 5-9); transgenic LAMD lines (lane10).

Southern Blot Analysis of Transgenic Plants (T0)

Southern blots were performed to confirm integration of the NS3 gene cassette utilizing two different DNA probes (Fig. 9 and Fig. 10). A 0.81 kb DNA fragment containing chloroplast-flanking sequences was used to probe a Southern blot to determine homoplasmy or heteroplasmy after bombardment with pLD-AB-NS3 (T0). This determination was also used to estimate chloroplasts genome copy number. BglII digested DNA from transformed plants produced a 5.2 kb and 2.7kb fragment when probed with the 0.81 kb probe that hybridizes to the trnI and trnA flanking sequences (Fig. 9). Untransformed plant DNA from both tobacco varieties produced only a 4.47 kb fragment, indicating no integration of foreign DNA. Transgenic plant DNA (T0) produced only the 5.2 and 2.7 kb fragment in all transgenic plants indicating homoplasmy (contained only transformed chloroplast genomes).

The second probe used was a 2.1kb 5'UTR/NS3 sequence that hybridized to a 2.7 kb fragment in transformed plants and no fragment was evident in untransformed plants (Fig. 10). All transgenic plants produced a 2.7 kb fragment corresponding to the NS3 sequence (Fig. 10).

A.



B. PLANTS: 1 2 3 4 5 6 7 8 9

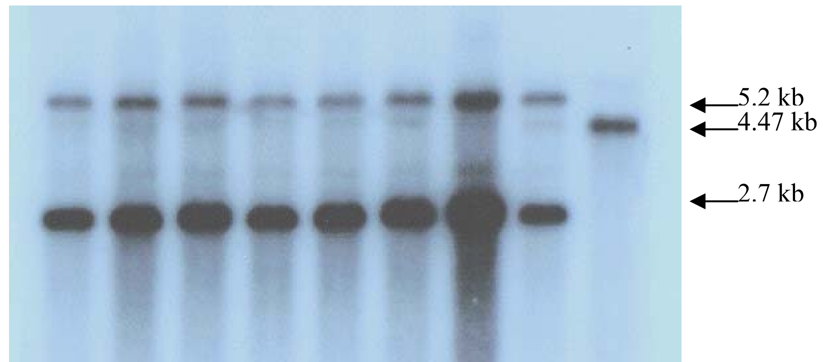


Figure 9: Southern Blot using Flanking Probe

Confirmation of Chloroplast Integration and Determination of Homoplasmy/Heteroplasmy in T_0 Generation.
A 810 bp probe containing chloroplast flanking sequences and DNA fragments of 4.47 kb indicate untransformed chloroplast.

A. DNA fragments of 5.2 and 2.7 kb indicate transformed chloroplasts of transgenic plants (lanes 1-8) and DNA fragments of 4.47 kb indicate untransformed chloroplasts of transgenic plants (lane 9).

PLANTS : 1 2 3 4 5 6 7 8 9

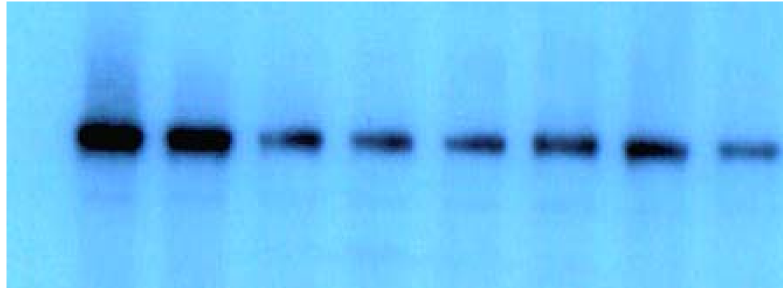


Figure 10: Southern Blot using NS3 gene specific probe

A 2.1 kb NS3 gene specific probe was used. All transformed plants (lanes 2-9) show 2.7 kb DNA fragment and the untransformed plant (lane1) does not show any DNA fragment.

Chloroplast-synthesized NS3 and Immunoblot Analysis

Petit Havana and LAMD were bombarded with pLD-AB-NS3. Western blot analysis was performed on the leaf cell extracts. The total plant protein was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The NS3 protein was detected by mouse monoclonal antibody against NS3. Western blots detected NS3 protein at 69 kDa using chemiluminescense (Fig.11A).

