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Seethamahalakshmi Chebolu
University of Central Florida

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EXPRESSION OF GAL/GALNAc LECTIN OF *ENTAMOEBA HISTOLYTICA* IN TRANSGENIC CHLOROPLASTS TO DEVELOP A VACCINE FOR AMEBIASIS

by

SEETHAMAHALAKSHMI CHEBOLU
M.Sc. Botany, Osmania University, India, 2001.

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ABSTRACT

Amebiasis, also defined as invasive intestinal and extra intestinal amebiasis, is caused by *Entameoba histolytica,* an invasive protozoan parasite. World Health Organization (WHO) has reported that approximately 50 million people are infected each year causing an estimated 40 to 100 thousand deaths annually. *Entameoba histolytica* ranks only second to malaria as a protozoan cause of death. Amebiasis occurs world wide but people living in Central and South America, Africa and Asia are the majority to suffer from morbidity and mortality. The enteric parasite has no zoonotic reservoirs and insect vectors for its transmission and infects humans and non-human primates. Therefore, anti-amebic vaccine could completely eradicate the disease.

Entamoeba histolytica invades tissue and causes the disease in series of events. The disease is caused when the cyst form of the parasite is ingested with contaminated food or water. After excysting in the small intestine to form the trophozoite, the parasite adheres to the colonic mucus and epithelial cells through interaction of Gal/GalNAc lectin, an amebic surface adhesin with the host glycoconjugates. The parasite then secrets the proteolytic enzymes that disrupt the intestinal mucus and epithelial barrier facilitating tissue penetration. The trophozoite then kills the host epithelial and immune cells. Also, it resists the host's immune response causing the prolonged infection called the invasive amebiasis and causes colon or liver abscess. The symptoms include gradual onset of abdominal pain, diarrhea and bloody stools. Also, it can form cysts that are excreted with stools to start new cycle. The parasite recognition of the host glycoconjugates plays an

important role in the pathogenesis. Therefore, the Gal/GalNAc lectin could be a possible vaccine candidate.

The Gal/GalNAc lectin is composed of a 260-kDa heterodimer of disulfide-linked heavy (170 kDa) and light (35 kDa) subunits, which is non-covalently associated with an intermediate sub-unit of 150 kDa. The only recognized Carbohydrate recognition domain (CRD) was found in the heavy sub-unit. The CRD of the lectin is the potential target for colonization blocking vaccines and drugs. Preliminary studies have shown that the recombinant fragments of cysteine-rich region of LecA (lectin) containing the CRD (carbohydrate recognition domain) of the GalNAc lectin conferred protection against amebiasis. Therefore, production of LecA in plants using chloroplast genetic engineering would result in low cost vaccine because of high expression levels of vaccine antigens, and elimination of the cold-chain (low temperature, storage & transportation), hospitals and health professionals for their delivery.

The LecA protein was expressed in transgenic chloroplasts of *Nicotiana tabacum var. Petit havana* by transforming the chloroplast genome using the LecA gene (1755 bp) by homologous recombination. The pLD-CtV has *trn*I and *trn*A genes that are used as flanking sequences for homologous recombination and the constitutive 16s rRNA promoter to regulate transcription. The *aad*A gene conferring spectinomycin resistance has been used for selection and gene10 regulatory sequence from T7 bacteriophage to enhance translation. The chloroplast integration of LecA was confirmed by PCR and Southern blot analysis. The expression of LecA protein in transgenic chloroplasts was analyzed by immunoblot analysis using anti-LecA antibodies. Maximum expression

levels of LecA up to 6.3 % of the total soluble protein were observed in the old leaves. The evaluation of the immune response in animal model is underway. This is the first report of expression of LecA in a plant system.

ACKNOWLEDGMENTS

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INTRODUCTION

Background and significance of Amebiasis

Diarrheal diseases continue to be the major causes of morbidity and mortality in children in developing countries. For example, in Bangladesh 1 in 30 children dies of diarrhea or dysentery by his or her fifth birthday (Haque *et al*. 2003). In developed countries, microorganisms causing diarrheal diseases remain a major concern for their potential use as bioterrorist agents. Amebiasis is defined as an invasive intestinal amebiasis or extra intestinal disease and ranks only second to malaria as a protozoan cause of death. Amebiasis is caused by *Entameoba histolytica,* an enteric protozoan parasite. The World Health Organization estimates that there are 50 million cases of colitis and liver abscess annually and about 100,000 deaths each year from *Entamoeba histolytica* infection (Huston and Petri 1998, Petri *et al*. 2002, Dodson *et al.* 1998). The infection occurs throughout the world but occurs mostly in the developing countries of Central and South America, Africa and Asia. There is no zoonotic reservoir and no intermediate vectors required for its transmission. The life cycle of *Entamoeba* is simple with an infectious cyst and an invasive trophozoite.

There are two morphologically identical but genetically distinct organisms that have been reclassified by Diamond and Clark based on cumulative biochemical, immunological and genetic data: *Entamoeba histolytica*, that which causes invasive intestinal amebiasis and the non-pathogenic intestinal parasite, *Entamoeba dispar*

(Huston and Petri 1998). *Entamoeba histolytica,* is one of the most potent cytotoxic cells known, named by Schaudinn in 1903 for its ability to destroy human tissues (McCoy *et al*. 1994). Molecular phylogeny places Entamoeba on one of the lowermost branches of the eukaryotic tree, closest to dictyostelium and the unusual features of entamoeba include polyploid chromosomes, multiple origins of DNA replication, closely placed genes that lack introns and unique endocytic pathways (Haque *et al.* 2003).

The infection initiates when the cyst form of the parasite is ingested with contaminated food or water (Petri *et al*. 2002, Stanley Jr. 1997). The infective cyst form of the parasite survives passage through the stomach and the small intestine. The cyst is resistant to gastric acidity, chlorination, and desiccation, and can survive in moist environment for several weeks. The cysteine-rich composition of the surface antigens may be important for the survival of the amebae in such harsh environment. Excystation occurs in the bowel lumen where motile and invasive trophozoites are formed. The trophozoites use the galactose and N-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin to adhere to colonic mucins and thereby colonize the large intestine. Colitis results when the trophozoite penetrates the intestinal mucous layer, which acts as a barrier to invasion by inhibiting amebic adherence to the underlying epithelium and by slowing trophozoite motility. It secretes proteolytic enzymes that disrupt the intestinal mucus and epithelial barrier and facilitate tissue penetration. The trophozoite then kills the host epithelial and immune cells causing characteristic flask shaped ulcers. Finally, the parasite resists the host's immune response and survives to cause prolonged extra intestinal infection such as amebic liver abscesses (Huston and Petri 1998). *Entamoeba*

histolytica utilizes multiple non-specific and specific means to evade host defenses and survive within the gut and extra intestinal sites of infection.

The overall population of *E.histolytica* is clonal as the reproduction of trophozoites has no sexual cycle. In most infections, the trophozoites aggregate in the intestinal mucin layer and form new cysts resulting in asymptomatic infection. Cysts excreted in the stool perpetuate the life cycle by further fecal-oral spread. In some cases, however, once the intestinal epithelium is invaded, extra intestinal spread to the peritoneum, liver and other sites may follow. Patients with amebic colitis typically show several week history of cramping abdominal pain, weight loss and watery or bloody diarrhea. Amebic liver abscess is 10 times as common in men as in women (Haque *et al*. 2003). Approximately 80 percent of patients with amebic liver abscess develop symptoms relatively quickly (typically within two-four weeks), which include fever, cough, and a constant, dull, aching abdominal pain in the right upper quadrant or epigastrium. Associated gastrointestinal symptoms, which occur in 10-35 percent of patients, include nausea, vomiting, abdominal cramping, abdominal distention and diarrhea. Extrahepatic amebic abscesses have occasionally been described in the lung, brain and skin and presumably may result from hematogenous spread. Since amoebae only infect humans and some higher non-human primates, an anti-amebic vaccine could theoretically eradicate the disease.

