

**EXPRESSION OF CHOLERA TOXIN B SUBUNIT-ROTAVIRUS
NSP4 ENTEROTOXIN FUSION PROTEIN IN TRANSGENIC
CHLOROPLASTS**

by

ANILA KALLURI

M.Sc. Biochemistry, Andhra University, India, 1998.

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Molecular Biology and Microbiology
in the Burnett College of Biomedical sciences
at the University of Central Florida
Orlando, Florida

Summer Term
2005

© 2005 Anila Kalluri

ABSTRACT

Rotavirus, the major cause of life-threatening infantile gastroenteritis, is a member of the *Reoviridae* family and is considered to be the single most important cause of virus-based severe diarrheal illness in infants and young children particularly 6 months to 2 years of age in industrialized and developing countries. Infection in infants and young children is often accompanied by severe life threatening diarrhea, most commonly following primary infection. Diarrhea is the major cause of death among children around the world. Responsible for 4 to 6 million deaths per year according to the World Health Organization (WHO), diarrhea is especially dangerous for infants and young children. Globally, it is estimated that 1.4 billion episodes of diarrhea occur in children less than five years of age annually.

In the United States alone, rotavirus causes more than 3 million cases of childhood diarrhea each year, leading to an estimated 55,000 to 100,000 hospitalizations and 20 to 100 deaths. And is a major cause of mortality for children in developing countries with approximately one million deaths annually. Rotaviruses belong to the family *Reoviridae* and are spherical 70-nm particles. The virus genome contains 11 segments of double-stranded RNA, each encoding a viral capsid or nonstructural protein. The identification of a rotavirus nonstructural protein gene (NSP4) encoding a peptide, which functions both as a viral enterotoxin and as a factor involved in the acquisition of host cell membrane during virus budding from cells, provides a new approach for mucosal immunization.

Protein expression through chloroplast transformation system offers a number of advantages like high level of transgene expression, transgene containment via maternal inheritance, lack of gene silencing and position effect due to site specific gene integration and also the possibility of multi gene engineering in single transformation event. It is also an environmentally friendly approach due to effective gene containment and lack of transgene expression in pollen.

To achieve an enhanced immune response to rotavirus infection, a fusion gene encoding the cholera toxin B subunit linked to rotavirus enterotoxin 90 aa protein (CTB-NSP4₉₀) was introduced into transgenic chloroplast and was transformed into chloroplast genome of *Nicotiana tabacum* by homologous recombination. The chloroplast integration of CTB-NSP4₉₀ fusion gene was confirmed in transgenic tobacco plants by PCR analysis. Southern blot analysis further confirmed site specific gene integration and homoplasmy. Immunoblot analysis of transformed chloroplast confirmed the expression of CTBNSP4₉₀ fusion protein both in monomeric and pentameric forms that retained the binding affinity to the enterocytes GM1 ganglioside receptor. Expression levels of CTB-NSP4 protein was quantified by GM1 ganglioside binding ELISA assay; mature leaves expressed CTB-NSP4 fusion protein to upto 2.45 % in total soluble protein, 100-400 fold higher than nuclear expression which was only 0.006%-0.026%. Antibody titration and virus challenge experiments will be performed in mice at Loma Linda University to evaluate the antigenic and protective properties of the chloroplast derived CTB-NSP4 fusion protein.

ACKNOWLEDGMENTS

I would like to thank Dr. Henry Daniell for providing me the opportunity to work on this project in his lab. I would also like to thank my other committee members Dr. Naser and Dr. White for serving on my committee. I would like to thank Dr William H R Langridge for the kind gift of CTB-NSP4 gene, rabbit anti-NSP4 antibodies and the purified CTB-NSP4₉₀ protein.

TABLE OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGMENTS	v
LIST OF FIGURES	ix
LIST OF ACRONYMS/ABBREVIATIONS	x
INTRODUCTION	1
Rotavirus: Major cause of infantile gastroenteritis	1
NSP4 an important factor in the pathogenesis of Rotavirus infection	1
CTB an efficient adjuvant and carrier of mucosal vaccine antigens	3
Humoral and Cell-Mediated Immune response to NSP4 enterotoxin of rotavirus	4
Plants As The Expression System For Vaccines And Biopharmaceuticals	5
Chloroplast Genetic Engineering As A Better Plant Based Expression System	6
Unique features of Chloroplast genome and the copy correction mechanism	7
Chloroplast and post-translational mechanism	9
Chloroplast transformation and Multigene engineering	9
Light regulated expression of transgene in chloroplast using psbA 5'UTR	11
Maternal inheritance and gene containment	12
Chloroplast engineering of agronomically important crops	15
Vaccine antigens Expressed Via the Chloroplast Genome	17
Bioencapsulation as protection to vaccine antigens for the oral delivery	18
Rationale and Approach	20
MATERIALS AND METHODS	22

General Protocols.....	22
Preparation of Ultra Competent cells (Rubidium Chloride Method).....	22
Transformation of the Competent E. coli XL1-Blue cells.....	23
Isolation of Plasmid DNA by Alkaline lysis method.....	24
Construction of pLD-5'UTR-HisCTBNSP4 vector for transformation of tobacco.....	25
Chloroplast.....	25
Bombardment of the pLD-5'UTR-Histag-CTBNSP4 vector.....	25
Preparation of gold particles.....	25
Preparation of tobacco tissue culture media.....	26
Preparation of Spectinomycin Solution.....	26
Bombardment protocol for tobacco leaves.....	27
Tissue regeneration and selection.....	28
Plant genomic DNA extraction procedure.....	29
Confirmation of transgene integration into the chloroplast genome.....	30
Southern blot analysis of transgenic plants.....	31
Restriction Digestion of plant genomic DNA.....	31
Agarose electrophoresis and DNA transfer.....	31
Generation of probes.....	33
Probe labeling.....	34
Prehybridization, hybridization and washing of membrane.....	35
Autoradiography.....	36
Characterization of expressed proteins.....	36

Extraction of Protein from Transformed E. coli Cells.....	36
Extraction of Protein from Transformed Tobacco Leaves	37
SDS-PAGE Buffers and Gels	38
Transfer of protein and analysis of Western Blot.....	39
GM1 Binding (ELISA) assay.....	40
Bradford assay for protein quantification (Bio-Rad manual).....	42
RESULTS	43
Construction of pLD-5'UTR-His-CTB-NSP4 vector for tobacco chloroplast.....	43
Transformation.....	43
Sequencing of pLD-5'UTR-Histag-CTB-NSP4(pLD-AK).....	46
Selection and Regeneration of Transgenic Lines.....	48
PCR Analysis of Confirmation of Chloroplast Integration of transgene.....	50
Southern Analysis of transgenic plants.....	54
Immunoblot analysis.....	57
Protein quantification and binding affinity using GM1 Binding assay ELISA.....	59
DISCUSSION.....	61
Future Studies	65
Assessing the immunogenicity of the plant derived CTB-NSP4 vaccine antigen.....	65
Development of transgenic carrots expressing CTB-NSP4.....	65
CONCLUSIONS	66
LIST OF REFERENCES.....	68

LIST OF FIGURES

Figure 1 Schematic representation of the cloning of pLD-5'UTR-Histag-CTB-NSP4....	45
Figure 2 Vector map of pLD-5'UTR-Histag-CTB-NSP4 (pLD-AK) with sequence confirmed.....	47
Figure 3 First Round of Selection.....	49
Figure 4 Second Round of Selection on RMOP media with spectinomycin.....	49
Figure 5 Propagation of Petit Havana Transgenic Lines.....	50
Figure 6 PCR analysis of Wild type and putative transformants of pLD-5'UTR-His-CTB-NSP4.....	53
Figure 7 Southern Blot analysis of CTB-NSP4 T0 plants.....	57
Figure 8 Immunoblot analysis of crude plant extracts expressing CTB-NSP4.....	58
Figure 9. Quantification of CTB-NSP4 fusion protein expression levels in transgenic plants (T0 generation).....	60
Figure 10. Chloroplast genome.....	61

LIST OF ACRONYMS/ABBREVIATIONS

NSP4 -Rotavirus non structural protein four enterotoxin.

CTB-NSP4 – Cholera Toxin B-Rotavirus NSP4 Enterotoxin Fusion protein.

INTRODUCTION

Rotavirus: Major cause of infantile gastroenteritis

Rotaviruses, members of the *Reoviridae* family, exhibit a marked tropism for the differentiated enterocytes of the intestinal epithelium, are the leading cause of acute viral gastroenteritis and severe diarrhea among infants and young children of less than 5 years of age worldwide. Almost all children are infected at least once with rotavirus by the age of 3, and accounts for half the cases requiring hospitalization. Infantile diarrhea is the major killer of children around the world, responsible for 4 to 6 million deaths per year according to World Health Organization (WHO). Globally it is estimated that 1.4 billion episodes of diarrhea occur in children less than 5 years of age annually. Each year, rotavirus infections causes approximately 111 million episodes of gastroenteritis requiring only home care, 25 million clinic visits, 2 million hospitalizations, and 352,000-592,000 deaths (median, 440,000 deaths) in children less the 5 years of age (Parashar *et al* 2003).

NSP4 an important factor in the pathogenesis of Rotavirus infection

Rotaviruses are large, complex non enveloped icosohedral spherical particles of 70nm round double shelled consisting of three outer capsid layers. The inner most core layer of capsid encloses the unique viral genome of double stranded RNA in the form 11

segments. Each of these segments of the viral RNA genome code for either structural viral proteins (VP) or nonstructural proteins (NSP) (Parashar *et al* 1998). Rotavirus infection spreads among the people by fecal-oral transmission. The rotavirus particles after ingestion are carried to the small intestine where they infect the mature enterocytes in the mid and upper part of the villi of the small intestine, leading to diarrhea. Structural changes in the intestinal epithelium are observed within 24 hours of rotavirus infection, the shape of the villus epithelium changes from columnar to cuboidal, and the villi become stunted and shortened. Among the various structural and non structural proteins of Rotavirus that were identified and studied, NSP4 has been implicated as the important factor in rotavirus pathogenesis (function rotavirus). Moreover NSP4 has been designated as the viral enterotoxin as it was demonstrated that a peptide derived from its cytoplasmic domain is enough to cause Diarrhea in 3-Day old mice (Ball *et al* 1996). Various critical functions of NSP4 at the molecular level have also been identified; it plays a major role in viral morphogenesis by functioning as an intracellular receptor to aid in the budding of subviral particles into the endoplasmic reticulum(ER) (Tian *et al* 1996). It has been demonstrated that NSP4 possesses membrane destabilization activity on ER by mobilizing intracellular calcium and hence increasing its levels in intestinal cells. It also affects the membrane trafficking from the ER to the Golgi complex with its ability to bind to the micro tubules (Xu *et al* 2000). NSP4 induced intracellular calcium mobilization may be responsible for some of the cellular aspects of rotavirus pathogenesis as this increase in intracellular calcium ultimately stimulates endogenous fluid secretory pathway in the intestinal mucosa (Dong *et al* 1997). The gastrointestinal

symptoms of rotavirus infection may be directly attributed to NSP4. Studies on crystal structure of NSP4 revealed that the C-terminal domain corresponding to residues 114-135 directly attribute to diarrhea as a consequence of efflux of chloride ions from intestinal cells via a calcium dependent signaling pathway. An overlapping region of NSP4 peptide from amino acid residues 95-137 have shown to mediate oligomerization (tetrameric core formation) via a α helical coiled coil interactions which binds to the microtubules and disrupts the ER-Golgi trafficking. The coiled coil oligomerization domains have found to be resistant to protease degradation. Hence these two domains together have been shown to be involved with the budding of immature capsid particles into the lumen of ER (Bowman *et al* 2000, Mirazimi *et al* 2003). These above attributes of C-terminal portion led to the use of the truncated form of NSP4 with 90 amino acids as a good candidate for rotavirus vaccine antigen instead of the full length NSP4.

CTB an efficient adjuvant and carrier of mucosal vaccine antigens

Cholera toxin B subunit (CTB) of *Vibrio cholera* is well studied candidate for oral subunit vaccine for cholera, a disease that causes acute watery diarrhea by colonizing the small intestine and producing the enterotoxin, cholera toxin (CT). CTB is a homopentamer of 5 identical subunits of 11.6 K da that form a ring like structure and have the ability to bind selectively to sugar-lipid GM1 ganglioside receptors present on the intestinal epithelial cell surfaces. This binding elicits a mucosal immune response to pathogens and enhances the immune response when coupled chemically to other antigens

(Daniell et al 2001). Hence CTB has been shown to function efficiently as a carrier molecule for foreign proteins and especially for mucosal vaccines. Direct linking of small antigens with CTB results in specific targeting of the antigens to the mucosal immune system through specific enterocytes attachment and also increases the local antigen concentration at the mucosal surface. Hence the immune response to CTB-NSP4 fusion protein is expected to be lot stronger (Yu *et al* 2001). And since rotavirus also specifically infects only mature enterocytes lining the duodenum and small intestine causing gastroenteritis in infants. Expression of the CTB-NSP4₉₀ enterotoxin fusion protein in tobacco chloroplast is considered to be a better vaccine candidate for rotavirus.

Humoral and Cell-Mediated Immune response to NSP4 enterotoxin of rotavirus

Rotavirus NSP4 a multifunctional enterotoxin has shown to elicit both B and T cell immune response. But the serotype specific humoral immune response is mainly elicited during the primary rotavirus infection. In mice it was observed that, 7 days after post infection the appearance of anti rotavirus IgA could be correlated to the primary rotaviral infection. However, successful immune response to the pathogen in mice lacking IgA was thought to be mediated by IgG. CD4 helper cells (T_H) also seem to play a vital role in the clearance of rotavirus infection by inducing the proper B cell response to rotavirus (McNeal *et al* 1997). Studies on CD8⁺ T cells mediating rotavirus immunity revealed that the mechanism may involve the cytokine Tumor necrosis factor (TNF). More investigation has to be done to establish the exact mechanism. It was also observed

that rotavirus specific CD8⁺ T memory cells (being low in number) cannot prevent reinfection but can limit the extent of second infection by accelerating resolution. Thus it can be concluded that the correlates of immunity to rotavirus infection involves the presence of both an antiviral IgA by rotavirus specific TH cell response as well as a rotavirus specific CTL response (Farnco *et al* 1995, 1999). Recent studies on natural rotavirus infection have shown similar NSP4 specific IgG and IgA antibody response in children with strains of different NSP4 serotypes. Hence NSP4 is considered to be a potential candidate for vaccine development for rotavirus as the magnitude of antibody response is not affected significantly by the genotype of the infection strain as it appears to be heterotypic in nature (Ray *et al* 2003).

Plants As The Expression System For Vaccines And Biopharmaceuticals

Various recombinant proteins such as biopharmaceuticals, antibodies and vaccines constitute the vast majority of biological macromolecules that are currently under high demand as human therapeutic proteins and also for protection against deadly pathogens. Many of these recombinant proteins have been produced using the various traditional transgenic expression systems available, such as cultured mammalian cells and bacterial and fungal expression systems. Though the mammalian system is ideal for correctly synthesizing the human proteins, the low yield of products and high maintenance and purification costs associated with mammalian, bacterial and fungal systems present potential challenges. Therefore scientists must look for more efficient

systems for large scale production of these proteins. Among the various protein expression systems available, genetically engineered plants are considered to be most economical. As lot of investment is needed to establish and maintain the industrial facilities using fermentation or bioreactors when compared to the technology that is already available for harvesting and processing plants and plant products on a large scale (Daniell *et al.*, 2001b). Plant-derived products are less likely to be contaminated with human pathogenic microorganisms than those derived from animal cells because plants don't act as hosts for human infectious agents (Giddings *et al.*, 2000). Recombinant proteins expressed in plant cells are naturally protected from degradation when taken orally (Kong *et al.*, 2001).

Chloroplast Genetic Engineering As A Better Plant Based Expression System

Most of the plant derived recombinant proteins have been produced by nuclear transformation and regeneration of the transgenic lines, for further extraction and purification of these proteins (Rainer 2004). Though the plant nuclear expression system provides a eukaryotic environment necessary for the proper folding and post translational modifications of human recombinant proteins there is a concern with the levels of expression. The levels of recombinant proteins expressed in transgenic plants by nuclear system have been observed to be less than 1% of total soluble protein which is considered to be commercially unfeasible for protein purification (Daniell *et al* 2001b). In addition, with nuclear expression, the foreign protein levels vary in transgenic lines because the

foreign gene can be inserted randomly into different locations (Bogorad, 2000). Other factors that are associated with lower expression levels are the gene silencing and position effects so often observed in nuclear transgenic plants (Daniell and Dhingra, 2002).