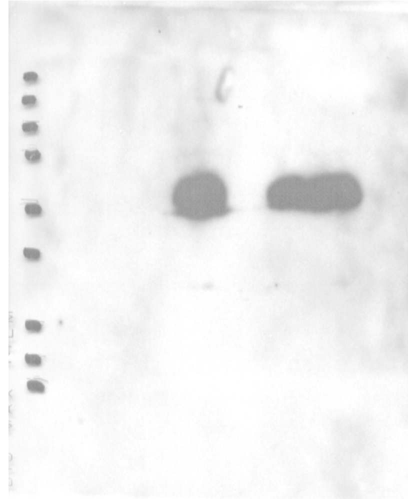


Figure 11: Western Blot of Transgenic plants expressing NS3

Plant tissue extracts separated on 10 % SDS-PAGE with NS3 detected by mouse monoclonal antibody against NS3. Protein Marker (lane1); untransformed plant (lane 2); Blank- Sample buffer (lane 3); transgenic PH plant (lane 4); Mutant PH plant not expressing NS3 (lane 5) ; transgenic PH plant (lane 6); ransgenic LAMD plant (lane 7).

Quantification of Chloroplast-synthesized NS3 by ELISA

To quantify the amount of NS3 in transgenic Petit Havana and LAMD leaf extracts, an indirect enzyme-linked immunosorbent assay (ELISA) was used. The purified NS3 protein was used to make a six -point standard curve. 1µl of the plant protein extracts were diluted into 20ul and 30ul of coating buffer to determine the dilution that would be in the linear range of NS3 standard curve. The primary antibody was anti-NS3 Mouse Monoclonal Antibody. The secondary antibody was Goat anti-mouse IgG conjugated to horseradish peroxidase. The addition of one step substrate (TMB) into the wells resulted in a color change that was eventually read on a plate reader with a 450nm filter. The total

soluble protein (tsp) in the plant leaf extracts was determined with a Bradford Bio-Rad Protein Assay. The levels of NS3 in transgenic LAMD were calculated as a percentage of the total soluble protein of leaf extracts (Fig. 12).

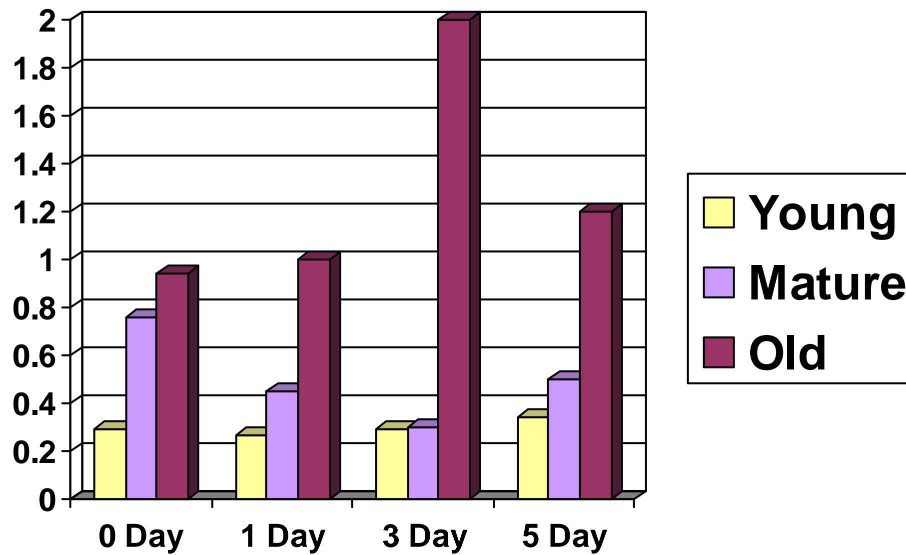


Figure 12: Quantification of NS3 in Transgenic chloroplasts

A. Protein quantification by ELISA in young, mature and old transgenic leaves of LAMD of plant in 16 h light and 8 h dark (day 0), 1, 3 and 5 day continuous illumination.