Several virulence factors have been implicated in host cell death, including Gal/GalNAc lectin and pore-forming "amoebapore" proteins (Leippe 1997, Mann 2002). Parasite recognition of the host glycoconjugates plays an important role in the

pathogenesis of amebiasis. Amebic adherence and contact-dependent cytolysis of target cells is mediated by amebic galactose/N-acetyl-D-galactosamine-inhibitable adhesin (Mann and Lockhart 1998). The Gal/GalNAc lectin plays roles in the cytolytic activity of the parasite, in invasion and in resistance to lysis by complement. The Gal/GalNAc lectin was first purified by Petri using galactose affinity chromatography (Petri *et al*.1987). The Gal/GalNAc lectin is a heterodimer with disulfide linked heavy (170 kDa) and light (35/31 kDa) subunits, which are non-covalently associated with an intermediate subunit of 150 kDa (Mann and Lockhart 1998, Huston and Petri 1998, Mann 2002, Petri *et al*. 2002). The genes encoding the heavy and light subunits are members of multigene families consisting of five to seven members. The heavy (170kDa) subunit gene sequence contains amino-terminal 15-amino acid hydrophobic signal sequence, an extra cellular cysteine-rich domain of 1209 amino acids containing sites for N-linked glycosylation, and transmembrane and cytoplasmic domains of 26 and 41 amino acids, respectively (Petri *et al*. 2002). Anti-lectin monoclonal antibodies directed against the cysteine-rich extracellular domain inhibit adhesion of *Entamoeba histolytica in vitro* (Huston and Petri 1998). The light subunit is encoded by multiple genes encoding isoforms with different posttranslational modifications. The major 35- and 31-kDa isoforms have nearly identical amino acid compositions. The 35 kDa isoform is highly glycosylated and lacks the acylglycosylphosphotidylinositol (GPI) anchor present on the 31-kDa isoform (McCoy *et al.* 1993, Ramakrishnan *et al*. 2000). The function of the 35- and 31-kDa subunits remains unclear.

The carbohydrate recognition domain (CRD) was identified in the heavy subunit of the Gal/GalNAc lectin and it has been demonstrated that an adherence-inhibitory antibody response against this domain protects against amebic liver abscess in an animal model (Dodson *et al*. 1998). Therefore, the CRD of the Gal/GalNAc lectin is the potential target for colonization blocking vaccines and drugs. Preliminary studies have shown that the recombinant fragments of cysteine-rich region of lectin (termed "lecA") containing the CRD of the Gal/GalNAc lectin conferred protection against amebiasis (Houpt *et al*. 2004, Mann *et al*. 1993). This recombinant fragment (LecA) is used for expression in plants via chloroplast genetic engineering.

Amebiasis can be prevented by eradicating the fecal contamination of food and water. However, massive monetary investments are required in providing safe food and water in developing countries. Instead, an effective vaccine would be much less costly and is a feasible goal. An effective expression system to express the vaccine antigen so as to provide the vaccine in cleaner form and at low costs is absolutely necessary.

Chloroplast genetic engineering

The concept of chloroplast genetic engineering was first conceived in the mid 1980's with the introduction of isolated intact chloroplasts into protoplasts (Daniell and Dhingra 2002). Later focus was laid on the development of chloroplast systems capable of efficient, prolonged protein synthesis and the expression of foreign genes (Daniell and McFadden 1987). The biolistics method of transformation has made it feasible to

transform plastids without the need to isolate them (Klein *et al.*1987). The first successful

chloroplast genome complementation was reported in 1998 for the unicellular green alga

having single chloroplast, *Chlamydomonas reinhardtii* (Boynton *et al*. 1988). For this,

the photosynthetically incompetent mutants that lacked *atpB* gene and chloroplast ATP

synthase activity were used. The wild-type *atpB* gene was introduced into the cells using

tungsten microprojectiles coated with the atp gene (Klein *et al*. 1987). The single large

chloroplast provided an ideal target for DNA delivery. Restoration of photoautotrophic

growth upon selection in the light demonstrated that the deletion mutant phenotype was

corrected with the introduction of wild type atp-B gene into the cells. After the successful

transformation of the unicellular algae *C. reinhardtii* through chloroplast genome, efforts

were made to transform the higher plants through chloroplast genome. Initially in higher

plants, foreign genes were introduced and expressed only in isolated but intact plastids

(Daniell and McFadden 1987). The first expression of a foreign gene in plastids of

cultured tobacco cells used autonomously replicating chloroplast vectors (Daniell *et al*.

1990). This work was repeated in wheat leaves, calli and somatic embryos (Daniell *et al*.

1991). Simultaneously, the *C. reinhardtii* chloroplast genome was transformed with the

*aad*A gene conferring spectinomycin or streptomycin resistance (Goldschmidt- Clermont

1991). This became a major breakthrough because the majority of higher plants

genetically transformed via the chloroplast genome now use aadA gene as selectable

marker. Stable integration of the *aad*A gene into the tobacco chloroplast genome was

then demonstrated (Svab and Maliga 1993). Initially, when the transgenes were

introduced via the chloroplast genome, it was believed that foreign genes could be

inserted only into transcriptionally silent spacer regions of the chloroplast genome (Zoubenko *et al.* 1994). However, Daniell *et al.* (1998) advanced forward the concept of inserting transgenes into functional operons and transcriptionally active spacer regions. This approach facilitated the insertion of multiple genes under the control of a single promoter, enabling the coordinated expression of transgenes (Daniell and Dhingra 2002). The trnI-trnA intergenic region is transcriptionally active because of the read-through transcription of the upstream 16S rRNA promoter capable of transcribing six native genes downstream (Dhingra *et al.* 2004).

Tobacco, a non-food/ feed crop is proven to be ideal for transformation because of its ease for genetic manipulation. Tobacco is an excellent biomass producer (in excess of 40 tons fresh leaf weight/acre based on multiple mowings per season) and a prolific seed producer (up to one million seeds produced per plant), which is ideal for the large-scale commercial production. It has been extensively used for the large-scale production of therapeutic proteins. It has been estimated that the cost of production of recombinant proteins in tobacco leaves will be 50-fold lower than that of *Escherichia coli* fermentation systems (Kusnadi 1997). Using the chloroplast transformation, tobacco has been used for hyper-expression of vaccine antigens and production of valuable therapeutic proteins like human elastin-derived polymers for various biomedical applications (Guda *et al.* 2000), vaccines antigens for cholera, anthrax, plague and tetanus (Daniell *et al.* 2004d), monoclonal antibody Guy's 13, a monoclonal antibody against *Streptococcus mutans* and which protects against dental carries has also been expressed in the chloroplast (Daniell *et al.* 2001b). Human therapeutic proteins, including human

serum albumin (Fernandez *et al*. 2003), magainin, a broad spectrum topical agent, systemic antibiotic, wound healing stimulant and a potential anticancer agent (DeGray *et al*. 2001), interferon (Daniell *et al*. 2004a) and insulin- like growth factor (Daniell 2004) have been expressed. Several other laboratories have expressed other therapeutic proteins, including human somatotropin (Staub *et al*. 2000) and interferon-GUS fusion proteins (Leelavathi and Reddy 2003) in transgenic chloroplasts.

The chloroplast transformation technology has been used to introduce various agronomic traits. Insect resistance has been achieved by expressing insecticidal proteins from *Bacillus thuringiensis* (Bt); the Cry2Aa2 protein had accumulated up to 46.1% tsp and this is by far the highest expressed foreign protein in transgenic plants to date (DeCosa *et al*. 2001). Similarly herbicide resistance against Glyphosate, a broad spectrum herbicide that non-selectively kills the weeds by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a nuclear-encoded chloroplast localized enzyme in the shikimic acid pathway of plants and microorganisms that is required for the biosynthesis of aromatic amino acids has been achieved. The plastid that was engineered with *EPSPS* gene in tobacco plants developed resistance to glyphosate over the wild type plants (Daniell *et al*. 1998). The antimicrobial peptide MSI-99, an analog of magainin has been expressed in the chloroplast genome of transgenic tobacco up to 21.5% TSP (DeGray *et al*. 2001). MSI-99, offers protection against prokaryotic organisms due to its high specificity for negatively charged phospholipids found mostly in bacteria. Extracts from MSI-99 transformed plants inhibited growth of *Pseudomonas aeruginosa*, a multi-drug resistant bacterium, which acts as an opportunistic pathogen in plants, animals, and

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humans. It is also biologically active against *Pseudomonas syringae*, a major plant pathogen (DeGray *et al*. 2001, Devine and Daniell 2004, Daniell *et al*. 2004a). The yeast trehalose phosphate synthase (TSP1) gene expressed in the chloroplast showed a high degree of drought tolerance (Lee *et al*. 2003).