One of the most attractive alternative means for higher expression of foreign proteins in plants is through the chloroplast transformation. Plastid transformation has been proven to be highly successful in tobacco. The popularity of tobacco is due to the availability of well defined regulatory elements for the transgene expression. Tobacco being a non food, non feed crop carries a reduced risk of transgenic maternal or recombinant proteins contaminating feed and human food chains. One of the other advantages of tobacco crop is its ability to yield high biomass with low maintenance and cost (Rainer *et al* 2004). For these attractive reasons tobacco has been a successful vehicle for the large scale production of human recombinant proteins and vaccines too.

Unique features of Chloroplast genome and the copy correction mechanism

Expression of the transgenes in chloroplast genome offers unique advantages that render this technology safe and acceptable to the public. Chloroplast genome is usually a circular molecule that is self-replicating and varies in size from 120 – 220 kb within different species of plants. A typical plant leaf cell contains about 100 chloroplasts and each chloroplast further harbors approximately 100 copies of the same genome. This implies that a single gene is represented by at least 10,000 copies in a single plant cell.

The chloroplast genome of most plant species possess two inverted repeat (IR) regions and usually any transgene introduced into one of these regions gets integrated into the other by a mechanism known as copy correction which doubles the copy number of the genes encoded by this region. This makes it quite appealing to introduce a transgene into the chloroplast genome and obtain high levels of expression, taking advantage of the high copy number (Daniell *et al* 2004c). It is possible to insert a foreign gene into the site-specific transcriptionally active spacer region between the functional genes of chloroplast genome using two flanking regions unlike random nuclear integration because chloroplast transgenes are integrated via homologous recombination (Daniell 2001). Chloroplast transformation vectors are thus designed with homologous flanking sequences on either side of the transgene and introduced into the chloroplast genome of plant cells via particle bombardment (Sanford *et al* 1993) or into the protoplasts by the process of PEG treatment (Golds *et al* 1993). After bombardment transformation is accomplished by integration of the transgene into a few genome copies initially followed by about 15-20 cell divisions under selection pressure thereby yielding homogenous population of plastid genomes. (Daniell *et al* 2004a, Daniell *et al* 2004c). Chloroplasts also possess the ability to accumulate any foreign proteins in large amounts that could otherwise be harmful if they were in the cytoplasm. For example CTB an oral subunit vaccine for cholera was not toxic when expressed in transgenic plastids in very high quantities which was otherwise toxin when expressed in leaves by nuclear transformation. Trehalose, a pharmaceutical industry preservative was toxic when

accumulated in cytosol where as was non toxic when compartmentalized in plastids by chloroplast expression system (Daniell *et al* 2005a).

Chloroplast and post-translational mechanism

Another important aspect for chloroplast is it can process eukaryotic proteins by ensuring the correct folding and disulphide bond formations. Chaperonins present within chloroplast are thought to aid in the folding and assembly of non native prokaryotic and eukaryotic proteins (Daniell *et al* 2001, Daniell *et al* 2005). The light signal sensed by chlorophyll is transferred via the photosynthetic electron flow to proteins called thioredoxins, which are very efficient in thio-disulfide interchanges with various protein disulfides (Ruelland and Miginiac-Maslow, 1999). Another mechanism for the simple, reversible activation of genes that regulate expression in the chloroplast is the Protein Disulfide Isomerase (PDI) system composed of chloroplast polyadenylate-binding proteins that specifically bind to the 5'UTR of the *psbA* mRNA and are modulated by redox status through PDI (Kim and Mayfield, 1997). The ability of chloroplasts to form disulfide bonds and properly fold foreign proteins eliminates a major part of the costly downstream processing.

Chloroplast transformation and Multigene engineering

Multigene engineering in a single transformation event was successfully demonstrated using chloroplast transformation as it can process polycistronic RNAs

arranged in an operon that is typically absent in a nuclear transformation (Daniell et al 2002). The transcript analyses performed on different transgenic lines with multigene operons like cry2Aa2, hsa, tsp1 and ctb showed that polycistrons as the most abundant transcript forms. Further, the polysome fractionation assays performed on the cry2Aa2 transcripts suggested that polycistrons are preferentially translated without processing. It was shown that ribosome associated polycistrons were in much higher abundance than the monocistronic transcripts proving that the heterologous operons are preferentially translated as a polycistronic unit. Hence it was shown that transgenes engineered as operons in chloroplast can be translated very efficiently and do not require processing into monocistrons in order to be translated. This approach has been highly successful for expressing proteins in an operon system downstream of *aadA* after inserting the cassette into *trnA*(tRNA Ile) –*trnI* (tRNA Ala) intergenic region of the chloroplast vector (Vargas Q T *et al* 2005). The *cry* operon from *Bacillus thurengiensis* (Bt), coding for the insecticidal protein delta-endotoxin, introduced into the chloroplast genome showed an expression of about 46% of the total soluble protein (DeCosa *et al.* 2001). Also two bacterial enzymes that confer resistance to two different forms of mercury-mercuric ion reductase (*merA*) and organomercurial lyase (*merB*)- expressed as an operon in transgenic chloroplast conferred resistance to very high levels of mercury and highly toxic organomercurial compounds. This study for the very first time proved that the chloroplast genome engineering was highly successful in enhancing the capacity of plants for phytoremediation, where a native bacterial operon was used for expression in plants without codon optimization (Ruiz *et al* 2003).

Light regulated expression of transgene in chloroplast using psbA 5'UTR

Chloroplast transgenic approach to hyper-express the foreign protein is achieved by using strong chloroplast promoters and untranslated sequences (UTR) that mediate the transcript stability and translation efficiency. The role of regulatory sequences like psbA 5' UTR was analyzed in chloroplast by fusing with GUS reporter gene (Staub *et al* 1994). Studies have revealed that the 5'UTRs of chloroplast harbor the cis-acting determinants to stabilize the plastid transcripts and also provide the platform for translation initiation. The very critical step in translation is initiation which is facilitated by 5'UTR, involved with the interactions between mRNA ribosome binding site and 16s rRNA 3' end. 5'UTR also has specific sites for binding to translational activation proteins that facilitates loading of mRNA on to ribosomes (Maliga 2003). Apart from the 5'UTR the 3'UTR has also been shown to have potential role in post-transcriptional stabilization by often forming a stem loop structure which binds to different RNA binding proteins (Daniell *et al* 2002, Nickelsen J 2003, Staub *et al* 1994). The psbA 5'UTR consists of essential light regulated translation elements that are target sites for enhancing the translation. The light driven photosynthetic electron transport chain generates the electrochemical gradient across the thylakoid membranes, the redox states of specific electron carriers, and stromal ADP/ATP ratio which are sensed by the translation regulators. These regulators aid in the binding of specific proteins to the ribosome binding sites of 5'UTR psbA which enhances the translation (Zerges 2004). Expression of human serum albumin HSA under the control of psbA 5'UTR was light dependent and the quantity was observed to be maximum up to 50 h of continuous light illumination (11.1% of tsp) in mature leaves and

a 2-4 fold decrease in expression was observed after the 8 h dark period. The levels of expression of HSA in mature plants under the translational control of SD sequence showed very low amount of accumulation when compared to expression under the control of psbA promoter and 5'UTR which resulted in a 500-fold increase in HSA accumulation as inclusion bodies in chloroplast which probably offered protection from proteolytic degradation (Fernandez-San Millan A *et al* 2003). The protein levels of Anthrax Protective antigen (PA) expressed in chloroplast under psbA 5'UTR was 2.7% under normal illumination pattern (16hrs light and 8hrs dark) where as reached a maximum of 18.10% and 13.40 % of TSP in mature leaves when exposed to continuous light for 5 and 3 days respectively (Watson *et al* 2004). Other chloroplast derived vaccine antigens and therapeutic proteins that showed increased expression under psbA 5'UTR include plague vaccine antigen (CaF1~LcrV) with 14.8% of TSP, canine parvovirus VP2 antigen expressed as fusion protein with CTB and green fluorescent protein GFP (CTB-2L21/ GFP-2L21) showed levels of expression of about 31.1% and 22.6% of TSP respectively. The expression levels of Insulin like growth factor (IGF-1) with high therapeutic value was about 33% of TSP and that of Interferon alpha 2b was about 19% of TSP (Daniell *et al* 2005, Daniell *et al* 2004a). This increase in expression in light is due to the presence of light dependent regulatory sequences in psbA 5'UTR.

Maternal inheritance and gene containment

The risk of transplastomic gene spread to wild relative crops has been of great concern with GM crops which limits their widespread acceptance. This could potentially

lead to the emergence of “superweeds” with resistance to herbicide or introducing undesired traits into related crops. These major concerns are overcome by the use of chloroplast genetic engineering as in most of the crops chloroplast genome are maternally inherited eliminating any potential outcross among GM crops or between GM crops and weeds. The maternal inheritance of chloroplast genome in plants is achieved during the male pollen development of generative cells which form the sperm cells. During the process of microspore mitosis all the plastids are distributed to vegetative cell and the generative cells are devoid of plastids or in some species generative cells get a few plastids which degenerate during maturation. The third type of plastid exclusion found in several cereal crops occurs during the fertilization process where the sperm nucleus alone is transmitted into the egg cell as they are stripped off the sperm nucleus just before or during the process of fertilization. This results in exclusion of chloroplast DNA during sexual fusion (Hagemann 2004). Chloroplast transgenic plants have successfully demonstrated the maternal inheritance of transgenes by preventing the gene flow through pollen making them an excellent choice as biofactories to produce functional biopharmaceuticals, vaccine antigens and biopolymers (Daniell 2002).

Engineering marker free transgenic plants is of high importance to avoid the potential risk of antibiotic resistance gene transfer to the environment or the gut microbes. Gene transfer between plants and bacteria residing in the gastrointestinal tract or soil is probably due to the presence of compatible protein synthetic machinery for chloroplast and bacteria and also the presence of thousands of copies of the antibiotic resistance genes per cell. Most of the antibiotic genes used for selection originate from

bacteria, hence to develop marker free transgenic plants the betaine aldehyde dehydrogenase enzyme (BADH) gene from spinach present only in chloroplast was used as selection marker. The process of selection is based on the ability of BADH to convert the toxic betaine aldehyde (BA) to a non toxic glycine betaine, which is also an osmoprotectant. The efficiency of chloroplast transformation was 25 fold higher in BA selection than with spectinomycin. Another advantage with BA selection was rapid regeneration; transgenic shoots appeared within 12 days of bombardment unlike the spectinomycin selection which took about 45 days. This successful chloroplast transformation with naturally occurring BADH gene from spinach for could ease the public concern regarding the use of antibiotic resistance genes for selection in GM crops (Daniell *et al* 2001a). Other procedures to develop marker free transgenic plants are also available, which include methods like using direct repeats of short DNA of 174 bp which allows removal of selectable marker by homologous recombination between the direct repeats (Iamthan and Day 2000) or by introducing lox sites on either side of the selectable marker gene which can be activated by introducing plastid targeted CRE site specific recombinase and gene of interest can be retained that is outside the lox sites thus generating marker free plants (Hajdukiewicz *et al* 2001, Cornielle *et al* 2001). Most recently cointegrated selection was used for complete removal of marker gene in the first generation, the mechanism is not well understood but most likely involves cointegrate loop out recombination event which leads to loss of selectable marker by placing the marker outside the flanks for homologous recombination (Klaus *et al* 2004).

Chloroplast engineering of agronomically important crops

Plastid genetic engineering is being currently applied to other useful crops such as potato, tomato, carrot, cotton and soybean by transforming different plastid genomes (Sidorov *et al.* 1999, Ruf *et al.* 2001, Kumar *et al.* 2004a, Kumar *et al.* 2004b, Dufourmantel *et al.* 2004). The rationale in trying plastid transformation in other food crops is to synthesize immunogenic proteins of major pathogens in plant tissues in order to use them as edible subunit vaccines to humans or commercially important animals. The practical aspects to deliver defined doses of vaccine in the development of edible vaccine are being explored (Daniell *et al.* 2001a). The limitations in extending the plastid transformation technology to major crops that regenerate via somatic embryogenesis include inadequate tissue culture protocols, lack of selectable markers and also the inability of expressing the transgenes in non-green tissues (Daniell *et al.* 2005). Successful engineering of carrot plastid genome was achieved by transforming the non-green tissue as explants and regenerating via somatic embryogenesis. Enhanced tolerance to salt stress in carrot was achieved by overexpression of betaine by manipulation of *badh* gene via carrot chloroplast genome. The toxic betaine aldehyde (BA) is converted to non-toxic glycine betaine by the chloroplast BADH enzyme. This glycine betaine also serves as an osmoprotectant and confers salt tolerance. The transgenic and non transgenic carrot can be distinguished easily just on the basis of color. Transgenic calli obtained from carrot cells expressing BADH were green in color even in the absence of selection and

the untransformed cells were yellow. A uniform source for carrot cell culture as somatic embryos can be derived from a single cell that multiply through recurrent embryogenesis, a homogeneous single source of origin highly essential for producing therapeutic proteins. Carrot is an ideal source for oral delivery of vaccine antigens since it has several different advantages like when the proteins are delivered through carrot there is no need of cooking hence the structural integrity of the protein is preserved. Carrot is a biennial crop and hence there is no flowering in the first year when the crop is harvested. Therefore, the maternal inheritance along with absence of flowering should help in prevention of cross contamination with other crops. This makes carrot as a excellent choice for genetic engineering as it is doubly protected against any transgene flow via pollen or seed germination (Kumar *et al.* 2004a).

Recently stable transformation of the cotton plastid genome and maternal inheritance has been demonstrated by Kumar *et al.* All the transgenic seeds obtained from self pollinated transgenic lines were resistant to kanamycin selection whereas the untransformed seeds were not, and no Mendelian segregation was observed among the transgenic seeds (Kumar *et al.* 2004b). Similarly, the first successful development of transgenic soybean plants was achieved by Dufourmantel *et al.* (Dufourmantel *et al.* 2004). Therefore, the successful plastid transformation of the above said crop plants was suggested to be due to the 100% homologous plastid DNA sequences used in the species-specific vectors. Even though the concept of universal vector was proposed several years ago, the use of species-specific vectors has demonstrated successful plastid transformation (Daniell *et al.* 2005a).

Vaccine antigens Expressed Via the Chloroplast Genome

Proteins of microbial and viral pathogens that are considered to be candidates for vaccine antigens have been feasibility expressed in chloroplast and this expression system has proven to be advantageous: subunit vaccines are not toxic even when expressed at high levels as they get accumulated in the chloroplast, Cholera toxin B subunit (CTB), candidate for oral subunit vaccine for cholera was non-toxic when accumulated in large quantities in chloroplast yet toxic when expressed in leaves via nuclear genome, even at very low levels (Daniell *et al* 2005a). The bacterial genes are comparatively rich in AT content allowing for high expression in the chloroplast as it provides a prokaryotic environment; and oral delivery of vaccines yields high mucosal IgA titers along with high systemic IgG titers, enabling the immune system to fight against germs at their portals of entry. Vaccines that have already been expressed in the chloroplast include the Cholera toxin B-subunit (CTB), which does not contain the toxic component that is in CTA (Daniell *et al.* 2001), the F1~V fusion antigen for plague (Singleton 2003), the 2L21 peptide from the Canine Parvovirus (CPV) (Molina *et al.* 2004), Anthrax Protective antigen (PA) (Watson *et al.* 2004), LecA protein as vaccine antigen for *Entamoeba histolytica* (Chebolu 2005), NS3 protein as vaccine antigen for hepatitis C (Bhati 2005), C terminus of *Clostridium tetani* (TetC) (Tregoning *et al.* 2003, Maliga 2003). CTB was expressed at 4 to 31% of Total Soluble Protein (TSP) and was effective in the GM1-ganglioside binding assay which indicates proper folding and formation of disulfide bonds to form pentamers (Daniell *et al.* 2001, Molina *et al.* 2004). The 2L21 peptide from the Canine Parvovirus (CPV) fused to GFP expressed 22% TSP

and CPV fused to CTB 31% TSP (Molina *et al.* 2004). When mice were immunized intraperitoneally with the leaf extracts from CTB-2L21, the developed anti-2L21 antibodies were able to recognize VP2 protein from CPV. Anthrax PA83 was expressed at 14.7% TSP in transgenic tobacco chloroplasts and elicited immunogenic response in the mice proving that plant derived PA is biologically similar to PA derived from *Bacillus anthracis* (Koya 2004). The C terminus of *Clostridium tetani* (TetC) was expressed at 25% TSP for AT rich and 10% TSP for GC rich sequences which shows that chloroplasts favor prokaryotic-AT rich sequences. TetC when administered intranasally produced both IgG and IgA and was immunoprotective against the toxin (Tregoning *et al.* 2003). To date, only two vaccine candidates derived from chloroplast genetic engineering have been tested for immunogenicity in mice and only one vaccine candidate has been tested for immunoprotective property. High expression is not only economically important, but for oral vaccines it is essential for the immune response.