Figure 13 : Maternal inheritance

Seeds were sterilized and grown in MSO plates with spectinomycin (500ug/ul).

DISCUSSION

HCV vaccine development began recently with the use of recombinant HCV proteins as the immunogenic material (Choo et al., 1994). The initial candidate HCV vaccine developed in 1994, derived from the envelope glycoproteins (gpE1/E2) of HCV, with muramyl dipeptide adjuvants, induced high levels of neutralizing antibodies in chimpanzees and provided protection in a proportion of animals challenged with low doses of the homologous strain (Choo et al., 1994; Houghton et al., 1997). In the chimpanzees that were infected, the risk of persistent infection seemed to be reduced. Little new information about this candidate vaccine is available. Additional studies of a recombinant E1/E2 protein and peptide vaccine produced in insect cells (Esumi et al., 1999) also suggested that induced antibodies could neutralize low-level challenge with homologous HCV in the chimpanzee. In one DNA vaccine study utilizing chimpanzees, a plasmid encoding the E2 HCV protein was used as immunogen and elicited antibodies and immune response but on challenge with homologous HCV, sterilizing immunity could not be achieved (Forns et al, 2000). Other approaches to vaccine development have included the incorporation of HCV proteins into recombinant viruses (Siler et al., 2002; Brinster et al., 2002), the synthesis of HCV-like particles in insect cells (Lechmann et al., 2001), expression of the hypervariable-1-region of E2 in tobacco plants (Nemchinov et al., 2000) and DNA-based immunization (Brinster et al., 2001; Forns et al., 2000). Plant synthesized recombinant TMV/HCV HVR1 epitope/CTB induced a strong immune response when mice were immunized intranasally (Nemchinov et al., 2001). Plants infected with a recombinant tobacco mosaic virus engineered to express the hypervariable region 1 (HVR1) of HCV, the HVR1/CTB chimeric protein elicited

both anti-CTB and anti-HVR1 serum which specifically bound to HCV virus- like particles. The HCV HVR1 epitope was also cloned into alfalfa mosaic virus (ALMV) coat protein and expressed in transgenic tobacco plants. The Plant–derived HVR1/ALMV-CP reacted with HVR1 and ALMV-CP specific monoclonal antibodies and immune sera from individuals infected with HCV (Nemchinov et al., 2001). A replication-deficient recombinant adenovirus expressing HCV NS3 protein was constructed. Mice immunized with this recombinant adenovirus were protected against challenge with a recombinant vaccinia virus expressing HCV polyprotein (Arribillaga et al, 2002).

The NS3 gene was introduced into pLD-Ct, the universal chloroplast expression vector, which was developed with unique features that facilitate chloroplast genetic engineering (Fig.2). The 5' untranslated region (UTR) of the plastid psbA gene and its promoter were used to increase translation efficiency. The 5'UTR is involved in mRNA – rRNA interactions (between the mRNA ribosome- binding site and 16S r RNA 3'end) and interactions with translational- activating proteins that facilitate loading onto ribosomes (Maliga, 2002). The psbA gene encodes the D1 protein of photosystem II and is rapidly turned over in the chloroplasts (Eibl et al., 1999). The psbA 5'UTR is about 200bp and contains a promoter. The 3' regulatory region (3'UTR) is important for mRNA stability and functions as an inefficient terminator of transcription. A unique short inverted repeat (IR) which can potentially fold into a stem loop structure at the 3'UTR probably act as a RNA processing signal rather than termination signal , playing a role in both RNA 3' end formation and stabilization (Hager and Bock, 2000). The pLD-Ct contains a chimeric aadA gene as a selectable marker, which encodes aminoglycoside