Different biomaterials, enzymes and amino acids also were expressed in the transgenic chloroplasts. For example, xylanase which is an industrially important enzyme when expressed through the nuclear transgenic plants showed cell wall degradation and affected plant growth. But when was expressed via the chloroplast, there were no such effects seen as in nuclear transgenics (Leelavathi *et al*. 2003, Daniell *et al*. 2004d). Polyhydroxy butyrate is polyester used as biodegradable plastic. It has three bacterial genes encoding for the operon and the operon was expressed via chloroplast genome (Lossl et al. 2003). Significant levels of expression of the polymer were achieved. Chloroplast genetic engineering technology is 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 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*. 2004d).

For the first time carrot plastid genome has been transformed using the non-green tissue as explants and regenerated via somatic embryogenesis (Kumar *et al.* 2004a). A useful plant trait (salt tolerance) has been expressed for the first time in a non-

solanaceous crop via the 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 calli obtained from cultured cells expressing BADH were green in color in the absence of selection and the untransformed cells were yellow. Somatic embryos of carrot are single cell derived and multiply through recurrent embryogenesis, which provides uniform source of cell culture and homogeneous single source of origin. Carrot is ideal 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.

Also, Kumar *et al*. have recently transformed the cotton plastid genome. The transgenic seeds obtained were resistant to kanamycin selection whereas the untransformed seeds were not (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*. 2004d).

Vaccine antigens Expressed Via the Chloroplast Genome

Expressing vaccine antigens via the chloroplast genome has proven to be advantageous: subunit vaccines are not toxic even when expressed at high levels, bacterial genes have high AT content allowing for high expression in the chloroplast; 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.* 2001a), 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), 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 GM_1-ganglioside binding assay which indicates proper folding and formation of disulfide bonds to form pentamers (Daniell *et al.* 2001a, 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 immunogenecity 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 for the oral delivery of vaccine antigens and protection of immunogens in the Gut

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. Inspite of lower expression in tobacco these antigens were immunogenic (Haq *et al.* 1995, Mason *et al.*

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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).

Advantages of Chloroplast Expression Over Expression in *E. coli*

Chloroplast expression system has several advantages over *E. coli* expression system. Production of recombinant proteins in microorganisms is expensive, requires stringent purification protocols, and scale-up requires building costly fermenters. Vaccine production in plants can circumvent these problems. First, farming of plants is straightforward, fairly inexpensive, and can be scaled-up at low cost in one season. The

cost of production of recombinant proteins in tobacco leaves will be 10 to 50 times cheaper than that of *E. coli* fermentation (with 20% expression levels in *E. coli*, Kusnadi *et al*. 1997). Second, plants provide a heat-stable environment for proteins, and the technology already exists for harvesting, storage, and purification of transgenic plant proteins. In addition, each transgenic plant generated can produce up to a million seeds per plant. Third, chloroplasts are capable of folding proteins and maintaining their natural conformation. Folding, assembly and production of disulfide bridges for CTB has already been demonstrated in chloroplasts (Daniell 2001a). Binding assays proved that chloroplast synthesized CTB binds to the GM-1 ganglioside receptor. Many antigens for vaccines that have been expressed in chloroplasts have been proven to be immunogenic (Tregoning *et al*. 2003, Molina *et al*. 2004). Lastly, the process of purification of a recombinant protein produced in *E. coli* is costly and time consuming. For example, for insulin production, chromatography accounts for 30% of the production cost and 70% of the set-up cost (Petrides *et al*. 1995). A transformed tobacco plant would still need purification. But an edible vaccine such as in the carrot or tomato would eliminate this cost. Expenses for delivery by injection would also be eliminated. Therefore, expression of LecA in the carrot plastids for oral vaccination would be beneficial. The transgenic chloroplast derived LecA will have to be tested to prove its functionality *in vivo*. This work would pave the way for the oral delivery vaccine in carrot.

Advantages of Chloroplast Transformation Over Nuclear Transformation

Cross Pollination with pollen from Genetically Modified crops with the wild type crop is a major concern. However, by genetically engineering the chloroplasts, the possibility of cross-pollination with pollen carrying transgenes is eliminated because chloroplast DNA is maternally inherited. Although pollen from plants was shown to exhibit maternal plastid inheritance through metabolically active plastids, the plastid DNA itself is lost during the process of pollen maturation and hence is not transmitted to the next generation. During fertilization, the paternal chloroplasts from pollen are disintegrated in a synergid cell and only the sperm nucleus enters the egg cell and fuses with the egg to form a zygote. Maternal inheritance thus offers the advantage containment of chloroplast transgenes due to lack of gene flow through pollen (Daniell 2002, Daniell and Parkinson 2003). The transgene inheritance thus occurs only via seeds. This environmental eco-friendly feature should eliminate all the concerns of cross contamination with wild type and relative crops. In order for plant production of proteins to be commercially feasible, expression levels greater than 1 % of the total soluble protein must be achieved in plants (Kusnadi *et al*. 1997). Nuclear transformation of plants has usually produced lower expression levels of antigens (Daniell *et al*. 2001a, May *et al*. 1996, Richter *et al*. 2000, Tacket *et al*. 2000, Ramirez *et al*. 2003, Devine and Daniell 2004, Daniell *et al*. 2004b, Daniell *et al*. 2005). For example, plant derived recombinant hepatitis B surface antigen was as effective as a commercial recombinant vaccine, but the levels of expression in transgenic tobacco were low (0.0066% of total soluble protein).

For an oral delivery vaccine, it may prove to be extremely important to have high expression levels in order to elicit the immune response. An alternative approach to nuclear transformation is to integrate foreign genes into the chloroplast genome, which is a powerful technique because of the number of copies of chloroplast genomes per cell (up to 10,000). This high polyploidy leads to high transcript levels and finally accumulation of abundant translated product resulting in high expression levels up to 47% of tsp (DeCosa *et al*. 2001). Chloroplast transformation occurs exclusively through site-specific homologous recombination. In contrast, nuclear transformation experiments frequently suffer from gene-silencing mechanisms resulting in unstable and inconsistent gene expression or complete loss of transgenic activity. The nuclear genome has mechanisms that may inactivate genes when regulatory sequences are inserted in a repetitive pattern. This occurs because integration of transgenes into the nuclear genome is random and not via homologous recombination (Daniell and Dhingra 2002). This random integration of transgenes may allow for insertion of the transgene into a region of the nuclear genome that is not highly transcribed. Due to the random position of the transgene in the nuclear genome, expression levels vary in different transgenic lines. However, neither gene silencing nor position effects have been observed in genetically engineered chloroplasts (Daniell and Dhingra 2002). Chloroplast transgenic lines with the accumulation of transcripts 169-fold higher than nuclear transgenic lines have shown no gene silencing (Lee *et al*. 2003, Dhingra *et al*. 2004). Likewise, chloroplast transgenic lines showed no transgene silencing at the translational level regardless of accumulation of foreign protein up to 47% TSP (DeCosa *et al*. 2001). Besides, the chloroplast genetic engineering offers

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attractive advantages of introducing multigenes in a single transformation step because of its ability to transcribe the operon with multigenes into polycistronic mRNA and translate the polycistronic mRNA (Ruiz *et al*. 2003). This saves a lot of time to create a transgenic plant expressing multigenes as opposed to the nuclear transformation where several independent transgenic lines have to be created followed by the laborious repetitive breeding (DeCosa *et al*. 2001, Daniell and Dhingra 2002, Lossl *et al*. 2003).

Advantages of Producing the Amebiasis Vaccine antigen in Plant Plastids

1. There are no known human or animal pathogens that affect plants (Streatfield *et al*. 2001), production of LecA protein in plants would yield a vaccine free of human pathogens.

2. The LecA gene has an A/T content of 67%, which is ideal for chloroplast expression of the protein. Because chloroplasts are prokaryotic in nature they tend to exhibit higher expression with higher AT content genes.

3. Bacterial proteins have been expressed at extraordinarily high levels in transgenic chloroplasts. This includes AT rich proteins such as Cry2a (67% AT) at 47% Total Soluble Protein (DeCosa *et al*. 2001), CTB (59% AT) at 33% TSP (Daniell *et al*. 2001a), and Human Serum Albumin (66% AT) up to 11.1% TSP, (Fernandez-San Millan *et al*. 2003). Disulfide bonds in the above examples were properly formed.

4. The LecA antigen is not glycosylated, which is good for our particular system because plastids do not glycosylate proteins.