Bioencapsulation as protection to vaccine antigens for the oral delivery

Mucosal vaccines are most sought after as a potential source for stronger immune responses usually directed to the site of interaction between the pathogen and the host. Bioencapsulation of pharmaceutical proteins within plant cells offers protection against digestion in the stomach yet allows for successful delivery (Walmsley and Arntzen 2000, Yu and Langridge 2001). In human clinical trials performed with plant derived vaccines, plant cells have proven sufficient for vaccinogen protection against digestion, and the vaccinogen has induced systemic and mucosal immune responses without the aid of

adjuvants (Tacket *et al.* 1998, Kapusta *et al.* 1999, Tacket *et al.* 2000, Walmsley and Arntzen 2000, Tacket *et al.* 2003, Tacket *et al.* 2004). Heat-labile enterotoxin B-subunit (LTB) from *E. coli* was expressed by nuclear transformation in tobacco (<0.01% Total soluble protein) and potato (0.19% TSP). The LTB expressed in potato was found to be immunoprotective when administered orally. Even with such low expression levels in tobacco these antigens were immunogenic (Haq *et al.* 1995, Mason *et al.* 1996, Tacket *et al.* 1998). The capsid protein of the Norwalk virus expressed in potato and tomato was immunogenic when administered orally (Mason *et al.* 1996 and Richter *et al.* 2000, Tacket *et al.* 2000). The envelope surface protein of hepatitis B virus was expressed by nuclear transformation in tobacco, potato and lupin. They all had less than 0.01% fresh weight expression but were still immunogenic (not protective) when administered orally (Richer *et al.* 2000, Kapusta *et al.* 1999). This again brings us to the need to ensure high expression of therapeutic proteins in plants. IFN-alpha given orally has biological activity in humans and other animals (Bocci 1999). Plant derived edible vaccines have also been proven in commercial animal and native animal trials (Castanon *et al.* 2000, Tuboly *et al.* 2000). Bioencapsulation is therefore protecting these antigens or therapeutic proteins from digestion so that they remain biologically active. Chloroplast genetic engineering is currently being applied to crops amiable to oral vaccines such as potato, tomato, carrot and soybean (Sidorov *et al.* 1999, Ruf *et al.* 2001, Kumar *et al.* 2004a, Dufourmantel *et al.* 2004).

Rationale and Approach

Presently there is no available vaccine for rotavirus included in national immunization systems, the only live tetravalent rhesus-human reassortant vaccine (RRV-TV; Rotashield) for rotavirus was licensed in 1998 in USA but withdrawn from market in 1999 for possible association with intussusception. Hence the main objective of this project is to express the surface antigen CTB-NSP4 fusion gene in plants using the chloroplast expression system to develop a low cost vaccine for rotavirus. As Chloroplast expression system has proven to have several different advantages over nuclear transformation which includes high expression levels under the control of light regulated psbA 5'UTR, proper folding of proteins, maternal inheritance of transgenes and lack of gene silencing and position effect. Also, plant systems provide opportunity for low cost production, the ability to carry out post-translational modifications and minimize the risk of contamination from potential human pathogens of the vaccine antigens. Oral vaccines have to be protected from the hostile environment of stomach and intestine and plant expression system provides the additional protection necessary for mucosal delivery due to bioencapsulation within plant cell walls and membrane compartments (Walmsley and Arntzen 2000). The delivery of plant derived vaccine antigens to mucosal tissues have shown to increase both mucosal and system immune response (Haq *et al.*1995., Mason *et al.*1996, Arakawa *et al.*, 1998). Also mucosal immunization with CTB-NSP4 will give higher protection to rotavirus infection at the site of interaction with host enterocytes of the intestinal epithelium, by producing higher s-IgA in the mucosal system. CTB proven to be an excellent adjuvant and an effective carrier

molecule for foreign proteins with its binding affinity to GM₁ receptors on intestinal epithelial cells makes it a good candidate for targeting mucosal vaccine antigens. For this reason organ specific targeting protein CTB was linked to enterocytes specific rotavirus enterotoxin epitope (NSP4₉₀). Tobacco has been chosen for plastid transformation for various advantages associated with it like low cost of recombinant protein production due to large biomass (40 metric tones of leaves in multiple harvests per year) and is considered as an ideal bioreactor. And also would not contaminate food sources as it is not a food or feed crop (Daniell *et al* 2004a). Thus tobacco chloroplast system was chosen to express CTB-NSP4 fusion protein to attain high levels of expression when compared to the earlier reports of only 0.006% to 0.26% of TSP in nuclear expression system. Therefore, expressed the vaccine antigen in chloroplast expression system. For this, the His-CTB-NSP4 gene was initially cloned downstream of psbA 5'UTR in pBluescript to enhance the translation. The whole gene cassette of 5'UTR-Histag-CTB-NSP4 was then cloned into the universal chloroplast transformation vector, pLD-Ctv which has flanking regions from the tobacco chloroplast genome so as to enable homologous recombination. The chloroplast derived CTB-NSP4 will be evaluated for its immunogenic response in an animal model. Once an immunogenic response is achieved, the animals will be pathogen challenged to test for the survival rates. This will encourage plastid transformation of carrots for oral delivery of the vaccine antigen.

MATERIALS AND METHODS

General Protocols

Preparation of Ultra Competent cells (Rubidium Chloride Method)

E.coli XL1-Blue MRF^{ab} Tc(Stratagene), a disabled non pathogenic, tetracycline resistant strain, has a history of safe laboratory use due to its inability to survive in the antibiotic environment and has been used to prepare the ultra competent cells. The rubidium chloride method (<http://www.nwfsc.noaa.gov/protocols-/rbcl.html>) was used to convert these *E.coli* XL1-Blue MRF^{ab} Tc cells to make them ultra competent for efficient transformation. The ultra competent cells are absolutely necessary for the transformation of bacterial cells with the plasmid vectors. The *E.coli* glycerol stock was streaked on to an LB agar plate containing 12.5 µg/ml tetracycline and incubated overnight at 37 °C. Single isolated colony was picked and grown in 5 ml of Psi broth (per liter- 5g Bacto yeast extract, 20g Bacto Tryptone, 5 g magnesium sulfate, pH 7.6) containing 12.5 µg/ml tetracycline and incubated at 37 °C for 12- 16 hrs in a horizontal shaker at 225 rpm. Approx. 1 ml of the overnight culture was inoculated into a fresh batch of 100 ml Psi broth and was incubated again at 37 °C for about 2 hours in a shaker at 225 rpm. The optical density (O.D) had to be checked at 550 nm first after two hours and subsequently for every half hour to an hour depending on the O.D value. The culture was allowed to grow until it reaches to a 0.48 O.D. The culture was then placed on ice for

15 minutes. The culture was then centrifuged at 3000g/5000rpm for 5 minutes in a sorvall centrifuge at 4°C. The supernatant was discarded and the pellet was resuspended in 0.4 volume (40 ml) of ice-cold TFB-I solution. The cells were re-pelleted by centrifuging at 3000g / 5000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in (0.04 volume) 4 ml of TFB-II solution and immediately iced for 15 minutes. This suspension was divided into 100 µl aliquots, then quick freezed in dry ice/liquid nitrogen and stored at - 80 °C.

Transformation of the Competent E. coli XL1-Blue cells

The competent cells of 100 µl aliquot, stored at -80 °C in the freezer were taken out and were immediately thawed on ice and transferred to a falcon tube. About one µl (100 ng) of plasmid DNA that has to be transformed was added to the competent cells in the falcon tube and mixed by gentle tapping. The mixture was incubated on ice for 30 minutes with gentle tapping every 10 minutes for the duration of incubation. Then, the mixture was incubated at 42° C in a water bath for about 90-120 seconds and then immediately put on ice for two minutes. Approx. 900µl of LB broth was added to cells and allowed to incubate at 37° C for 45 minutes in a horizontal shaker at 225 rpm. The cells were then pelleted by spinning at 13,000 rpm for 30 seconds. The eight hundred µl of supernatant was discarded leaving 100µl, followed by resuspension of the cells. Two samples, 50µl and 100µl, of the suspension were inoculated on the agar plate with appropriate selection agent and spread with a glass rod (<http://www.nwfsc.noaa.gov/protocols-/rbcl.html>).

Isolation of Plasmid DNA by Alkaline lysis method

A single isolated colony was picked from the desired LB agar plate and inoculated in about 3-5 ml of LB broth containing the appropriate antibiotics; the culture was then grown for 12-16 hours at 37 °C in a shaker at 225 rpm. 1.5 ml of the cell culture was put into an eppendorf tube and centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the pellet was resuspended in 150 µl of Solution I (50 mM Glucose, 10 mM EDTA, 25 mM Tris, pH- 8) and the mixture was vortexed to resuspend the cells uniformly in Solution I. One µl of RNase (100 mg/ml) is then added to each tube and pulse vortexed so that any RNA present in the suspension gets degraded as our aim is to isolate plasmid DNA. One hundred fifty µl of Solution II (0.2N NaOH, 10% SDS) was added to each tube and mixed by gently inverting the tube 6-8 times. Further, one hundred fifty µl of Solution III (60 ml of 5M Potassium Acetate, 11.5 M glacial acetic acid, 28.5 ml sterile dH₂O) was added immediately after adding Solution I and mixed by gently inverting the tube about 6-8 times. A cloudy white precipitate is observed, the mixture is then centrifuged at 13,000 rpm for 10 minutes at 4 °C. The supernatant was transferred into a fresh, eppendorf tube taking care to exclude the white debris (which is bacterial chromosomal DNA/SDS/membrane proteins) that settles at the bottom of the tube after centrifugation. Then, 900 µl of ice-cold ethanol (95%) was added to the supernatant to precipitate the plasmid DNA. The supernatant containing the ice cold ethanol was centrifuged at 13,000 rpm at 4 °C for 10 minutes in order to obtain the plasmid DNA as a pellet. The supernatant was removed and discarded, taking care not to dislodge the plasmid DNA pellet at the bottom. To remove the salts from DNA, 400 µl of

70 % chilled ethanol was added and (without mixing) centrifuged for 5 minutes. The ethanol was discarded and the pellet was subsequently dried in the speedvac on medium heat for 3-5 minutes. Dried DNA pellet was resuspended in TE (pH 8.0). Concentration and quality plasmid of DNA was measured by spectrophotometer. DNA was stored at -20 °C. The DNA samples were run on a 0.8% agarose gel for 40 minutes at 80 volts to confirm the successful isolation of plasmid (Sambrook *et al.* 1989).

Construction of pLD-5'UTR-HisCTBNSP4 vector for transformation of tobacco

Chloroplast

Initially the gene cassette Histag-CTB-NSP4 was cloned downstream to 5'UTR b in p-bluescript between EcoRV and EcoRI sites. Then the final gene cassette containing the 5'UTR and His-CTB-NSP4 (approximate size 0.7 kb) were digested with EcoRV and XbaI and cloned into tobacco universal vector pLD-Ctv between EcoRV and XbaI.

Bombardment of the pLD-5'UTR-Histag-CTBNSP4 vector

Preparation of gold particles

50mg of gold particles (0.6 µm in size) and 1ml of 70% EtOH were placed in a micro centrifuge tube. The mixture was vortexed for 3-5 minutes and then incubated for 15 minutes at room temperature. A quick centrifugation was done to pellet the gold particles. The supernatant was then discarded and 1ml of dH₂O was added to the particles and vortexed. The particles were allowed to settle for 1 minute and then pulse centrifuged

for 3 seconds, the supernatant was discarded. The previous step was repeated three times. At the end of the last wash the gold particles are stored in 50% glycerol stock at -20°C (Kumar and Daniell 2004).

Preparation of tobacco tissue culture media

The RMOP media containing MS basal salt mixture (one pack), 30 grams of Sucrose, 100mg of myo-inositol, 1ml of benzylaminopurine (BAP: 1mg/ml), 100 μl of α -naphthalene acetic acid (NAA: 1mg/ml), 1ml of thiamine hydrochloride (1mg/ml), and Water (1 liter) was used to regenerate and select the transgenic plants after bombardment. The pH was adjusted to 5.8 using 1N KOH. About six grams of phytagar per liter was added to the media and autoclaved. The autoclaved media is allowed to cool down till the temperature reaches to about 40°C and antibiotic is added if the media has to be used for selection. The medium is then poured into the deep Petri dish (100" x 25"). The MSO media containing 30g sucrose, 1 packet of MS basal salt mixture, and water to 1-liter was used to generate roots. The pH was calibrated to 5.8 with 1N KOH and 6g per liter of phytagar were added before autoclaving (Kumar and Daniell, 2004).

Preparation of Spectinomycin Solution

The spectinomycin solution was made by dissolving 1 g of spectinomycin into 10 mL of distilled water for a final concentration of 100 mg/mL. Then, the solution was filter sterilized under the hood and stored at -20°C until ready to use.

Bombardment protocol for tobacco leaves

The bombardment media was prepared as described previously (Daniell 1997). The most important aspect for bombardment is to maintain a highly aseptic condition to avoid any kind of contamination during the process. For this all the essential equipments were sterilized. The stopping screens, macro carrier holders, forceps, Whatman filter paper, Kim wipes were autoclaved prior to bombardment. The macro carriers and the rupture discs were sterilized under hood by immersing them in 95% ethanol for 15 minutes followed by drying. Fifty μl of gold particles that were prepared previously are placed in a micro centrifuge tube and 10 μl of DNA (1 $\mu\text{g}/\mu\text{l}$) were added. Fifty μl of 2.5M CaCl_2 , 20 μl of 0.1M spermidine-free base were added sequentially to the mixture to ensure proper binding of DNA to the gold particles. Vortexing was done after addition of each component to ensure proper mixing of components and binding of DNA to the gold particles. The mixture was then vortexed for 20 minutes at 4 °C. Two hundred μl of absolute ethanol was added to the vortexed mixture at room temperature and followed by a quick spin at 3000 rpm in a microfuge for 30 seconds, supernatant was removed and this wash procedure was repeated four times. Finally, the gold particles were resuspended in thirty μl of 95% ethanol. The gold particles with DNA were placed on ice to be used in next two hours. Aseptic tobacco plant *Nicotiana tabacum* var. *Petit havana* green healthy leaves were cut from a young plant growing in jars containing MSO media and were placed on a petri dish (100 x 15) containing RMOP media with no selection and a Whatman filter paper on the top of media. The leaves were placed with the abaxial side upwards. The gene gun (Bio-Rad PDS-1000/He) was sterilized in the inside chamber

with 70% ETOH prior to bombardment. The macro carriers were placed on the macro carriers holders. The gold particles lying on ice were vortexed and five μl of gold particles containing the DNA were placed on top of the macro carrier. Vortexing is an important step while placing the gold particles on the macro carriers. The rupture disc, stopping screens and macrocarrier holders containing the macrocarrier, and the leaf were put in place and secured to proceed with the bombardments. The gene gun and the vacuum pump were turned on, and the helium tank was turned to the open positions and the valve is turned on till the pressure reaches 1350 psi. The vacuum in the gene gun was allowed to build to 28 psi, and was then held briefly and then fired (the fire switch was held until the rupture disk broke at ~ 1100 psi). After the bombardment, the vacuum was released, and the petri dish with the leaf was taken out and covered. After the samples were finished they were covered with aluminum foil (to keep them dark) and incubated for 48 hours at room temperature (Kumar and Daniell 2004).

Tissue regeneration and selection

After the two day incubation period in dark the leaves were transferred to 100 x 25 petri dish with RMOP media containing 500 $\mu\text{g/ml}$ of spectinomycin final concentration as explained by Daniell, 1997. After four to six weeks, the shoots that appeared were cut in 5mm² pieces and transferred to fresh RMOP plus spectinomycin for the second round of selection. Before transferring the shoots to secondary selection, total DNA was extracted and a PCR analysis was performed to confirm the integration into the

chloroplast on these putative transgenic lines. Finally, after 4 weeks on secondary selection, the shoots were transferred to a jar that contained MSO media with 500 µg/ml spectinomycin (Daniell 1997), this step is called the third selection (Kumar and Daniell 2004).

Plant genomic DNA extraction procedure

The Qiagen DNeasy Kit was used to isolate plant genomic DNA as described in the Qiagen manual. 100mg of tissue sample was taken from the plant using aseptic techniques, placed into a micro centrifuge tube, and grinded by using a micro pestle in 400 µl of buffer AP1 and 4 µl of RNase A (stock solution 100mg/ml). The mixture was incubated for 10 minutes at 65 °C and mixed about 2-3 times during incubation by inverting the tube. 130 µl of buffer AP2 were added to the lysate, vortexed and incubated for 5 minutes on ice. Following, a centrifugation was done at maximum speed for 5 minutes and the supernatant was transferred to a Qiashredder spin column (lilac) sitting in a 2ml collection tube. The centrifugation was performed at full speed 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 µl of the mixture was applied to a DNeasy mini spin column (clear) and centrifuged for 1 minute at 8000 rpm. The flow through was discarded and the collection tube reused to repeat the previous step with the rest of the sample. The tube with the flow through was discarded and the column was placed in a supplied 2 ml tube. 500 µl of buffer AW were added to the column and centrifuged for 1minute at 8,000 rpm. The flow through was discarded, and the tube was

reused. The DNeasy column was washed once again by using 500 µl of AW buffer and by centrifuging it for 2 minutes at maximum speed. The Column was transferred to a clean 1.5ml tube and 100 µl of preheated (65 °C) buffer AE were directly delivered into 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.