3'-adenylyltransferase. This enzyme catalyzes the covalent modification of aminoglycoside-type antibiotics and thereby inactivates them. The aadA protein catalyses the covalent transfer of an AMP residue from ATP to spectinomycin, thereby converting the antibiotic into an inactive from (adenyl-spectinomycin) that no longer inhibits protein biosynthesis on prokaryotic 70S ribosomes present in the chloroplast. The aadA gene is driven by a portion of the constitutive promoter of the chloroplast 16S rRNA operon (Prrn). The pLD-AB vector integrates the 16S rRNA promoter, aadA gene, 5'UTR, NS3 gene and 3'UTR cassette into the Inverted Repeat (IR) regions of the chloroplast genome between the homologous flanking sequences, trnI and trnA genes. The trnI and trnA intergenic spacer regions are highly conserved among higher plants (Guda et al., 2000). The pLD-Ct vector was constructed with a multiple cloning site downstream of the aadA gene and upstream of the TpsbA portion and flanked by chloroplast transfer RNA genes for isoleucine and alanine (trnI and trnA respectively). The plasmid can replicate autonomously because it contains a unique chloroplast origin of replication (Daniell, 1990; Kumar et al. 2004a, b) and ColE1 origin of replication that operates in E.coli (Glick and Pasternak, 1998). The translational apparatus of chloroplasts very much resembles that of prokaryotes, in that tRNAs, rRNAs, ribosomal proteins and the initiation and elongation factors exhibit strong similarity with their counterparts in E.coli (Brixey et al., 1997). As a way of testing the integrity of the NS3 cassette and its potential for protein expression, E.coli was transformed with pLD-AB-NS3. Western blot analysis performed on the E.coli cell lysates indicated the presence of NS3 protein at the expected size of 69 kDa (Fig. 3), while the untransformed E.coli cell lysates showed no protein. Since the protein synthetic machinery of chloroplasts is

similar to that of E.coli (Brixey et al., 1997), the positive expression of NS3 suggested that it could be successfully expressed within transgenic chloroplasts. Two varieties of tobacco were bombarded with gold particles coated with pLD-AB-NS3 (Daniell, 1993). Petit Havana is the model tobacco variety because it is amenable to genetic engineering. The second variety of tobacco bombarded with pLD-AB-NS3 was LAMD-609. This tobacco hybrid contains 0.06% nicotine (Collin et al., 1974), which is at least 50-fold lower than the Petit Havana tobacco (3-4%). Tobacco is the easiest plant to genetically engineer and is widely used to test suitability of plant-based systems for bioproduction of recombinant proteins. Tobacco is ideal for transformation because of its ease for genetic manipulation and is an excellent biomass producer and a prolific seed producer (up to one million seeds produced per plant). Bombarded leaves were placed on RMOP medium containing no antibiotics and allowed to recover from bombardment in the dark for 48 hours (Daniell, 1993; Daniell, 1997; Daniell et al. 2004a).

After the recovery period, bombarded leaf discs were placed on selective plant medium containing 500 µg/ml of spectinomycin. Green shoots that emerged from the part of the leaf disc in contact with the medium were considered putative transformants because growth indicated that the aadA gene had been integrated into the chloroplast genome and was expressing functional enzyme. Each shoot (transgenic event) was subjected to a second round of selection (500 µg/ml of spectinomycin) in an effort to ensure that only transformed genomes existed in the cells of the transgenic lines (homoplasmy). A heteroplasmic condition is unstable and will result in loss of the transgene when the cell divides without selective pressure (Hager and Bock, 2000). A PCR method of screening putative transformants was utilized to distinguish chloroplast transformants from mutants

and nuclear transformants (Daniell et al. 2004a). Only those transgenic lines with the appropriately sized PCR products were used in further characterizations. The Southern blot analysis utilized the integrity of DNA complimentary hybridization to identify specific sequences in the various plant genomes. Different positive transgenic lines (T0) were tested to confirm site-specific integration and to determine homoplasmy or heteroplasmy (Fig.12&13). The 810 bp flanking sequence probe confirmed that the NS3 gene cassette had been integrated into the chloroplast genome. An enzyme-linked immunosorbent assay (ELISA) utilizing 96-well microtiter plates, was used to quantify the amount of NS3 in transgenic LAMD-609 leaf extracts. The highest percentage of NS3 was 2 % of total soluble protein, observed in the old leaves. In conclusion, this study reports successful expression of the HCV NS3 antigen in transgenic chloroplasts and the plant derived recombinant HCV vaccine antigen can potentially reduce expenses normally associated with the production and delivery of conventional vaccines and is a safe and inexpensive source for the production of HCV vaccine antigen.

Further studies

Animal studies to test the immunogenecity of the chloroplast derived HCV NS3 will be performed using chloroplast derived NS3 antigen.

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