5. The technology to sow, harvest, store, and transport crops already exists.

6. Plants have up to 100 chloroplasts per cell, each containing about 100 chloroplast genomes. This provides up to 10,000 genomes per cell to efficiently produce the antigens.

7. Using the chloroplasts eliminates cross-pollination of the transgene by pollen because chloroplast's DNA is maternally inherited in most of the plants (Daniell 2002, Daniell and Parkinson 2003).

8. Transgene integration is specifically targeted to intergenic spacer sites in the chloroplast genome; this eliminates gene silencing and the position effect that is seen in nuclear transformation (Daniell *et al*. 2002, Daniell and Dhingra 2002).

9. AT rich CTB has been produced in transgenic chloroplasts in our lab and has been shown to have native conformation via the GM_1-ganglioside binding assay. It was produced 410 and 3100-fold higher in transgenic chloroplasts than nuclear transgenic plants at 4.1 and 31% total soluble protein (Daniell *et al*. 2001a, Monila *et al*. 2004).

10. Vaccine antigens have already been expressed in plants and have shown to be protected in the stomach through bioencapsulation. Also, they have been proven immunogenic when administered orally in clinical trials (Tacket *et al*. 1998, Kapusta *et al*. 1999, Tacket *et al*. 2000, Castanon *et al*. 2000, Tuboly *et al*. 2000, Walmsley and Arntzen 2000, Tacket *et al*. 2003, Tacket *et al*. 2004).

11. Using plastid transformation technology, large quantities of vaccine can be produced.

12. Proving that this vaccine is immunogenic when produced in tobacco chloroplasts could justify the expense to engineer oral vaccines by transforming plastids of carrot. Delivery of plant-derived vaccine to mucosal tissues has been proven to induce both a

mucosal and a systemic immune response (Haq *et al.*1995, Mason *et al.*1996, Arakawa *et al.* 1998).

Rationale and Approach

The objective of this project is to express the surface antigen of *E. histolytica* in plants using the chloroplast expression system to develop a low cost vaccine for amebiasis. Chloroplast expression system has several different advantages over nuclear transformation which include high expression levels, 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. Therefore, we went ahead and planned to express the vaccine antigen in chloroplast expression system. For this, the LecA gene was modified using the Polymerase chain reaction to create the start and stop codons using the primers as described in Materials and Methods. The LecA gene was then cloned into the universal chloroplast transformation vector, pLD-Ctv which has flanking regions from the tobacco genome so as to enable homologous recombination. The gene10 regulatory sequence from T7 bacteriophage was cloned upstream of LecA to enhance the translation. The chloroplast derived LecA 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)

The ultra competent cells were prepared using rubidium chloride method (http://www.nwfsc.noaa.gov/protocols-/rbcl.html). The ultra competent cells are absolutely necessary for the transformation of bacterial cells with the plasmid. *E.coli* XL1-Blue MRF ab (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 *E.coli* glycerol stock was streaked on the LB agar plate containing 12.5 µg/ml tetracycline and incubated at 37 °C overnight. Single isolated colony was picked and grown in 5 ml of Psi broth 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 in 100 ml of Psi broth and was incubated at 37 °C for about 2 hours in a shaker at 225 rpm. The optical density (O.D) was checked at 550 nm after two hours and subsequently after each half hour or an hour depending on the O.D value. The culture was continued to grow until it reaches to 0.48 O.D. The culture was kept on ice for 15 minutes. The cells were pelleted by centrifugation at 3000g/5000 rpm for 5 minutes in a sorvall centrifuge. 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 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 frozen in dry ice/liquid nitrogen and stored at - 80 °C.

Transformation of the Competent *E. coli* XL1-Blue cells

The competent cells of one hundred µl aliquot were taken from -80 °C and immediately thawed on ice and transferred to a falcon tube. About one µl (100 ng) of plasmid DNA was added to the competent cells and was mixed by gentle tapping. The mixture was incubated on ice for 30 minutes with gentle tapping at after first 15 min. Then, the mixture was incubated at 42° C in a water bath for 90-120 seconds and then immediately put on ice for two minutes. Approx. 900µl of LB broth was added to cells and were incubated at 37° C for 45 minutes in a horizontal shaker at 225 rpm. The cells were pelleted by spinning at 13,000 rpm for 30 seconds. The eight hundred µl of supernatant was discarded leaving 100µl, followed by resuspending 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

A single isolated colony was picked from the LB agar plate and was grown in LB medium 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. One µl of RNase (100 mg/ml) was added to each tube and pulse vortexed. 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 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 dH_2O) was added and mixed by gently inverting 6 times. The mixture was centrifuged at 13,000 rpm for 10 minutes at 4 °C. The supernatant was transferred into a fresh, eppendorf tube taking care to exclude white debris (which is bacterial chromosomal DNA/SDS/membrane proteins). Then, 900 µl of ice-cold ethanol (95%) was added to the supernatant to precipitate the plasmid DNA. The supernatant was centrifuged at 13,000 rpm at 4 °C for 10 minutes. 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

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were run on a 0.8% agarose gel for 40 minutes at 80 volts to confirm the successful isolation of plasmid (Sambrook *et a*l. 1989).

Construction of pLD-g10-LecA vector for transformation of tobacco Chloroplast

Polymerase Chain Reaction was used to introduce start and stop codons at the N-terminal and C-terminal of the LecA gene respectively using primers forward 5'-GGAATTGAATTCCATATGTGTGAGAACAGA-3' and reverse 5'-AGAATTGCCTCTAGACTATTCTGAAAC-3' (Invitrogen). The plasmid pcDNA 3.1-LecA was used as the template and the PCR amplified product of approximately 1.7 kb containing Nde I restriction site the 5' end and Xba I at the 3' end is obtained. For a 50 μl reaction volume, the PCR was set as follows: 100ng of plasmid DNA, 5 μl of 10X Pfu buffer, 5 μl of 2.5 mM dNTP, 1 μl of each 10mM primer, 0.5 μl Pfu DNA polymerase and H$_2$O to make up the final volume. The PCR amplification was carried for 30 cycles as following: 94 °C for 45 sec, 50°C for 45sec, and 72 °C for 45sec. Cycles were preceded by denaturation for 5 min at 94 °C and followed by a final extension of 7 min at 72 °C. The 5 μl of each PCR product including the controls were loaded onto a 0.8% gel to analyze the PCR product. The PCR product was purified using PCR purification kit (Qiagen). The PCR product was cloned into TOPO vector. The PCR product was digested from TOPO vector with NdeI and NotI and cloned into p-bluescript containing gene10 T7 bacteriophage UTR. Then the final product containing the gene10 and the

LecA PCR product (approximate size 1.8 kb) are digested with HincII and NotI and cloned into tobacco universal vector pLD-Ctv between EcoRV and NotI.

Bombardment of the pLD-gene10-LecA 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. To pellet the gold particles, a quick centrifugation was done. The supernatant was discarded and 1ml of dH_2O was added to the particles and vortexed. The particles were allowed to settle for 1 minute and then a pulse centrifugation was performed for 3 seconds, the supernatant was discarded. The previous step was repeated three times. 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. Six grams per liter of phytagar was added to the media and autoclaved followed by cooling down and pouring into plates. 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).

Bombardment protocol for tobacco leaves

The bombardment media was prepared as described previously (Daniell 1997). For the bombardment, it is most important to maintain the aseptic conditions. 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 was placed in a micro centrifuge tube and 10 µl of DNA (1 µg/µl) 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 ul 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

The leaves, after two days incubation period, were transferred to 100 x 25 petri dish with RMOP media containing 500 µg/ml of spectinomycin final concentration as explained by Daniell, 1997. After four to six weeks, the shoots that appeared were cut in 5mm^2 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. a*l. 2001a) and to confirm the integration of gene of interest PCR was performed using primer pairs 5P (5'-CTGTAGAAGTCACCATTGTTGTGC-3') and 2M (5'-GACTGCCCACCTGAGAGC-

GGACA-3') (Daniell *et al*. 2001a). 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 H_2O 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 T_0 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 dH$_2$O 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.

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 Havan*a) 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-gene10-LecA) with BglII and PvuII. The 400 *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 dH$_2$O 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

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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, H_2O, 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 dH_2O. 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 H_2O was used. The gene specific probe was made by cutting out the 400bp fragment from pLD-

gene10-LecA in a reaction as follows: 2μg (5μl) of pLD-gene10-LecA, 2 μl of 10X buffer, 1 μl each of BglII and PvuII (NEB) and 11 μl of dH$_2$O. The reaction was incubated at 37 °C for 3 hrs. The fragment of interest (400bp) was eluted from the agarose gel and checked for the concentration on the spectrophotometer.