Confirmation of transgene integration into the chloroplast genome

To confirm the transgene cassette integration into the chloroplast genome, PCR was performed using the primer pairs 3P (5'-AAAACCCGTCCTCGTTCGGATTGC-3')-3M (5'-CCGCGTTGTTTCATCAAGCCTTACG-3') (Daniell *et. al.* 2001) and to confirm the integration of gene of interest PCR was performed using primer pairs 5P (5'-CTGTAGAAGTCACCATTGTTGTGC-3') and 2M (5'-GACTGCCCCACCTGAGAGC-GGACA-3') (Daniell *et al.* 2001). Positive control (known transgenic plant DNA sample) and Negative control (Wild type *Petit havana* DNA sample) were used to monitor the PCR reaction. For a 50 ul reaction volume, the PCR was set as follows: 150ng of plant DNA, 5 µl of 10X buffer, 4 µl of 2.5 mM dNTP, 1 µl of each primer from the stock, 0.5 µl Taq DNA polymerase and H₂O to make up the total volume. The amplification was carried during 30 cycles with a program timed in the following way: 94 °C for 30 sec, 65 °C for 30sec, and 72 °C for 30sec for the 3P-3M primer pair and 72 °C for 1min for the 5P-2M primer pair. Cycles were preceded by denaturation for 5 min at 94 °C and

followed by a final extension for 7 min at 72 °C. The 5 ul of each PCR products including the controls were loaded into a 0.8% agarose gel to confirm the results.

Southern blot analysis of transgenic plants

Restriction Digestion of plant genomic DNA

The total plant DNA was extracted from transgenic T₀ plants as well as from untransformed tobacco plants following the protocol previously explained using Qiagen DNeasy Plant Mini Kit. These steps were performed as described in (Daniell *et al.*, 2004c). The total plant DNA was digested with HincII in a reaction containing: 2ug of DNA, 2 µl of 10X buffer (New England Biolabs), 2 µL of HincII enzyme (New England Biolabs) and sterile dH₂O to make up the volume up to 20 ul. The reaction was incubated overnight at 37 °C. All the samples must contain equal quantity of DNA. The plant DNA samples digested with HincII were used for probing with Chloroplast flanking probe. The total plant DNA was also digested with ClaI in the similar manner and the digested samples were run on 0.7% agarose gel and probed with CTB-NSP4 gene specific probe.

Agarose electrophoresis and DNA transfer

The total 20 µl reaction volume was loaded on a 0.7% agarose gel for each of the transgenic plant DNA samples. The digested DNA of wild type plant (*Nicotiana tabacum*

var. *Petit Havana*) acts as negative control and (unlabeled probe) acts as the positive control. The positive control (unlabeled probe) was prepared by digesting the plasmid DNA (pLD-5'UTR-CTB-NSP4) with NdeI and EcoRI. The 700 bp fragment was diluted 20 times and then 1 µl of the diluted probe was loaded onto the gel. The gel was run for 2.5 hours at 50 volts. After the run was completed, the DNA was transferred by capillary action to a nylon membrane. The parts of the gel that were not needed were removed, and the upper right corner was cut to help as a guide. The gel was then depurinated by immersing it in 0.25M HCl (depurination solution) for 15 minutes (until the color of the dye became yellow). Following, the gel was washed twice in dH₂O for 5 minutes, and then equilibrated in transfer buffer (0.4N NaOH, 1M NaCl, filled to 1 liter with water) for 20 minutes. The four pieces of Whatman paper and the membrane were cut to fit the size of the gel then the upper right corner of the membrane was removed and washed briefly in water. Following, the membrane was equilibrated by immersing it in transfer buffer for 5 minutes. In a glass tray, a stack of two sponges was placed and enough transfer buffer was added to cover the sponge that is in touch with the tray. On top of the sponge two pieces of Whatman paper were placed and some transfer buffer was poured to soak the paper and to remove any air bubbles. The gel was placed facing down on the Whatman paper and then the nylon membrane was placed with the cut corner touching the cut corner of the gel. Some transfer buffer was added to remove any bubbles. Two Whatman papers, and then a stack of paper towels were placed on top of the membrane. A 500g weight was placed on the paper towels to help the capillary transfer. 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. Following, the membrane was allowed to dry on a Whatman paper for 5 minutes and then cross-linked using the Bio-Rad GS Gene Cross Linker at setting C3 (150 m joules). The membrane was wrapped in saran wrap and stored in a dry place until use.

Generation of probes

The flanking sequence probe was obtained from the plasmid pUC-Ct vector that contains the chloroplast flanking sequences for the trnI and trnA. The digestion reaction was setup as follows: 15 µl of pUC-Ct vector DNA, 2 µl of 10X buffer, 1 µl of BamHI (NEB), 1 µl of BglII and 1 µl of dH₂O. The reaction was performed for 3 hrs at 37 °C and then run in an agarose gel to obtain the desired fragment of 0.8kb. The band was cut out and eluted from the gel as explained before. For the final DNA elution, 50 µl of H₂O was used. The gene specific probe was made by cutting out the 700bp fragment of CTB-NSP4 from pLD-5'UTR-Histag-CTB-NSP4 in a reaction as follows: 2µg (5µl) of pLD-5'UTR-Histag-CTB-NSP4, 2 µl of 10X buffer, 1 µl each of NdeI and EcoRI (NEB) and 11 µl of dH₂O. The reaction was incubated at 37 °C for 3 hrs. The fragment of interest (700bp) was eluted from the agarose gel and checked for the concentration on the spectrophotometer.

Probe labeling

The probes were denatured (45 μ l of the DNA) by incubating the tube at 94 °C for 5 minutes, immediately placing the tube on ice for 2-3 minutes and then pulse centrifuging to bring down any droplets. Following, the probe was added to the ready mix (Quant[™] G-50 Micro columns, Amersham) and mixed by flicking. 5 μ l of α ³²P was added to the tube and the mixture was incubated for 1 hour at 37 °C. Once the incubation period was complete, a G50 column was taken and the resin was re-suspended by vortexing, then the cap was loosened about ¼ and bottom plug was removed. Then, the column was placed into a micro centrifuge tube with the top cut off and centrifuged for 1 minute at 3,000 rpm. The collection tube was discarded and the column was transferred to a new 1.5ml tube. The DNA probe was added in the center of the resin and spun at 3,000 rpm for 2 minutes and the column was discarded. From the labeled probe, 1 μ l was mixed with 98 μ l of STE buffer. The mixture was aliquoted into 50 μ l samples and to each sample 3ml of Opti-Fluor was added. The activity of the radioactive probe was measured in a Beckman LS 5000TD. The two samples plus a blank containing 3ml of Opti-Fluor were placed into the machine holder. The readings were taken by using the auto-read mode of the machine. The amount of probe to be used was determined by calculating the amount of probe needed to yield 2.5×10^6 cpm/2ml. The amount of probe was calculated as follows: Reading value (502050) was equal to 0.50×10^6 cpm/ μ l, and then multiplied by 50 μ l of total volume of sample for a total of 35×10^6 cpm/ μ l.

Because 5ml of hybridization solution was used, we needed 6.25×10^6 cpm, therefore 6.25×10^6 cpm divided by 0.7×10^6 cpm is equal to 12.5 μ l of labeled probe needed.

Prehybridization, hybridization and washing of membrane

The Quick-Hyb solution from Stratagene was mixed and incubated for 5 to 10 minutes at 68 °C. Following, the blot was placed into the hybridization bottle with the top facing in toward the solution and 5ml of the preheated pre-hybridization solution was added. The bottle with the membrane was incubated in the Fisher Biotech Hybridization Incubator for 1 hour at 68 °C. One hundred μ l of salmon sperm DNA was added to the probe and then heated for 5 minutes at 94 °C. Following, 1ml of pre-Hyb solution from the bottle containing the membrane was withdrawn and added to the probe solution. Immediately, the content was returned to the bottle. The hybridization bottle was incubated for 1 hour at 68 °C. After the hour of incubation, the quick-Hyb solution was discarded into the liquid radioactive waste container. The membrane was washed twice as follows: 50ml of wash solution number 1 (2X SSC and 0.1% SDS) was poured and incubated at room temperature for 15 minutes. The liquid was discarded in the liquid waste container and the step was repeated. A second round of washes was performed twice by pouring 50ml of solution number 2 (0.1X SSC and 0.1% SDS) and incubating it for 15 minutes at 60 °C to increase the stringency. The liquid of these washes were discarded into the radioactive liquid container. The radioactive membrane was wrapped around with saran wrap and kept in a radioactive container in the radioactive hood.

Autoradiography

The blots were placed into the film cassette and then taken to the dark room. Using the safe light (red light), the X-ray film was placed into the cassette on top of the blot and the intensifier screen was placed on top of the X-ray film. The cassette with the blot and the film was placed into a black bag to protect against light and then incubated overnight at -80°C . The next day the cassette was taken out from the -80°C , allowed to thaw, and then moved to the dark room where the film was developed.

Characterization of expressed proteins

Extraction of Protein from Transformed E. coli Cells

E. coli, XL1-blue cells that have tetracycline resistance gene in their genome, were transformed with pLD-5'UTR-CTB-NSP4 and were grown in 5 ml of Terrific broth with ampicillin ($100\ \mu\text{g}/\mu\text{l}$) and tetracycline ($50\ \mu\text{g}/\mu\text{l}$) at 37°C for 14-16 hrs. Untransformed *E. coli* cells were also cultured for use as a negative control. The buffers and gels used in this study were made from protocols in SDS-PAGE Buffer System below (Laemmli 1970). After the boiling step (as described below), samples were immediately loaded into polyacrylamide gels. The $800\ \mu\text{l}$ of cultured cells were centrifuged for 1 minute at 13,000 rpm. Supernatant was discarded from pelleted *E. coli* cells then washed with 1ml of 1x Phosphate-Buffered Saline (PBS: 140mM NaCl,

2.7mM KCl, 4mM Na₂HPO₄, 1.8mM KH₂PO₄, pH 7.2). Pellet was resuspended, followed by centrifugation for 1 minute at 13,000 rpm. Supernatant was discarded. 50 µl of 1x PBS was added and mixed well to resuspend the pellet. A mixture containing 475µl 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, q.s. with dH₂O to 9.5 ml, and the 25 µl of β-mercaptoethanol was prepared. Equal amounts of sample and the mix of sample loading buffer and β-mercaptoethanol was taken and boiled for 5 minutes, then immediately loaded samples onto gels (Sambrook *et al.* 1989).

Extraction of Protein from Transformed Tobacco Leaves

In order to confirm expression of CTB-NSP4 in tobacco plants, extracts were made from *Petit havana* plants. These steps were performed as described in (Daniell *et al.*, 2004c). Approx. 100 µg of plant leaf tissue was weighed and ground with a mortar and pestle in liquid nitrogen and put into a micro centrifuge tube. Two hundred µl of extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl-pH8, 0.05% Tween-20, 0.1% SDS, 14 mM BME, 400 mM sucrose, 2 mM PMSF) was added and the samples were mixed for 3 minutes with a micro pestle. The samples are centrifuged at 13,000 rpm for 5min 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.

SDS-PAGE Buffers and Gels

To detect the CTB-NSP4 protein expression in the crude extract obtained from *E. coli* cells or crude extract obtained from the transgenic plant tissue extract transformed with pLD-5'UTR-CTB-NSP4, SDS-PAGE gels were made in duplicate utilizing buffer solutions: (1) Bio-Rad (cat#161-0158), 30% Acrylamide/Bis solution according to the ratio 37:5:1. (2) The resolving buffer (5M Tris-HCl, pH 8.8), was used to make the resolving portion of the gel. (3) The stacking buffer (0.5M Tris-HCl, pH 6.8), was used to make the stacking gel layer, concentrated the samples at top of the resolving gel to improve resolution. (4) 10x Electrode buffer: 30.3g Tris base, 144.0g glycine and 10.0g SDS to 1000 ml dH₂O and stored at 4 °C, (5) 2x loading buffer, also called the Sample buffer and the SDS Reducing Buffer: see previous section. (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 dH₂O in a micro centrifuge tube and stored at 4 °C for about a month. The 10% resolving gel was made by the following method: Added 3.3 ml of 30% Acrylamide/Bis, 2.5ml of resolving buffer, 4.1 ml dH₂O and 100 µl of 10% SDS to a 50 ml flask. Added 50 µl of 20% APS (#8 above) and then 10 µl of TEMED and used to cast the gel mixture between the two, vertical, glass plates (Mini-Protean 3 Cell gel system, Bio-Rad) leaving about 1.5 cm at the top of glass plates for the stacking gel. The gel is allowed to polymerize for 20 minutes. To make the 4% stacking gel, 1.3 ml of 30% Acrylamide/Bis, 2.5 ml of the stacking buffer, 6.1 ml dH₂O and 100 µl of 10% SDS are taken together into a flask followed by 50 µl of 20% APS and 10 µl of TEMED. The 4%

gel mixture is 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 the gel was put vertically into PAGE apparatus containing 1x Electrode (running) buffer. 20 µl of protein extract along with the sample-loading buffer was loaded along with CTB-NSP4₉₀ protein standard, and 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.

Transfer of protein and analysis of Western Blot

The separated proteins were transferred onto a 0.2 µm Trans-Blot nitrocellulose membrane (Bio-Rad) by electroblotting in Mini-Transfer Blot Module at 85V for 45 minutes in Transfer buffer (360 ml of 10x Electrode buffer, 360 ml of methanol, 0.18 grams of SDS, 1080 ml distilled dH₂O). For Western blotting, the proteins were transferred to nitrocellulose membranes and then blocked for one hour in P-T-M (PBS [12 mM Na₂HPO₄, 3.0 mM NaH₂PO₄-H₂O, 145 mM NaCl, pH 7.2], 0.5% Tween 20, and 3% Dry Milk) followed by transfer to P-T-M containing diluted (1:3000) rabbit anti-NSP4₉₀ antibody (provided by Dr Langridge, Univ of Loma Linda). Membranes were then washed with distilled water and transferred to P-T-M containing diluted (1:5000) donkey derived anti-rabbit IgG antibody conjugated with Horseradish peroxidase (Sigma, national immunization systems St. Louis, MO). Blots were washed three times with PBST for 15 minutes each time. Then washed with PBS for 10 minutes, followed by

addition of chemiluminiscent substrate ((Pierce, Rockford, IL) for HRP and incubating at room temp for 5 min for the chemiluminescence. Later the X-ray films were exposed to chemiluminescence and the films were developed in the film processor to visualize the bands.