Probe labeling

The probes were prepared by the random primed ^{32}P-labeling (Ready-to-go DNA labeling beads, Amersham Pharmacia). For this, the probes denaturation (45 μl of the DNA) was made 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 Quant tm G-50 micro columns (Amersham) and mixed by flicking. 5 μl of α ^{32}P 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 μl was mixed with 98 μl of STE buffer. The mixture was aliquoted into 50 μl 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

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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/μl, and then multiplied by 50 μl of total volume of sample for a total of 35×10^6 cpm/μl. 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 μl 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 μl 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. col*i, XL1-blue cells that have tetracycline resistance gene in their genome, were transformed with pLD-gene10-LecA and were grown in 5 ml of Terrific broth with ampicillin (100 µg/µl) and tetracycline (50 µg/µl) 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 µl 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 Na_2HPO_4, 1.8mM KH_2PO_4, 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 dH_2O 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 LecA in tobacco plants, extracts were made from *Petit havana* plants. These steps were performed as described in (Daniell *et al.*, 2004c). Approx. 100 mg 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 LecA protein expression in the crude extract obtained from *E. coli* cells or crude extract obtained from the transgenic plant tissue extract transformed with pLD-gene10-LecA, 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 dH$_2$O 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 dH$_2$O 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 dH$_2$0 and 100 µl of 10% SDS to a 50 ml flask. Added 50 µl of 20% APS (#8 above) and then 10 µl of TEMED and used to cast the gel mixture between the two, vertical, glass plates (Mini-Protean 3 Cell gel system, Bio-Rad) leaving about 1.5 cm at the top of glass plates for the stacking gel. The gel is allowed to polymerize for 20 minutes. To make the 4% stacking gel, 1.3 ml of 30% Acrylamide/Bis, 2.5 ml of the stacking buffer, 6.1 ml dH$_2$0 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 LecA 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 dH_2O). For Western blotting, the proteins were transferred to nitrocellulose membranes and then blocked for one hour in P-T-M (PBS [12 mM Na_2HPO_4, 3.0 mM NaH_2PO_4-H_2O, 145 mM NaCl, pH 7.2], 0.5% Tween 20, and 3% Dry Milk) followed by transfer to P-T-M containing goat anti-lecA antibody. Membranes were then washed with distilled water and transferred to P-T-M containing rabbit derived anti-goat IgG antibody conjugated with Horseradish peroxidase (Sigma, 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.

Enzyme Linked Immuno Sorbant assay (ELISA)

The quantification of LecA in the plant crude extract was done using the enzyme linked immunosorbent assay (ELISA). 100mg of transgenic leaf samples (young, mature, old) and the wild type leaf samples (young, mature, old) were collected. The young

leaves are small, tender from the top of the plant, mature are dark, large leaves from the middle and old are the bleached leaves at the bottom. The leaf samples were collected from plants exposed to regular lighting pattern (16 h light and 8 h dark). 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 Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, 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 Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, pH 9.6). The standards ranging from 100 to 1000 ng/ml were made by diluting purified LecA in coating buffer. The standards and protein samples (100 µl) were coated to 96-well polyvinyl chloride microtiter plate (Cellstar) for 1 h at 37°C followed by 3 washes with PBST and 2 washes with water. Blocking was done with 3% fat-free milk in PBS and 0.1% Tween and incubated for 1h followed by washing. The primary goat anti-LecA antibody (provided by Dr. Mann, Univ. of Virginia) diluted (1:2000) in PBST containing milk powder was loaded into wells and incubated for 1h followed by washing steps and then again incubated with 100 µl of anti-goat IgG-HRP conjugated antibody made in rabbit (American Qualex) (1: 5000) diluted in PBST containing milk powder. The plate was then incubated for 1h at 37 °C. After the incubation the plate was washed thrice with PBST and twice with water. The wells were then loaded with 100 µl of 3,3,5,5-tetramethyl benzidine (TMB from American Qualex) substrate and incubated

for 10-15 min at room temperature. The reaction was terminated by adding 50 µl of 2N sulfuric acid per well and the plate was read on a plate reader (Dynex Technologies) at 450 nm. (Modified form of protocol from Ausubel *et a*l., 4 [th] edition).

Bradford assay for protein quantification (Bio-Rad manual).

The Bradford assay was used to determine the total protein from the plant extracts prepared as described above. This was used to determine the percent of LecA 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 Na_2CO_3, 35 mM $NaHCO_3$, 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-gene10-LecA vector for tobacco chloroplast Transformation

The start and stop codons were introduced to the LecA gene along with NdeI and XbaI using the Polymerase Chain Reaction. PCR amplified product of approximate size 1.7 kb was obtained with NdeI site at the 5' terminal and XbaI site at the 3' terminal by performing the site directed mutagenesis. The PCR product contains NdeI site, start codon, LecA gene sequence and stop codon as shown in fig.1A. The PCR product was purified using the purification kit (Qiagen). The PCR product was TOPO cloned and the transformed colonies were selected by the blue white selection. Most of the colonies contained the desired plasmid ligated with the PCR product. The plasmid pCR 2.1-LecA was isolated using miniprep and the PCR product was sequenced. The PCR product sequence was compared to the sequence sent by Dr. Barbara Mann. The PCR product was then digested with NdeI and NotI and cloned into p-bluescript containing the gene10 as shown in fig. 1B and named as p-bluescript-gene10-LecA (gene 10 5' UTR is a regulatory sequence from T7 bacteriophage and is used to enhance the translation). The p-bluescript containing the gene10-LecA was then digested with HincII and NotI and was cloned into EcoRV and NotI sites of the universal chloroplast transformation vector, pLD-Ctv designated as pLD-SC as shown in fig.1C. The pLD vector contains the homologous recombination sequences (flanking sequences) that allowed the homologous