GM1 Binding (ELISA) assay

The quantification and binding affinity of chloroplast derived CTB-NSP4 for GM1-ganglioside receptor in the plant crude extract was done using the GM1 ganglioside binding affinity (ELISA) as described by (Arakawa *et al* 1997). 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), 1Day, 3 Day and 5Day continuous light exposure. The leaf samples were finely ground in liquid nitrogen, followed by collection of leaf powder into the eppendorf tube. To extract the protein from the plant leaf powder, plant protein extraction buffer (15mM Na₂CO₃, 35mM NaHCO₃, 3mM NaN₃, pH 9.6, 0.1% Tween, and 5mM PMSF) was used to resuspend the ground mixture. The mechanical pestle was used to agitate the leaf tissue so that the all the soluble protein comes out of the chloroplast and cytosol into the buffer. 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 microtiter plate was coated initially with (100 µl/well) with monoganglioside-GM1 (Sigma) (3.0 µg/ml in bicarbonate buffer pH 9.6) and incubated overnight at 4°C followed by washing three times with PBST (PBS and

0.05% Tween 20) and two times with dH₂O. As control, BSA (3.0 µg/ml in bicarbonate buffer pH 9.6) was coated in some wells. The wells were then blocked with 1% BSA in 0.01M phosphate buffer saline (PBS) (300 µl/well) for 2h at 37°C or incubate overnight at 4°C followed by 3 washes with PBST and 2 washes with dH₂O. The standards ranging from 50 to 350 ng/ml were made by diluting bacterial CTB-NSP4₉₀ purified from *E.coli* BL21 cells (Kim et al 2003) in 1X PBS. The standards and protein samples (100 µl) were coated to 96-well polyvinyl chloride microtiter plate (Cellstar) for 1 h at 37°C or incubate overnight 4°C followed by 3 washes with PBST and 2 washes with water. The primary rabbit anti-NSP4 antibody (provided by Dr. Langridge, Univ. of Loma Linda) diluted (1:1500) in 0.5% BSA in 1XPBS was loaded into wells and incubated for 2h at 37°C followed by washing steps and then again incubated with 100 µl of donkey anti-rabbit IgG-HRP conjugated antibody made in goat (American Qualex) (1: 3000) diluted in 0.5% BSA in 1XPBS. The plate was then incubated for 2h at 37 °C. After the incubation the plate was washed thrice with PBST and twice with water. The wells were then loaded with 200 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 on a plate reader (Dynex Technologies) at 450 nm. (Modified form of protocol from Ausubel *et al.*, 4th 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 CTB-NSP4 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 1:10 and 1:20 with extraction buffer. 10 µl of each standard and 10 µl of each plant dilution was added to the wells of a 96 well microtiter plate (Cellstar) 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. 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-His-CTB-NSP4 vector for tobacco chloroplast

Transformation

The pRSET vector with Histag-CTB-NSP4 cloned in its multiple cloning sites was a gift from Dr William H R Langridge, Loma Linda Univeristy School of Medicine. The goal was to clone the cassette into the universal chloroplast transformation vector, pLD-Ctv under the control of light regulated psbA 5'UTR regulatory sequence which enhances the translation of the genes. The Histag-CTB-NSP4 gene cassette in pRSET vector was digested with Nde I and EcoRI and cloned into p-bluescript containing the 5'UTR regulatory sequence as shown in fig 1C named as p-bluescript-5'UTR-Histag-CTB-NSP4. The p-bluescript containing the 5'UTR-Histag-CTB-NSP4 cassette was then digested with EcoRV and XbaI and was cloned into the universal chloroplast transformation vector, pLD-Ctv within the EcoRV and XbaI sites and was designated as pLD-AK as shown in fig.1D. The pLD vector contains the homologous recombination sequences (flanking sequences) that allowed the homologous recombination of the gene cassette (*aadA*, 5'UTR-His-CTB-NSP4) in between the *trnI* and *trnA* of the chloroplast genome (Daniell *et al.* 2001). Downstream to the *trnI*, the vector provided the constitutive 16S rRNA promoter, which regulates the expression of *aadA* gene (aminoglycoside 3' adenylyltransferase) that confers resistance to spectinomycin-streptomycin and the 5'UTR-His-CTB-NSP4 gene encoding the cholera toxin B subunit- rotavirus NSP4 enterotoxin

fusion protein. Upstream to the *trnA*, the vector contains the 3'UTR which is a transcript stabilizer derived from the *psbA* gene.

Fig 1A.

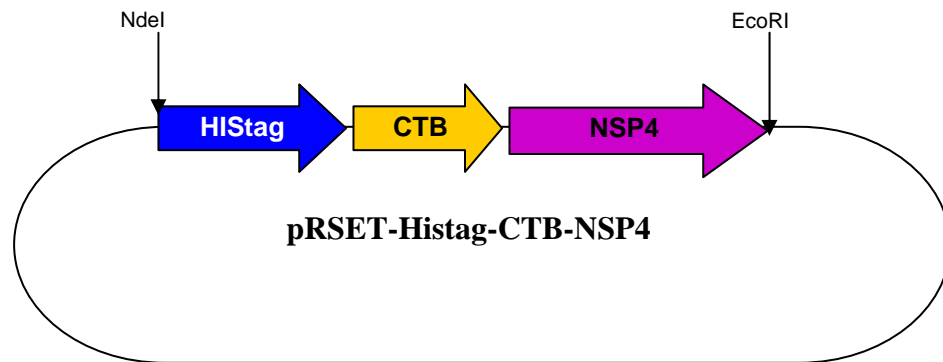


Fig 1 B.

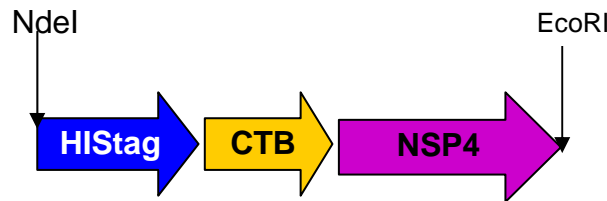


Fig 1C.

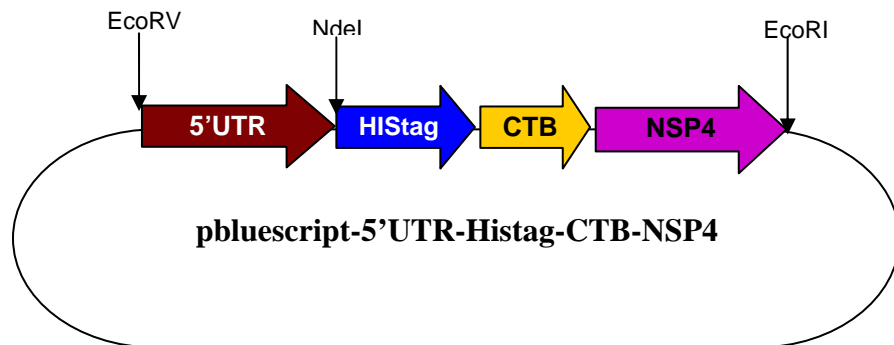


Fig 1 D.

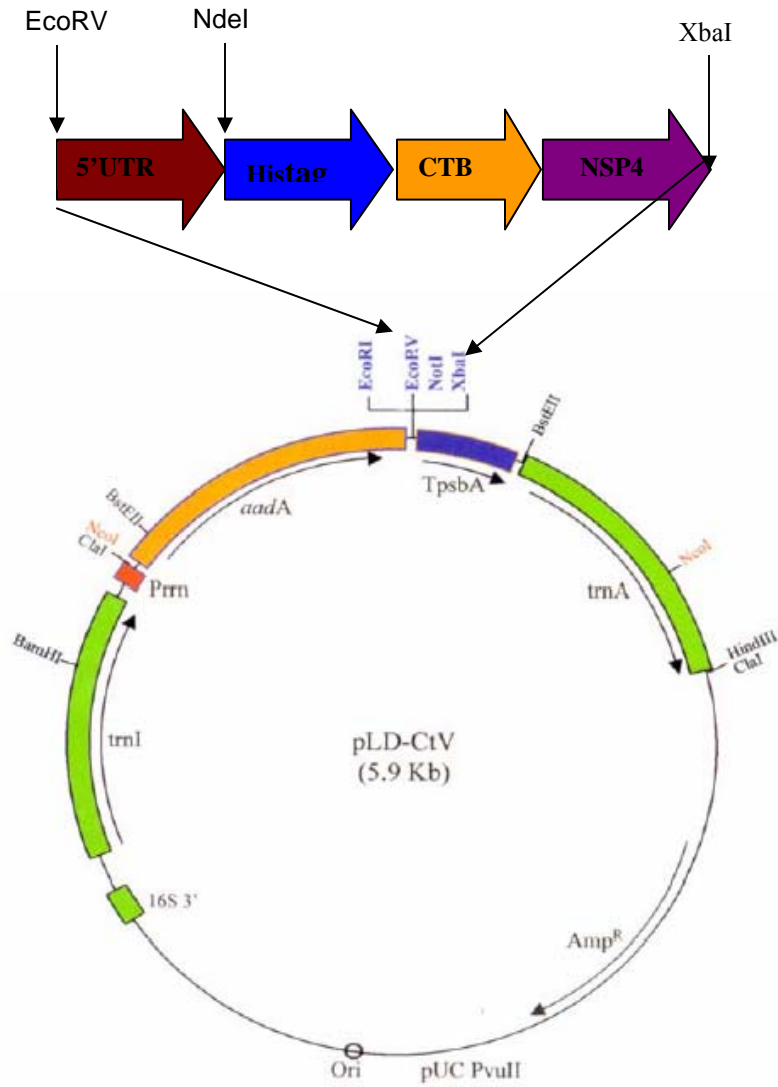


Figure 1 Schematic representation of the cloning of pLD-5'UTR-Histag-CTB-NSP4.

- A:** pRSET vector showing restriction sites within Histag-CTB-NSP4 gene cassette
- B:** Histag-CTB-NSP4 with NdeI and EcoRI sites
- C:** Cloning of Histag-CTB-NSP4 in pbluescript downstream of 5'UTR using NdeI and EcoRI sites
- D:** Cloning of in pLD-Ctv using EcoRV and XbaI sites (pLD-AK).

Sequencing of pLD-5'UTR-Histag-CTB-NSP4(pLD-AK)

The proper alignment and integration of 5'UTR-HIStag-CTB-NSP4 in pLD vector was confirmed by sequencing pLD-AK vector as template with specially designed set of primers. The forward primer (5'GATCACCAAGGTAGTCGGCAAATA 3') was complimentary to the 3' end of aadA gene. The reverse primer (5'TGCAGCCCAAACAAATACAAAATC 3') was designed to be complimentary to the 3' end of 3'TpsbA gene. The sequencing was done in the Beckman Coulter CEQ 2000 XL DNA analysis system using Beckman Coulter CEQ DTCS - Quick start kit for the preparation of DNA sequencing reaction.

Fig 2.

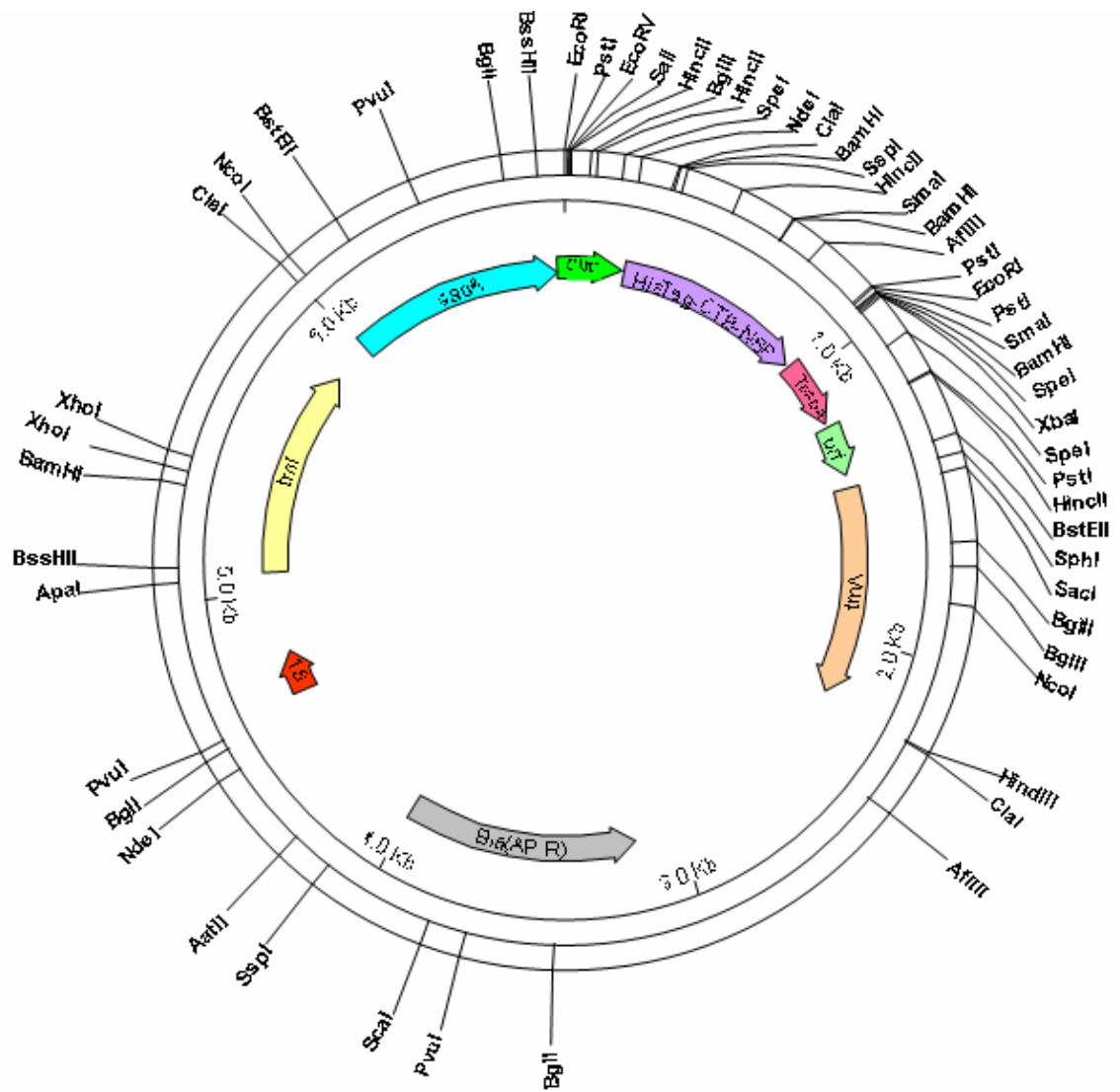


Figure 2 Vector map of pLD-5'UTR-Histag-CTB-NSP4 (pLD-AK) with sequence confirmed.

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, for the first round of selection as described in Daniell (1997). From 10 bombarded Petit Havana leaves, about 20 green shoots appeared after 4 weeks. Untransformed cells appeared bleached on the antibiotic because they did not contain the aadA gene (Fig.3). For second round of selection the leaves were cut into 2mm² pieces and then transferred to fresh RMOP plates with 500 µg/ml spectinomycin for Petite Havana.

The shoots that appeared during the second round of selection were tested positive for cassette integration into the chloroplast genome by PCR analysis, were grown in sterile jars containing fresh plant MSO medium with spectinomycin until the shoots grew to fill the jars. 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.

Fig 3A

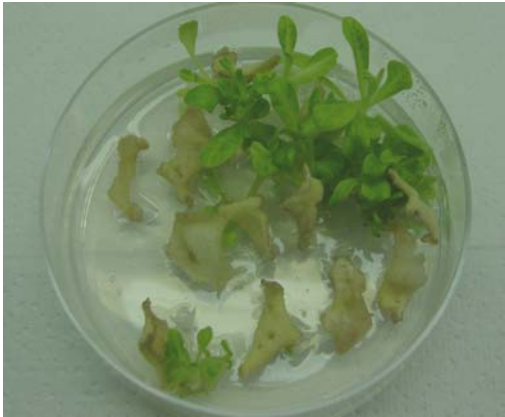


Fig 3B

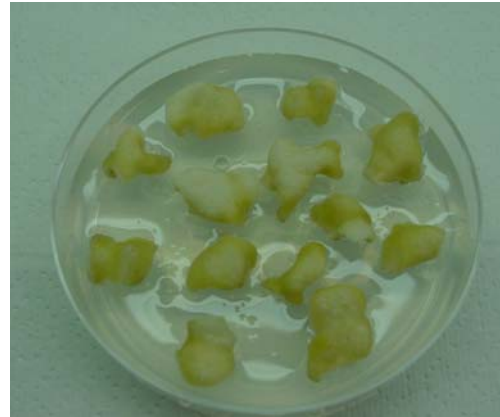


Figure 3 First Round of Selection

- A.** Shoots from bombardment of Petit Havana leaves (on RMOP medium with 500ug/ml spectinomycin) appeared within 4 weeks.
- B.** Wild type Petit Havana leaves as control did not grow on the selection medium.

Fig 4A

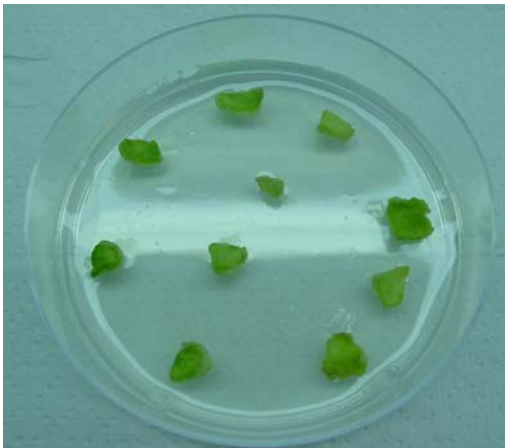


Fig 4B



Figure 4 Second Round of Selection on RMOP media with spectinomycin.

- A:** leaves from primary selection cut into 2mm² pieces and then transferred to fresh RMOP medium with 500 µg/ml spectinomycin.
- B:** Shoots of transgenic CTB-NSP4 plants after secondary selection.

Fig 5A



Fig 5B



Figure 5 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 Confirmation of Chloroplast Integration of transgene

After bombardment of tobacco leaves with gold particles coated with plasmid DNA (pLD-5'UTR-His-CTB-NSP4), about 5 shoots/ plate appeared after a period of 5-6 weeks. The shoots that were obtained on the RMOP selection medium could be due to any one of the three possible and two types of integration: chloroplast transgenic, nuclear transgenic or mutant shoots. Spontaneous mutation of the 16S rRNA gene, which confers resistance to spectinomycin in the ribosome, could allow plants to grow on

spectinomycin without integration of the gene cassette which will result in the mutant shoot growth. The *aadA* gene in the gene cassette confers resistance to spectinomycin and hence the shoots with the integration of the gene cassette in either nuclear or chloroplast genome grow on the selection medium. True chloroplast transformants were distinguished from nuclear transformants and mutants by PCR analysis. Two primers, 3P and 3M were used to test for chloroplast integration of transgenes (Daniell *et al.* 2001). 3P primer lands on the native chloroplast DNA in the 16S rRNA gene region and the 3M primer lands on the *aadA* gene as shown in fig 6A. Nuclear transformants were eliminated because 3P will not anneal and mutants were eliminated because 3M will not anneal. The 3P and 3M primers upon chloroplast integration of transgene will yield a product of 1.65kb size fragment as shown in figure 6B.

The Integration of the *aadA*, 5'UTR-His-CTB-NSP4 gene and 3'psbA UTR, were additionally tested by using the 5P and 2M primer pair for the PCR analysis. The 5P and 2M primers annealed to the internal region of the *aadA* gene and the internal region of the *trnA* gene respectively as shown in fig 6A (Daniell *et al.* 2001). The product size of a positive clone is of 2.5 kb for CTB-NSP4, while the mutants and the control do not show any product. Figure 6C shows the result of the 5P/2M PCR analysis. After PCR analysis using both primer pairs, the plants were subsequently transferred through different rounds of selection on spectinomycin media to obtain a mature plant and reach homoplasmy.

Fig 6A

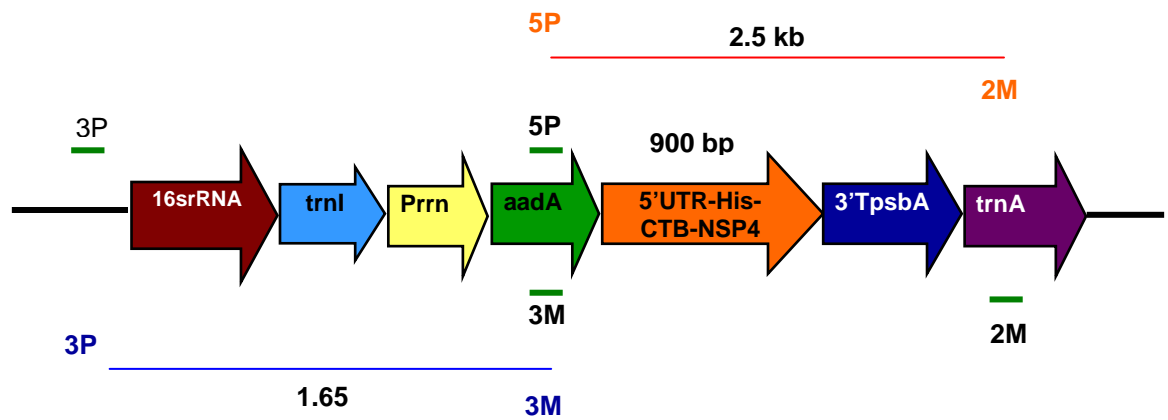


Fig 6B

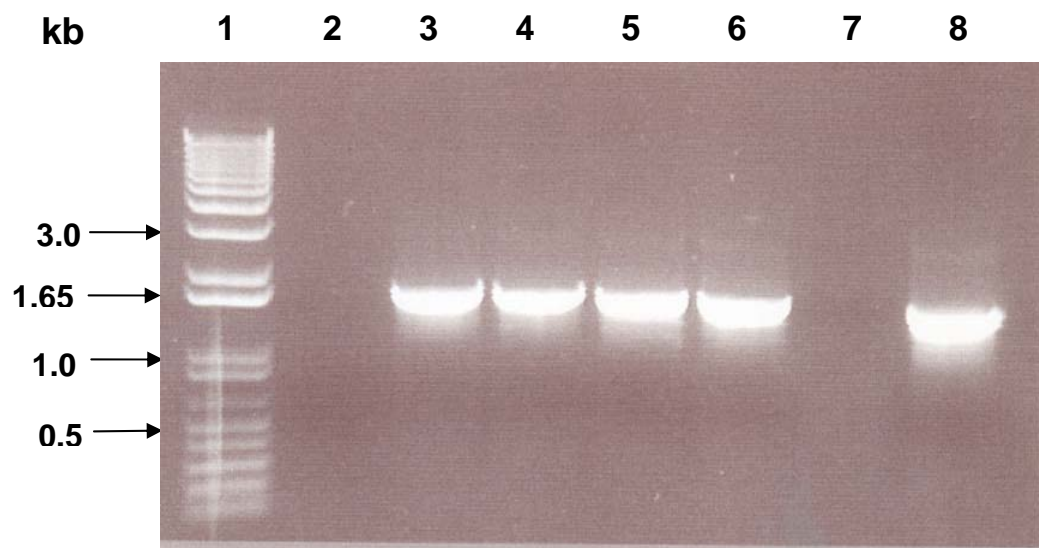


Fig 6C

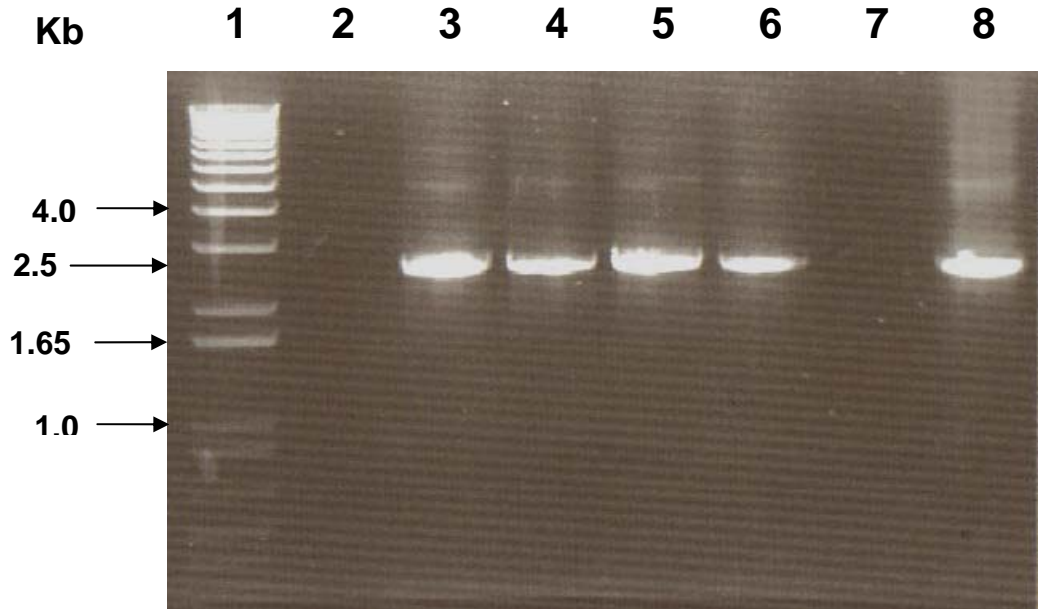


Figure 6: PCR analysis of Wild type and putative transformants of pLD-5'UTR-His-CTB-NSP4.

A: PCR using specific primers land within the native chloroplast genome (3P/3M) to yield a 1.65 kb product and 5P/2M primers to yield 2.5 kb product.

B: Lane 1: 1kb plus DNA ladder, **Lane2:** Negative control (Wild type) **Lane 3-6:** Transgenic lines of HisCTB-NSP4, **Lane 7:** Empty, **Lane 8:** Positive control (Interferon clone).

C: Lane 1: 1 kb plus DNA ladder, **Lane 2:** Negative control(wild type), **Lanes 3-6:** Transgenic lines of HisCTB-NSP4, **Lane 7:** Empty, **Lane 8:** Positive control pLD 5'UTR-HisCTB-NSP4 plasmid.

Southern Analysis of transgenic plants

The plants that tested positive for the PCR analysis were moved through three rounds of selection and were then tested by Southern analysis for site specific integration of the transgene and homoplasmy. The DNA of the full regenerated clones growing in jars (third selection) was extracted and used for the Southern analysis. The flanking sequence probe of 0.81 kb in size allowed detection of the site-specific integration of the gene cassette into the chloroplast genome; this was obtained by double digesting the pUC-Ct vector that contained the *trnI* and *trnA* flanking sequences (fig.7A) with BamHI and BglII (Daniell *et al.* 2001). Figure 7B shows the HincII sites used for the restriction digestion of the plant DNA for pLD-5'UTR-Histag-CTB-NSP4. The transformed chloroplast genome digested with HincII produced fragments of 4.3 kb and 2.0 kb for pLD-5'UTR-Histag-CTB-NSP4, while the untransformed chloroplast genome that had been digested with HincII resulted in a 5.0 kb fragment (Fig 7D). The flanking sequence probe can also show if homoplasmy of the chloroplast genome has been achieved through the three rounds of selection. The plants expressing CTB-NSP4 showed homoplasmy as there is no wild type band seen in transgenic lines within the levels of detection. The gene specific probe CTB-NSP4 of size approx.0.7 kb was used to show the specific gene integration producing a fragment of 11 kb when CTB-NSP4 transgenic plant DNA was digested with ClaI as shown in fig 7C and 7E.

Fig 7A.

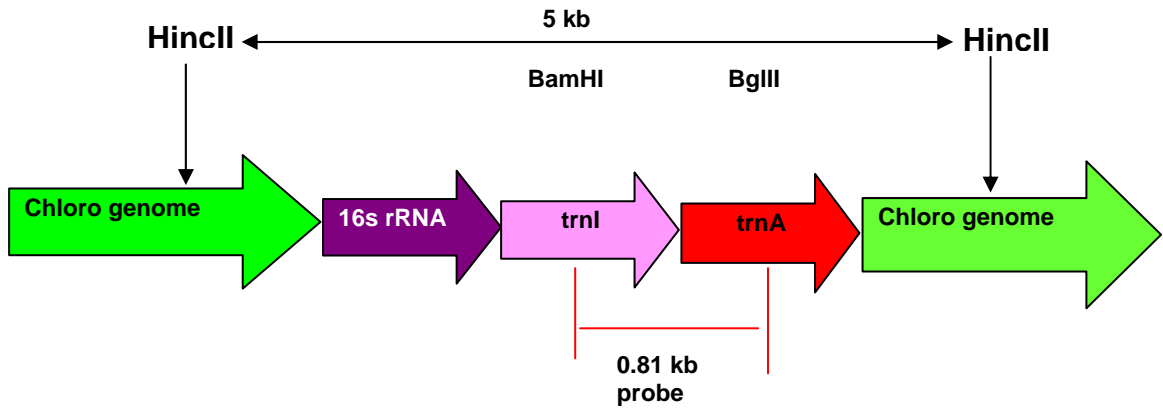


Fig 7B.

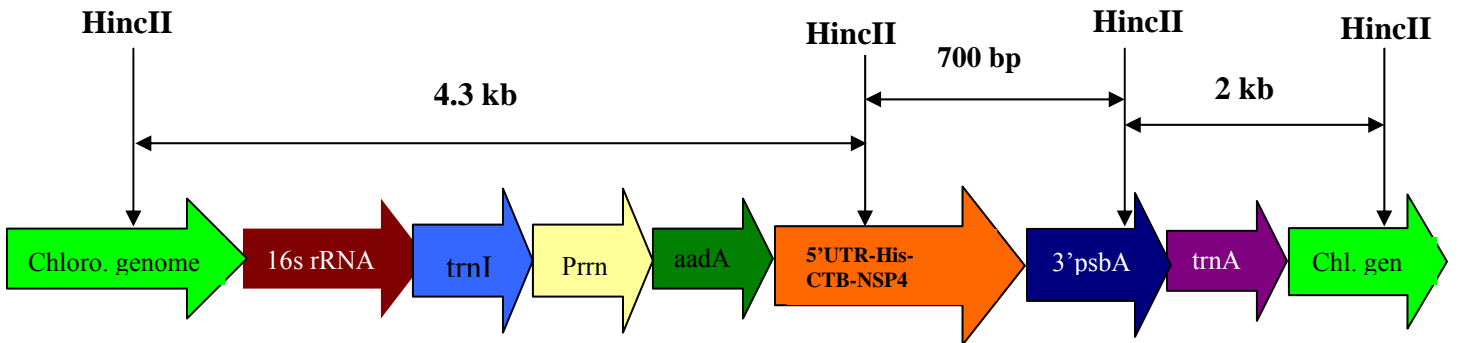


Fig 7C.

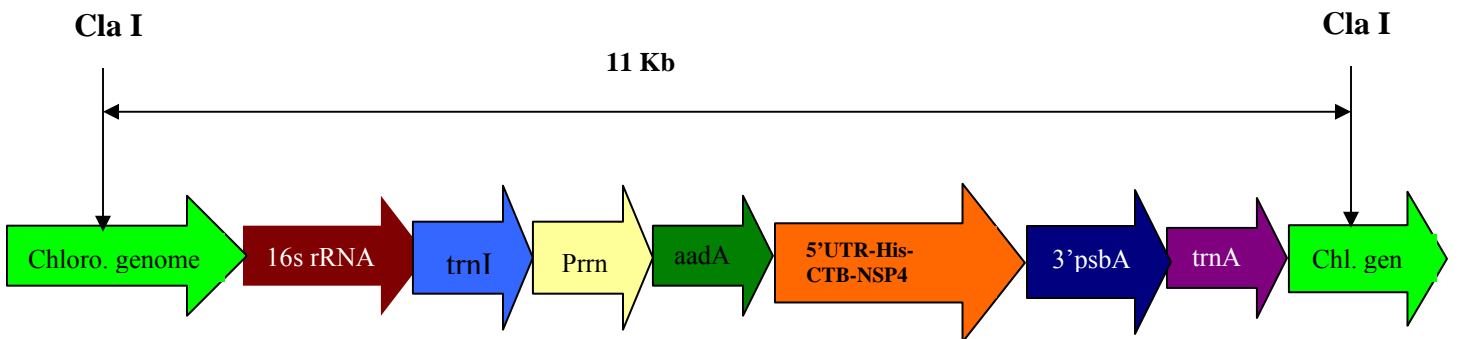


Fig 7D.

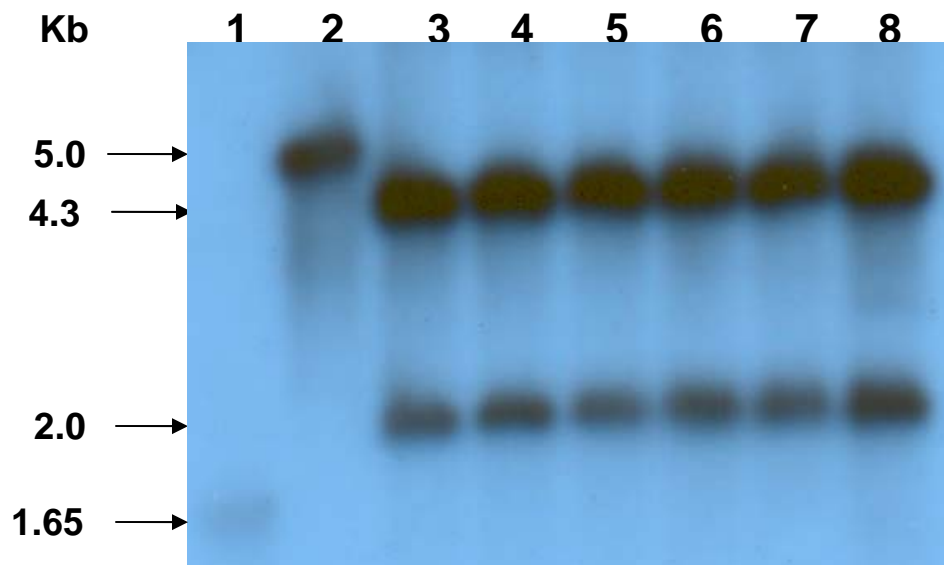


Fig 7 E.

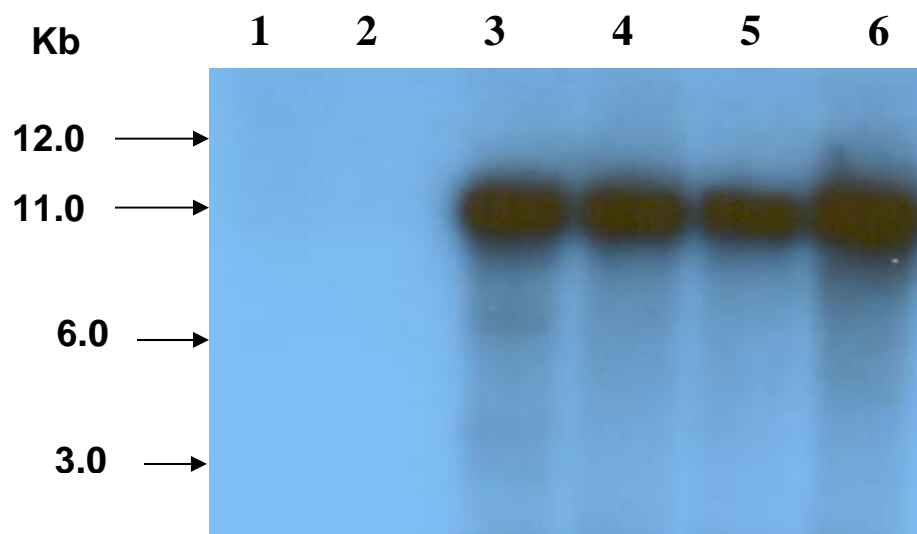


Figure 7 Southern Blot analysis of CTB-NSP4 T0 plants.