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recombination of the gene cassette (*aadA*, gene10-LecA) in between the *trnI* and *trnA* of the chloroplast genome (Daniell *et al*. 2001a). Downstream to the trnI, the vector provided the constitutive 16S rRNA promoter, which regulates the expression of *aadA* gene (aminoglycoside 3' adenyltransferase) that confers resistance to spectinomycin-streptomycin and the gene10-LecA gene encoding the *Entamoeba histolytica* lectin antigen (Goldschmidt-Clermont 1991). Upstream to the *trnA*, the vector contains the 3'UTR which is a transcript stabilizer derived from psbA gene.
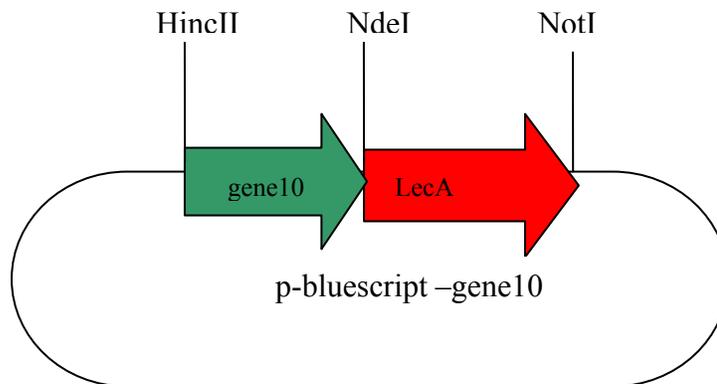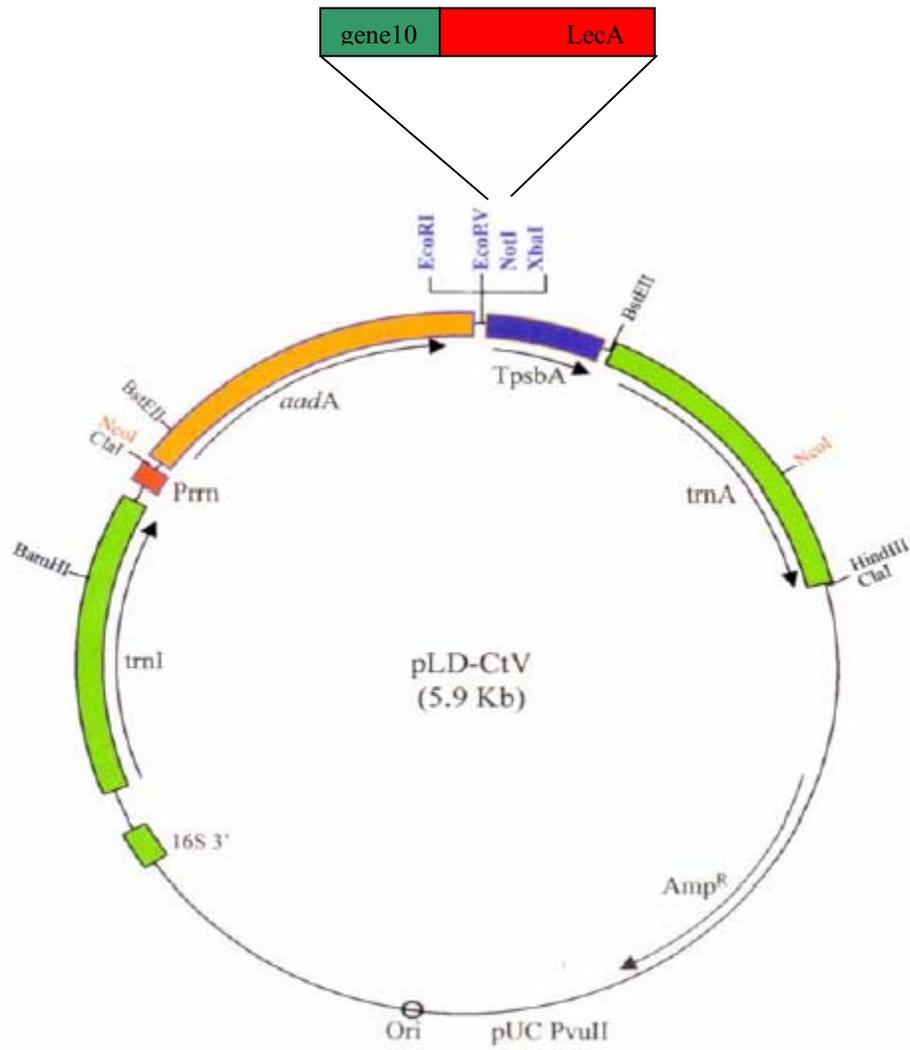
Fig. 1A
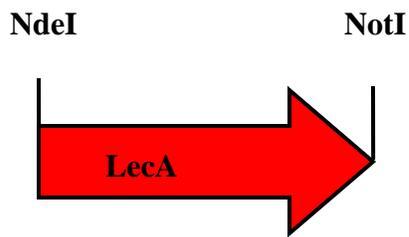
Fig.1B

Fig.1C

Figure 1. Schematic representation of the cloning of pLD-gene10-LecA.

A: PCR product showing the NdeI and Not I sites.
B: Cloning of the PCR product in p-bluescript using NdeI and NotI sites.
C: Cloning of LecA with gene10 from p-bluescript in pLD-Ctv using EcoRV and NotI sites (pLD-SC).

PCR Analysis of Confirmation of Chloroplast Integration of transgenes

After bombardment of tobacco leaves with gold particles coated with plasmid DNA (pLD-gene10-LecA), about 5 shoots/ plate appeared after a period of 5-6 weeks. There were 3 possibilities for obtaining shoots on the selection media: 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. The *aadA* gene in the gene cassette confers resistance to spectinomycin. True chloroplast transformants were distinguished from nuclear transformants and mutants by PCR. Two primers, 3P and 3M were used to test for chloroplast integration of transgenes (Daniell *et al*. 2001a). 3P primer landed on the native chloroplast DNA in the 16S rRNA gene. 3M landed on the *aadA* gene as shown in fig 2A. 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 2B.

The Integration of the *aadA*, gene10 -LecA gene and 3'psb*A*, 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 2A (Daniell *et al*. 2001a). The product size of a positive clone is of 3.3 kb for LecA, while the mutants and the control do not show any product. Figure 2C 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 to obtain a mature plant and reach homoplasmy.
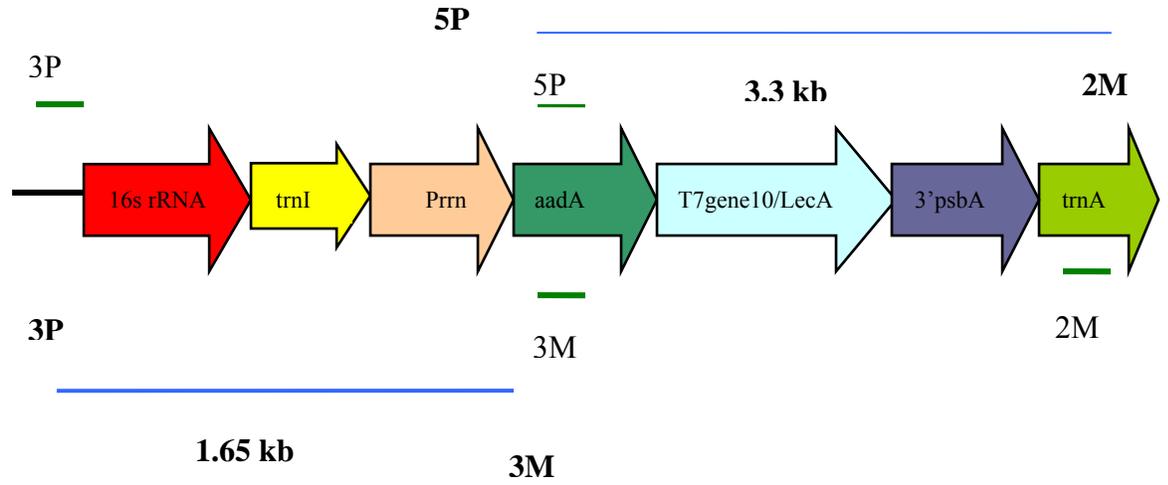
Fig. 2A