Schematic diagram of the products obtained from digestions of

A: Wild type untransformed plants show a DNA fragment of 5 kb.

B: Two DNA fragments of 4.3 kb and 2 kb indicate plants that are transformed with pLD-5'UTR-HisCTB-NSP4.

C: A DNA fragment of 11 Kb is seen for transgenic lines with gene specific probe

D: Southern with flanking sequence probe of CTB-NSP4 transgenic plants showing homoplasmy. **Lane 1:** 1 kb plus DNA ladder, **Lane 2:** Wild type, **Lanes 3-8:** CTB-NSP4 transgenic lines

E: CTB-NSP4 gene specific probe showing the presence of CTB-NSP4 gene in the transgenic plants. **Lane 1:** 1 kb plus DNA ladder, **Lane 2:** Wild type, **Lanes 3-6:** CTB-NSP4 transgenic lines

Immunoblot analysis

Crude protein extract of 20 ug, was loaded in each well of the SDS-PAGE. The rabbit anti-NSP4₉₀ antibodies (provided by Dr. Willam Langridge, Loma Linda Univ. of Loma Linda) were used to detect the 27 kDa and 135kDa monomeric and pentameric forms CTB-NSP4 fusion protein (figure 8). The wild type plant (*Petit havana*) did not show any bands indicating that the anti- NSP4 antibodies did not cross react with any other proteins in the crude extract. As the CTB-NSP4 expression level was 2.45% of TSP in mature leaves, which indicates that there is 2.4 ug of the fusion protein in 100 ug of TSP. the total crude protein extract loaded in each well is 20ug and so the expected amount of CTB-NSP4 fusion protein present in 20ug of TSP will be about 0.6ug. Hence each of the wells contains approximately about 0.6 ug of the CTB-NSP4 protein detected by the CTB-NSP4 antibodies.

Fig 8.

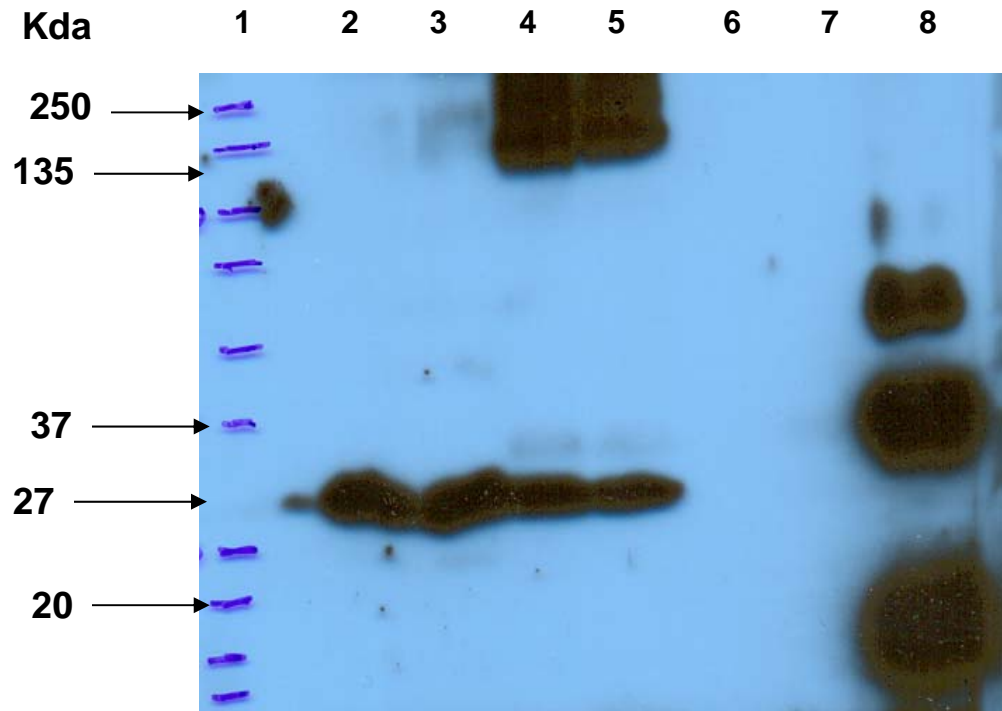


Figure 8: Immunoblot analysis of crude plant extracts expressing CTB-NSP4.

Lane 1: Molecular weight markers, **Lane 2-3:** Boiled T0 transgenic plant samples, only the 27 Kda CTB-NPS4 monomeric form of fusion protein was detected, **Lane 4-5:** Unboiled T0 transgenic plant samples (20 ug of crude plant extract was loaded), 27 Kda CTB-NSP4 fusion protein monomeric form and 135 Kda pentameric form were detected, **Lane 6:** Wild type, **Lanes 7:** Empty, **Lane 8:** bacterial CTB-NSP4₉₀ fusion protein purified from E. coli BL 21 cells (0.9ug).

Protein quantification and binding affinity using GM1 Binding assay ELISA

The levels of pentameric CTB-NSP4 fusion protein in transformed tobacco plants and its affinity for GM1- ganglioside was evaluated by quantitative GM1 ELISA. The standard curve has been obtained using different dilutions of purified CTB-NSP4. The dilutions were made in 0.01M phosphate buffered saline (PBS). The primary antibody used was Rabbit antibody raised against NSP490 protein expressed and purified from Ecoli BL21 cells and secondary antibodies were donkey anti-rabbit antibodies peroxidase conjugated. The percentage of CTB-NSP4 expressed was as a percent of total soluble protein calculated using the Bradford assay i.e. the percentage of CTB-NSP4 is inversely proportional to the TSP values. The CTB-NSP4 expression levels reached a maximum of 2.45 % of the total soluble protein in the mature leaves after 1Day of continuous light exposure due to increase in translation obtained under the control of light regulated psbA 5,UTR . The increased expression in mature leaves is due to more number of chloroplasts and high number of chloroplast genomes (up to 10,000 copies/cell) in the mature leaves. Also, the large size and more number of mature leaves per plant contributed to the higher levels of CTB-NSP4 in mature leaves.

Fig 9.

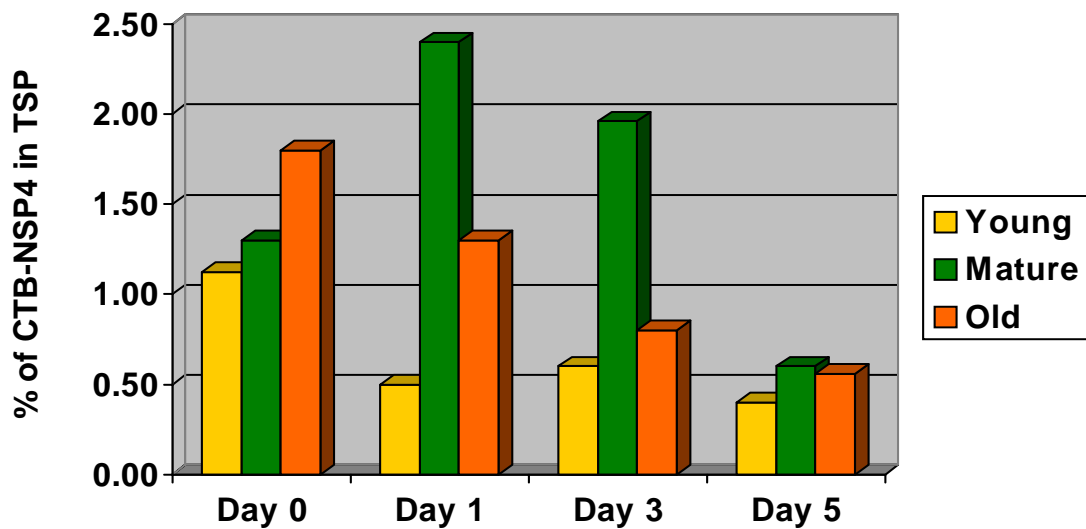


Figure 9. Quantification of CTB-NSP4 fusion protein expression levels in transgenic plants (T₀ generation).

Expression levels in % total soluble protein (TSP) of CTB-NSP4 expressed in Young, Mature and Old leaves under continuous light illumination observed for 0 to 5 days. The CTB-NSP4 expression levels reached a maximum of 2.45% of TSP in mature leaves by day 1 under continuous light and the expression levels declined to 0.6 % of TSP by Day 5.

DISCUSSION

The pLD-5'UTR-Histag-CTB-NSP4 vector was derived from the universal transformation vector, pLD-CtV (Daniell H. 2001). The pLD-5'UTR-Histag-CTB-NSP4 chloroplast transformation vector containing the *aadA* gene, CTB-NSP4 coding region and 3' *psbA*, integrates the transgene cassette into the transcriptionally active *trnI*-*trnA* spacer region of the chloroplast genome via homologous recombination(Fig.6).

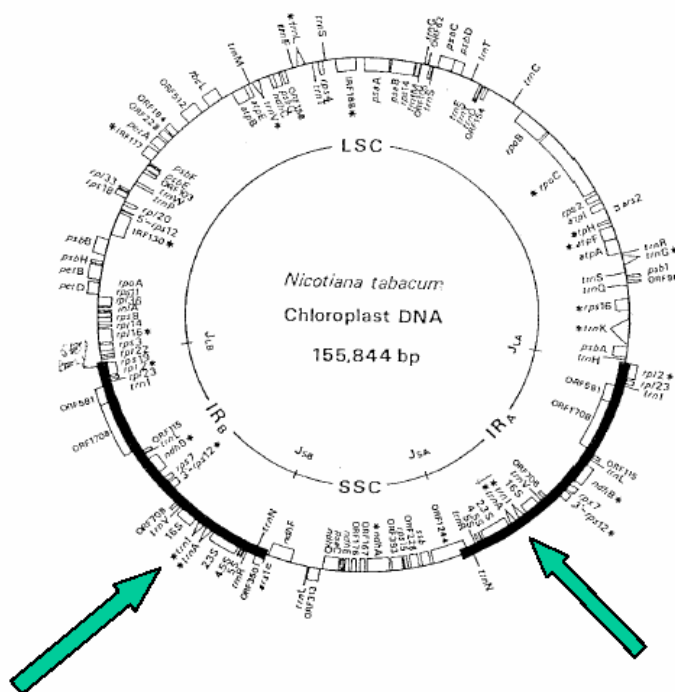


Figure 10. Chloroplast genome

Arrows point to the *trnI* and *trnA* where homologous recombination occurs between the chloroplast and the pLD-5'UTR-Histag-CTB-NSP4 vector.

The site directed insertion of CTB-NSP4 into the chloroplast genome is achieved by homologous recombination between trnI-trnA regions of pLD-5'UTR-His-CTB-NSP4 and plastid genome which prevents any random integration of transgene that is usually observed with nuclear transformation. Achieving high expression of the CTB-NSP4 recombinant fusion protein in the chloroplast depends on various factors. First, the pLD-His-CTB-NSP4 vector is designed to integrate into the inverted repeat region of the chloroplast genome via homologous recombination. When the CTB-NSP4 fusion gene is inserted into the IR region, the copy number of the transgene gets doubled by a phenomenon known as copy correction that recruits the introduced transgene into another IR region (Devine and Daniell 2004). Increased copy number results in increased transcript levels resulting in higher protein accumulation (Guda *et al.* 2000, Daniell *et al.* 2001a). Second, the psbA 5' UTR typically has stem loop structure which aid in transcript stability and is also a binding site for translation activation factors to enhance the binding of ribosomes to the mRNA for efficient translation. Also the translation of psbA mRNA is stimulated by light proposed to be mediated by a nuclear encoded protein. The binding of the nuclear encoded (RB) protein and psbA is directly dependent light which there by enhances the initiation of translation. The redox potential generated by light reactions of photosynthesis is used by chloroplast Protein Disulfide Isomerase system and thioredoxin which then activate the binding of translation activation factors to the ribosome binding sites in psbA 5' UTR thereby enhancing the translation in the presence of light (Zerges 2000). The expression of CTB-NSP4 in transgenic plant under continuous light showed an increase in expression at Day 1. The psbA 3'untranslated

region (UTR) used for the regulation of transgene expression has potential role in post transcriptional stabilization by binding to different RNA binding proteins and help in enhancing translation of the foreign protein (Kumar *et al.* 2004b). Third, the pLD-His-CTB-NSP4 vector consists of a OriA site for origin of replication within trnI flanking region allowing to attain homoplasmy even in the first round of replication by increasing the number of templates for integration into the chloroplast genome (Daniell *et al* 2004c, Devine and Daniell 2004). To obtain an optimal production of the CTB-NSP4 fusion protein and transgene stability, it is essential to achieve homoplasmy through several rounds of selection on media containing spectinomycin. If homoplasmy is not achieved, it could result in heteroplasmy which leads to changes in the relative ratios of the two genomes upon cell division. The presence of heteroplasmic condition in a transgenic plant might retrograde back to the wild type eliminating the transgene in the absence of selection pressure in subsequent generations. The chimeric, aminoglycoside 3' adenylyl transferase (*aadA*) gene, conferring resistance to spectinomycin was used as a selectable marker and its expression is driven by the 16S (*Prrn*) promoter (Daniell *et al.* 2001, Svab and Maliga 1993). Spectinomycin binds the 70S ribosome and inhibits translocation of peptidal tRNA's from the A site to the P site during protein synthesis. The *aadA* gene codes for the enzyme aminoglycoside 3' adenylyltransferase, which transfers the adenylyl moiety of ATP to spectinomycin and inactivating it. Fourth, chloroplast translation system provides the necessary enzymes for proper folding and disulphide bond formation. Chaperonins present in chloroplast are thought to aid in the folding and assembly of non native prokaryotic and eukaryotic proteins (Daniell *et al* 2001, Daniell *et*

al 2005). Reversible activation of genes that regulate expression in the chloroplast is the Protein Disulfide Isomerase (PDI) system composed of chloroplast polyadenylate-binding proteins that specifically bind to the 5'UTR of the *psbA* mRNA and are modulated by redox status through PDI (Kim and Mayfield, 1997). The ability of chloroplasts to form disulfide bonds and properly fold foreign proteins eliminates a major part of the costly downstream processing. The chloroplast expressed CTB-NSP4 fusion protein folded properly into functional pentameric form which was clearly seen on the immunoblot. The positive result in GM1 binding assay with CTB-NSP4 has reconfirmed the pentamer forms of CTB-NSP4 from transgenic tobacco chloroplasts.

Chloroplast transformants were distinguished from the nuclear transformants and mutants by PCR analysis. Southern blot analysis with gene specific CTB-NSP4 probe and flanking probe for chloroplast genome was done to confirm the site-specific integration of the gene cassette and also to determine the homo or heteroplasmy. High protein expression levels were obtained in the mature leaves after Day 1 of continuous light exposure of up to 2.4 % of the total soluble protein which was quantified using the GM1 binding assay.

The present study reports the successful expression of the CTB-NSP4 fusion protein as pentameric forms. This opens the doors for the expression of CTB-NSP4 in carrot plastids so as to enable oral delivery of the vaccine antigen. The immunogenicity of the vaccine antigen needs to be tested in an animal model which is underway.

Future Studies

Assessing the immunogenicity of the plant derived CTB-NSP4 vaccine antigen

The immunization experiments to test the functionality of the vaccine antigen *in vivo* are underway. Mice will be tested for the production of antibodies against the vaccine antigen CTB-NSP4. Once the antibody titers have been established, the studies can further be extended to pathogen challenge and the survival rates of the mice will be assessed.

Development of transgenic carrots expressing CTB-NSP4

Development of transgenic carrot expressing CTB-NSP4 will open the door for the oral delivery of the vaccine and develop mucosal immune response. An ideal vaccine for Rotavirus should induce both mucosal and systemic protection. If both subcutaneous and oral prove to be immunoprotective, priming both the mucosal and systemic systems may prove not only to be the cheapest way but also the most effective method of vaccination against any pathogen that attacks both the mucosal and systemic systems.

CONCLUSIONS

Currently there is no rotavirus vaccine included in national immunization systems. In USA a live orally administrated, tetravalent rhesus-human reassortant vaccine (RRV-TV; Rotashield) was licensed in 1998. But in 1999 Rotashield was withdrawn from the market due to intussusception associated with it. Intussusception is a problem with the intestine, wherein a portion of the bowel slides into the distal part leading to blockage and venous congestion which results in arterial obstruction, necrosis, perforation of bowel and possibly death (Kombo *et al* 2001). NSP4 enterotoxin of rotavirus being the major cause of infection has been chosen as the best candidate for rotavirus vaccine antigen. CTB-NSP4 fusion protein was expressed in transgenic potato but the levels of expression were only 0.006% to 0.026% (Kim *et al* 2003). The immunological studies performed on mice with CTB-NSP4 fusion protein from transgenic potato developed highest anti-NSP4 titer and the cytokine expression pattern clearly indicated a Th 1 lymphocyte mediated immune response (Yu *et al* 2001). Hence the expression of CTB-NSP4 fusion protein in tobacco chloroplast is highly advantageous as it results in low cost vaccine because of high levels of expression, and elimination of the cold-chain (low temperature, storage & transportation), hospitals and health professionals for their delivery (Daniell *et al.* 2005). Also, the technology exists to harvest and store plant produced proteins making the cost of production of recombinant proteins in tobacco leaves up to 10 to 50 times cheaper than that of *E. coli* fermentation (Kusnadi *et al.*1997).

The chloroplast integration of CTB-NSP4 was confirmed by PCR and homoplasmy was confirmed by Southern blot analysis. The expression of CTB-NSP4, fusion protein as a functional pentameric (135 kDa) form in transgenic chloroplasts was confirmed by Immunoblot analysis using rabbit anti NSP4₉₀ antibody. Maximum accumulation of the CTB-NSP4 protein up to 2.5% of tsp was observed in the mature leaves after Day 1 of continuous light exposure. The binding affinity of transgenic chloroplast expressed CTB-NSP4 to enterocytes GM1 ganglioside receptor was proved by GM1 ganglioside binding ELISA assay; mature leaves expressed CTB-NSP4 fusion protein to a maximum of 2.5 % in total soluble protein, 100-400 fold higher than earlier reports. Hence the high expression and successful assembly of CTB-NSP4 fusion protein into biologically active pentameric structures in tobacco chloroplast provides a valuable approach for targeting plant chloroplast synthesized vaccine antigen to mucosal immune system. Antibody titration and virus challenge experiments will be performed in mice to evaluate the antigenic and protective properties of the chloroplast derived CTB-NSP4 fusion protein.

LIST OF REFERENCES

- Arakawa T, Cong DKX, Langridge WHR. (1998) Efficacy of a food-plant based oral cholera toxin B subunit vaccine. *Nat Biotechnol.* 15:248-252. 16 292-297
- Bocci V. (1999) The oropharyngeal delivery of interferons: where are we and where do we need to go? *J Interferon Cytokine Res.* 19 (8): 859-61.
- Ball J M, Tian P, Zeng C Q Y, Morris A P, Estes M K (1996). Age dependent diarrhea induced by rotavirus nonstructural glycoprotein. *Science.* 272: 101-104.
- Bhati A. (2005) Expression of Hepatitis C viral non structural 3 protein in transgenic chloroplast Master's thesis, University of Central Florida.
- Bogorad, L. (2000). Engineering chloroplasts: an alternative site for foreign gene, proteins, reactions and products. *TIBTECH.* 18:257-263.
- Bowman D G, Nodelman M I, Levy O, Lin L S, Tian P, Zamb J T, Udem A S, Venkataraghavan B, Schutt E C. (2000) Crystal structure of the oligomerization domain of NSP4 from rotavirus reveals a core metal-binding site. *J Mol Biol* 304:861-871.
- Chebolu S. (2005) Expression of GAL/GALNAc lectin of *Entamoeba Histolytica* in transgenic chloroplast to develop a vaccine for amebiasis. Master's thesis, University of Central Florida.
- Cornielle S, Lutz K, Svab Z and Maliga P (2001) Efficient elimination of selectable marker genes from plastid genome by CRE-lox site specific recombination system. *Plant J.* 27: 171-178.
- Daniell, H. (1997). Transformation and foreign gene expression in plants mediated by microprojectile bombardment. *Meth Mol Biol.* 62:453-488.
- Daniell H. (2002) Molecular strategies for gene containment in transgenic crops. *Nat Biotechnol.* 20: 581-586.
- Daniell H. (2004) Medical molecular pharming: therapeutic recombinant antibodies, biopharmaceuticals and edible vaccines in transgenic plants engineered via the chloroplast genome. *Encyclopedia of Plant and Crop Science R.M. Goodman, ed. Marcel Decker, New York* 704-710.
- Daniell H, Carmona-Sanchez O, Burns BB. (2004b) Chloroplast derived antibodies,

- biopharmaceuticals and edible vaccines. In R Fischer and S Schillberg eds, *Molecular Farming* Weinheim: WILEY-VCH Verlag, pp.113-133.
- Daniell H, Chebolu S, Kumar S, Singleton M, Falconer R. (2005) Chloroplast-derived vaccine antigens and other therapeutic proteins. *Vaccine* 23:1779-1783.
- Daniell H, Cohill P, Kumar S, Dufourmantel N, Dubald M. (2004a) Chloroplast genetic engineering. H Daniell and C Chase, eds, *Molecular biology and biotechnology of plant organelles*, Kluwer Academic Publishers, Dordrecht, pp. 423-468.
- Daniell H, Datta R, Varma S, Gray S, Lee SB. (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nat Biotechnol.* 16: 345–348.
- Daniell H, Dhingra A. (2002) Multi gene engineering: dawn of an exciting new era in biotechnology. *Curr Opin Biotechnol.* 13: 136-141.
- Daniell H, Khan M, Allison L. (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci.* 7: 84–91.
- Daniell H, Krishnan M and McFadden BF (1991).Expression of β - glucuronidase gene in different cellular compartments following biolistic delivery of foreign DNA into wheat leaves and calli. *Plant Cell Rep.* 9: 615–619.
- Daniell H, Lee SB, Panchal T, Wiebe PO. (2001) Expression of cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *J Mol Biol.*311: 1001–1009.
- Daniell H, McFadden BA. (1987) Uptake and expression of bacterial and cyanobacterial genes by isolated cucumber etioplasts. *Proc Natl Acad Sci. USA*, 84: 6349-6353.
- Daniell H, Muthukumar B, Lee S B. (2001a) Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. *Curr Genet.* 39:109-116.
- Daniell H and Parkinson CL. (2003) Jumping genes and containment. *Nat Biotechnol.* 21:374-375.
- Daniell H, Ruiz ON, Dhingra A. (2004c). Chloroplast genetic engineering to improve agronomic traits. *Methods in Molecular Biology* 286: 111-137.
- Daniell H, Kumar S, Dufourmantel N (2005a) Breakthrough in chloroplast genetic engineering of agronomically important crops. *Trends Biotechnol.*23:238-45

- Daniell H, Streatfield SJ, Wycoff K. (2001b) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends Plant Sci.* 6:219-26.
- Daniell H, Vivekananda J, Nielsen BL, Ye GN, Tewari KK and Sanford JC. (1990). Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proc Natl Acad Sci. USA.* 87: 88–92.
- DeCosa B, Moar W, Lee SB, Miller M, Daniell H. (2001) Over expression of the *Btcry2Aa2* operon in chloroplasts leads to formation of insecticidal crystals. *Nat. Biotechnol.* 19: 71–74.
- DeGray G, Rajasekaran K, Smith F, Sanford J, Daniell H. (2001) Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. *Plant Physiol.* 127:852–862.
- Devine AL, Daniell H. (2004) Chloroplast genetic engineering for enhanced agronomic traits and expression of proteins for medical/industrial applications. In SG Møller, ed, *Plastids*, Vol. 13. Blackwell publishing, Oxford, pp. 283-323.
- Dong Y, Zeng C Q-Y, Ball J M, Estes M K, Morris A P. (1997) The rotavirus enterotoxin mobilizes intracellular calcium in human intestinal cells by stimulating phospholipase C-mediated inositol 1,4,5-triphosphate production. *PNAS* 94:3960-65.
- Fernandez-San Millan A, Mingeo-Castel AM, Miller M, Daniell H. (2003) A chloroplast transgenic approach to hyper-express and purify human serum albumin, a protein highly susceptible to proteolytic degradation. *Plant Biotechnol J.* 1: 71–79.
- Franco, M. A., and Greenberg, H. B. (1995). Role of B cells and Cytotoxic T Lymphocytes in clearance of and immunity to rotavirus infection in mice. *J. Virol.* 69: 7800–7806.
- Franco, M. A., and Greenberg, H. B. (1999). Immunity to rotavirus infection in mice. *J Infect Dis.* 179:466-469
- Giddings G, Allison G, Brooks D, Caryer A. (2000). Transgenic plants as factories for biopharmaceuticals. *Nat Biotechnol.* 18:1151-55.
- Golds, T. Maliga, P. and Koop, HU. (1993) Stable plastid transformation in PEG-treated protoplasts of *Nicotiana tabacum*. *Bio/Technology*, 11, 95-97

- Goldschmidt-Clermont M. (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of *Chlamydomonas*. *Nucl Acids Res.* 19: 4083–4089.
- Guda C, Lee SB, Daniell H. (2000) Stable expression of biodegradable protein based polymer in tobacco chloroplasts. *Plant Cell Rep.* 19: 257–262.
- Hagemann R. (2004) The sexual inheritance of plant organelles. H Daniell and C Chase, eds, *Molecular biology and biotechnology of plant organelles*, Kluwer Academic Publishers, Dordrecht, pp. 93-113.
- Hajdukiewicz P T, Gilbertson L, Staub J M (2001). Multiple pathways for Cre/lox mediated recombination in plastids. *Plant J.* 27: 161-170.
- Iatham S, Day A (2000) Removal of antibiotic resistance genes from transgenic tobacco plastids. *Nat Biotechnol* 18: 1172-6.
- Kapusta J, Modelska A, Figlerowicz M, *et al.* (1999) A plant-derived edible vaccine against hepatitis B virus. *FASEB J.* 13: 1796–1799.
- Kim T G, Langridge W H R. (2003). Assembly of cholera toxin B subunit full length rotavirus NSP4 fusion protein oligomers in transgenic potato. *Plant Cell Rep.* 21:884-890.
- Kim, J., Mayfield, S. (1997). Protein Disulfide Isomerase as a Regulator of Chloroplast Translational Activation. *Science.* 278:1954-1957.
- Kombo L A, Gerber M A, Pickering L K, Atreya C D, Breiman R F. (2001) Intussusception, Infection, and Immunization: summary of a workshop on rotavirus. *Pediatrics.* 108: 2.e37.
- Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y (2001)..Oral immunization with hepatitis B surface antigen expressed in transgenic plants. *Proc Natl Acad Sci.* 20:11539-44

- Koya V (2004) Evaluation of immunogenicity of transgenic chloroplast derived protective antigen of *Bacillus anthracis*. Master's thesis, University of Central Florida.
- Kumar S, Daniell H. (2004) Engineering the chloroplast genome for hyper-expression of human therapeutic proteins and vaccine antigens in recombinant protein protocols. *Methods Mol Biol.* 267: 365-383.
- Kumar S, Dhingra A, Daniell H. (2004a) Plastid-expressed *betaine aldehyde dehydrogenase* gene in carrot cultured cells, roots, and leaves confer enhanced salt tolerance. *Plant Physiol* 136: 2843-2854.
- Kumar S, Dhingra A, Daniell H. (2004b) Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. *Plant Mol Biol* 56:203-216.
- Kusnadi A, Nikolov G, Howard J. (1997) Production of recombinant proteins in plants: practical considerations. *Biotechnol Bioeng.* 56: 473–484.
- Lee SB, Kwon HB, Kwon SJ *et al.* (2003) Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. *Mol Breed.* 11: 1–13.
- Leelavathi, S. Gupta, N. Maiti, S. Ghosh, A. and Reddy, V.S. (2003) Overproduction of an alkali- and thermo-stable xylanase in tobacco chloroplasts and efficient recovery of the enzyme. *Mol Breed.* 11: 59-67.
- Leelavathi S, Reddy VS. (2003) Chloroplast expression of His-tagged GUS-fusions: a general strategy to overproduce and purify foreign proteins using transplastomic plants as bioreactors. *Mol Breed.* 11: 49-58.
- Maliga P. (2003) Progress towards commercialization of plastid transformation technology. *Trends Biotechnol.* 21: 20-28.
- McNeal M M, Rae M N, Ward R L. (1997). Evidence that Resolution of Rotavirus Infection in Mice Is due to Both CD4 and CD8 Cell-Dependent Activities. *J Virol.* 71:8735-42.
- Mirazimi A , Magnusson K E, Svensson L. (2003) A cytoplasmic region of the NSP4 enterotoxin of rotavirus is involved in retention in the endoplasmic reticulum. *J Gen Virol* 84: 875–883.
- Molina A, Herva-Stubbs S, Daniell H, Mingo-Castel AM, Veramendi J. (2004) High yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. *Plant Biotechnol. Journal* 2:141-153.

- Parashar U D, Bresee J S, Gentsch J R, Glass R I. (1998) Rotavirus. *Emerg Infect Dis* 4:561-570.
- Parashar U D, Hummelman E G, Bresee J S, Miller M A, Glass R I. (2003) Global illness and deaths caused by rotavirus disease in children. *Emerg Infect Dis* 9:565-572.
- Ray P, Malik J, Singh R K, Bhatnagar S, Bahl R, Kumar R, Bhan M K. (2003) Rotavirus Nonstructural Protein NSP4 Induces Heterotypic Antibody Responses during Natural Infection in Children. *J Infect Dis.* 187:1786–93.
- Ruelland, E., Miginiac-Maslow, M. (1999). Regulation of chloroplast enzyme activities by thioredoxins: activation or relief from inhibition? *Trends Plant Sci.* 4, 136-141.
- Ruf S, Hermann M, Berger I, Carrer H, Bock R. (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nat Biotechnol.* 19: 870–875.
- Ruiz ON, Hussein H, Terry N, Daniell H. (2003) Phytoremediation of organomercurial compounds via chloroplast genetic engineering. *Plant Physiol.* 132: 1344-1352.
- Sanford, J.C. Smith, F.D. and Russell, J.A. (1993) Optimizing the biolistic process for different biological applications. *Methods Enzymol.* 217, 483-509.
- Sambrook, Frish, Maniatis. Molecular cloning. A laboratory manual. 2nd edition. Cold spring harbor laboratory press.1989.
- Sidorov VA, Kasten D, Pang SZ, Hajdukiewicz PT, Staub JM. (1999) Technical advance: stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. *Plant J.* 19: 209–216.
- Singleton ML. (2003) Expression of CaF1 and LcrV as a fusion protein for development of a vaccine against *Yersinia pestis* via chloroplast genetic engineering. Master's thesis, University of Central Florida.
- Staub JM, Garcia B, Graves J. *et al.* (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nat Biotechnol.* 18: 333–338.
- Svab Z, Maliga P. (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc Natl Acad Sci USA.* 90: 913–917.
- Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM. *et al* (1998) Immunogenicity in humans of a recombinant bacterial-antigen delivered in transgenic potato. *Nat Med.*4: 607–609.

- Tackett CO, Mason HS, Losonsky G, Estes MK, Levine MM. *et al.* (2000) Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. *J Infect Dis.* 182: 302–305.
- Tackett CO, Pasetti MF, Edelman R, Howard JA, Streatfield S. (2004) Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn. *Vaccine.* 22:4385-4389.
- Tackett CO, Sztein MB, Losonsky GA, Wasserman SS, Estes MK (2003). Humoral, mucosal, and cellular immune responses to oral Norwalk virus-like particles in volunteers. *Clin immunology.* 108:241-247.
- Tregoning JS, Nixon P, Kuroda H, Svab Z, Clare S. *et al* (2003) Expression of tetanus toxin fragment C in tobacco chloroplasts. *Nucleic Acids Res.* 31: 1174-1179.
- Tian P, Ball J M, Zeng C Q Y, Estes M K.(1997) The rotavirus nonstructural glycoprotein NSP4 possesses membrane destabilization activity. *J. Virol.* 70:6973-81.
- Vargas Q T, Ruiz N R, Daniell H. (2005). Characterization of Heterologous Multigene Operons in Transgenic Chloroplasts: Transcription, Processing and Translation. *Plant Physiol Vol 138:* 1746-1762.
- Walmsley AM and Arntzen CJ. (2000) Plants for delivery of edible vaccines. *Curr Opin Biotechnology.* 11: 1179-1184.
- Watson J, Koya V, Leppla S, Daniell H.(2004) Expression of *Bacillus anthracis* protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop. *Vaccine* 22: 4374-4384.
- Xu A, Bellamy R A, Taylor A J. 2000) Immobilization of the early secretory pathway by a virus glycoprotein that binds to microtubules. 19:6465-6474
- Yu J, Langridge HR. (2001) A plant-based multicomponent vaccine protects mice from enteric diseases. *Nat Biotech.* 19: 548-552.
- Zerges W (2000) Translation in chloroplasts. *Biochimie* 82:583-601.
- Zerges W (2004) Regulation of translation in chloroplast. H Daniell and C Chase, eds, Molecular biology and biotechnology of plant organelles, Kluwer Academic Publishers, Dordrecht, pp. 347-383.