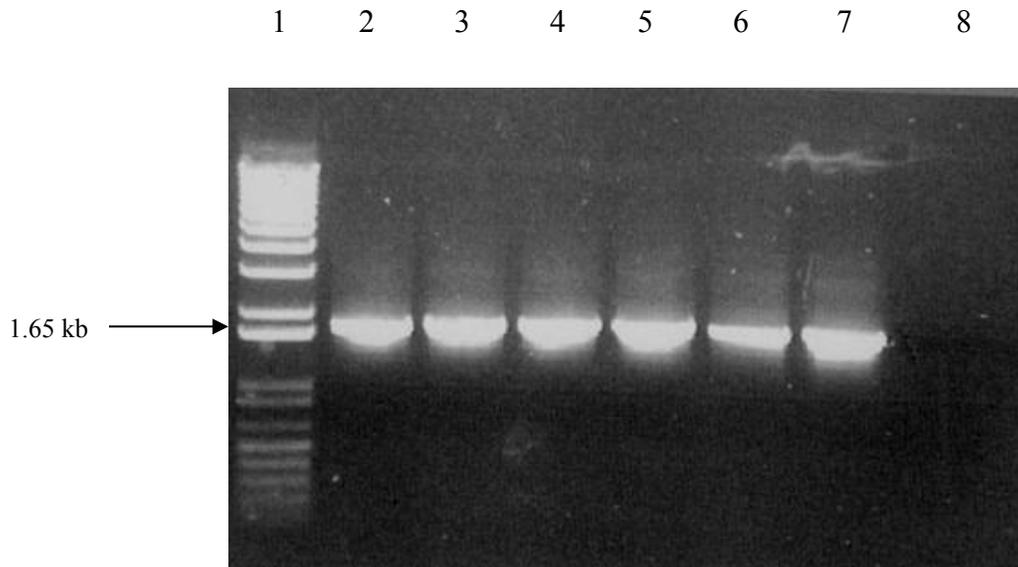
Fig. 2B

Fig. 2C

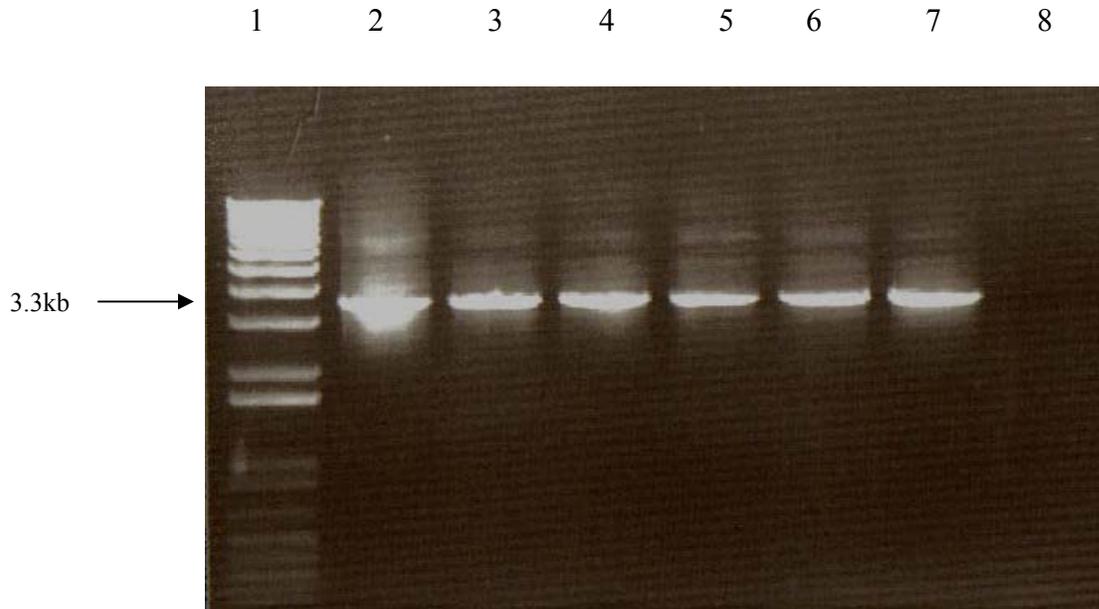1 2 3 4 5 6 7 8

3.3kb →

Figure 2. PCR analysis of Wild type and putative transformants of pLD-gene10-LecA.

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 3.3 kb product. **B) Lane 1:** 1kb plus ladder, **Lane2:** Positive control (Interferon clone), **Lane 3-7:** Transgenic lines pLD-gene10-LecA (2, 6, 8*, 14, 17), **Lane 8:** Negative control (Wild type). **C) Lane 1:** 1 kb plus DNA ladder, **Lane 2:** Positive control (pLD-gene10-LecA plasmid), **Lanes 3-7:** Transgenic lines pLD-gene10-LecA (2, 6, 8*, 14, 17), **Lane 8:** Negative control (Wild type).

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

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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.3A) with BamHI

and BglII (Daniell *et al*. 2001a). Figure 3B shows the HincII sites used for the restriction

digestion of the plant DNA for pLD-gene10-LecA.The transformed chloroplast genome

digested with HincII produced fragments of 6.0 kb and 2.0 kb for pLD-gene10-LecA (Fig

3C), while the untransformed chloroplast genome that had been digested with HincII

formed a 5.0 kb fragment. 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 LecA showed homoplasmy as there is no wild type band seen in

transgenic lines. The gene specific probe of size approx.0.4 kb was used to show the

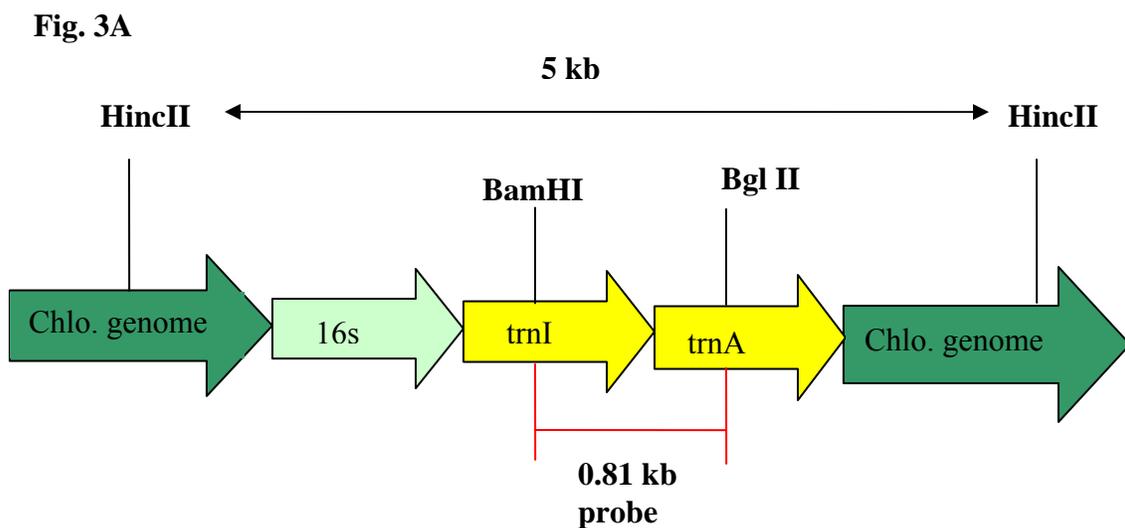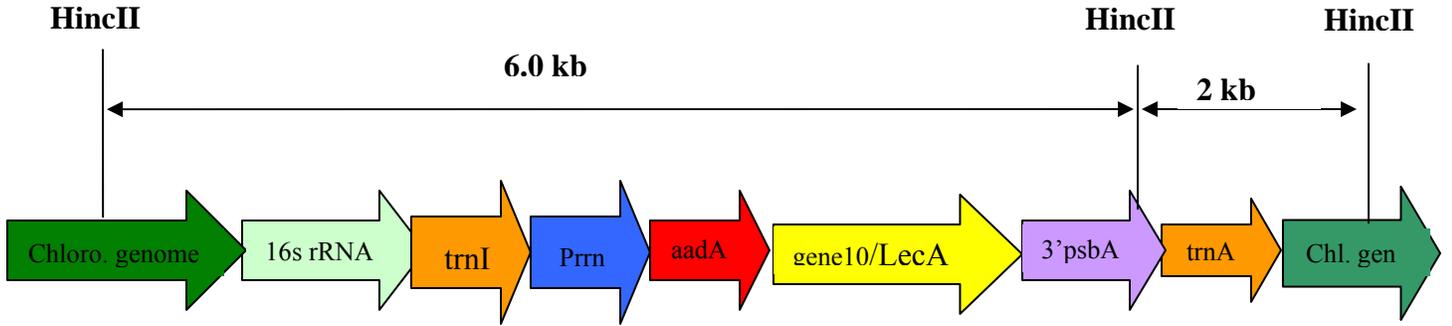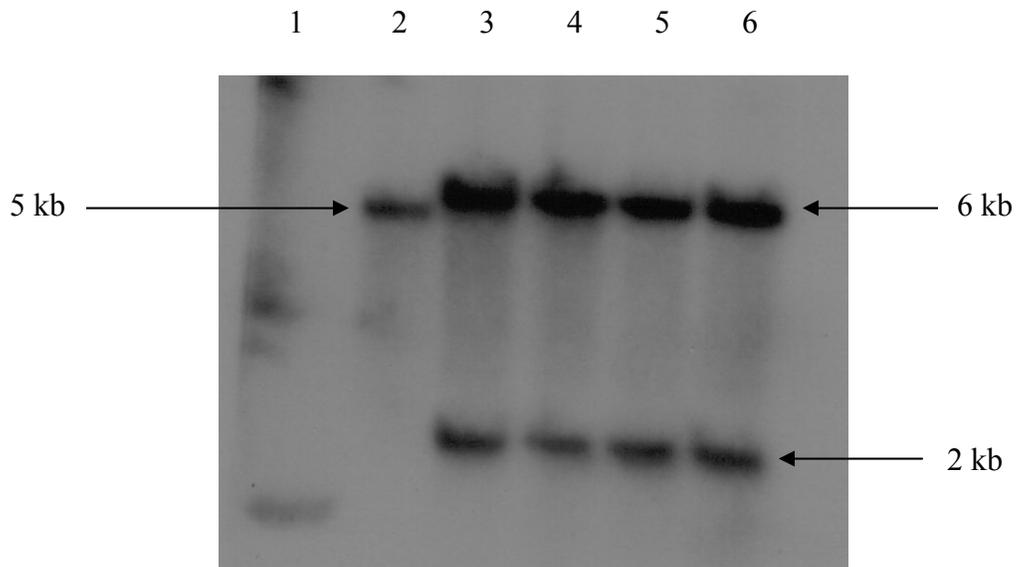specific gene integration producing a fragment of 6 kb as shown in fig 3D.
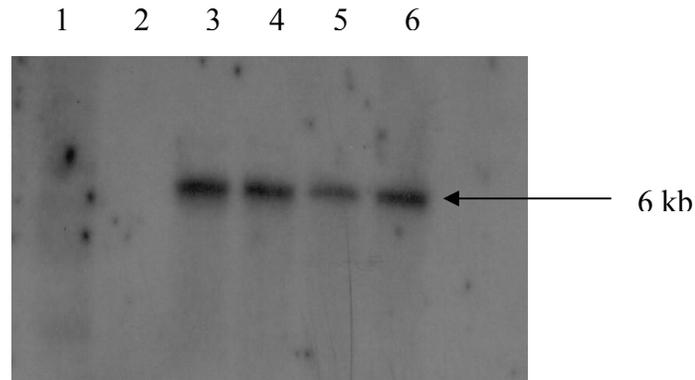
Fig. 3A

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Fig. 3B

Fig. 3C

Flanking sequence probe

Fig. 3D

Gene specific probe

Figure 3. Southern Blot analysis of pLD-gene10-LecA.

Schematic diagram of the products obtained from digestions of **A)** Wild type untransformed plants **B)** Plants transformed with pLD-gene10-LecA **C)** Southern with flanking sequence probe of pLD-gene10-LecA transgenic plants showing homoplasmy. **Lane 1:** 1 kb plus DNA ladder, **Lane 2:** Wild type, **Lanes 3-6:** pLD-gene10-LeA transgenic lines (8*, 17) **D)** LecA gene specific probe showing the presence of LecA in the transgenic plants. **Lane 1:** 1 kb plus DNA ladder, **Lane 2:** Wild type, **Lanes 3-6:** pLD-gene10-LecA transgenic lines (8*, 17).

Immunoblot analysis

Crude protein extract of 28 ug (10ul), was loaded in each well of the SDS-PAGE. The goat anti-LecA polyclonal antibodies (provided by Dr. Barbara Mann, Univ. of Virginia) were used to detect the 64 kDa protein (figure 4). The wild type plant (*Petit havana)* did not show any bands indicating that the anti- LecA antibodies did not cross react with any other proteins in the crude extract. The T1 generation plants also showed

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good levels of expression. Each of the wells contains around 1.5 ug of the LecA protein

detected by the LecA antibodies. The lower bands seen could probably be the degraded

LecA protein and the higher bands probably are the LecA protein aggregates.
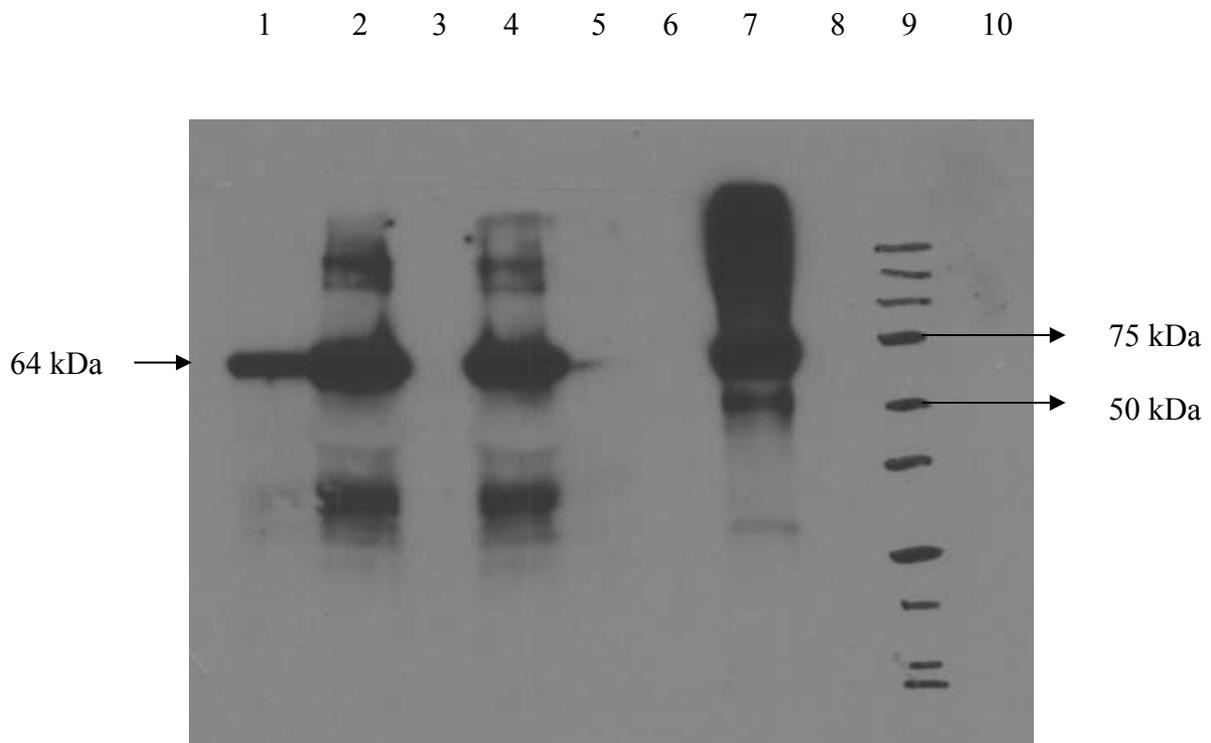
Figure 4

Figure 4.Immunoblot analysis of crude plant extracts expressing LecA.

Lane 1: T_1 generation transgenic plant**, Lanes 2& 4:** T_0 generation transgenic plant (28
ug of crude plant extract was loaded), **Lane 6:** Wild type, **Lane 7:** Standard protein (1
ug), **Lane 9:** Marker, **Lanes 3, 5, 8, 10**: Empty.

Protein quantification using ELISA

The standard curve has been obtained using different dilutions of purified LecA. The dilutions were made in coating buffer. The primary antibody used was Goat polyclonal antibodies against LecA and secondary antibodies were rabbit anti-goat IgG peroxidase conjugated. The percentage of LecA expressed was as a percent of total soluble protein calculated using the Bradford assay i.e. the LecA percent is inversely proportional to the TSP values. The LecA expression levels reached a maximum of 6.3 % of the total soluble protein in the old leaves when compared to 2.6 % TSP in young leaves and 5.2 % TSP in mature leaves. The maximum LecA expression was observed in old leaves when compared to young and mature leaves (Fig 5a). Based on the fresh weight calculations, the amount of LecA obtained from young, mature and old leaves is 0.67mg, 2.32 mg and 1mg per leaf respectively (Fig 5b). The difference in the LecA expression levels observed when calculating as percentage of TSP and based on fresh weight is due to the consideration of total soluble protein. The total soluble protein in old leaves was less when compared to the mature leaves and this could be due to degradation of the other soluble proteins in comparison to LecA as the leaves grew old. The expression levels of LecA were higher in mature leaves when compared to old leaves. When calculated based on fresh weight, the trend clearly shows higher expression of LecA in mature leaves. 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 LecA in mature leaves.
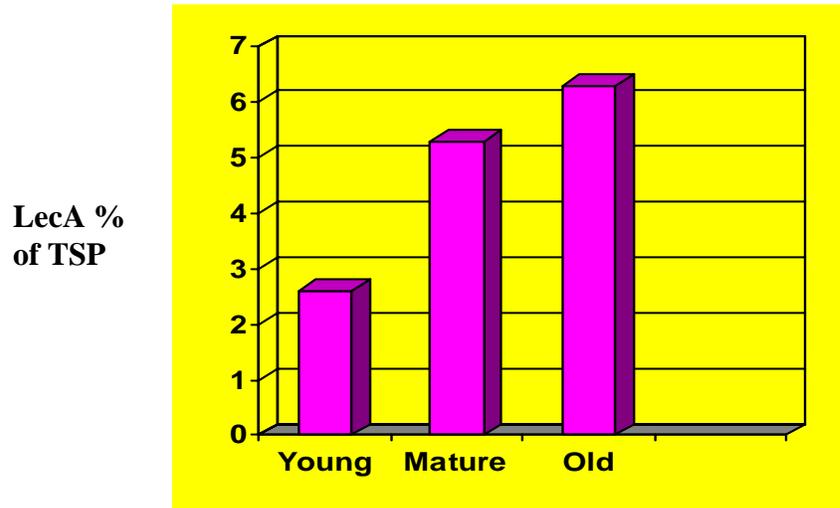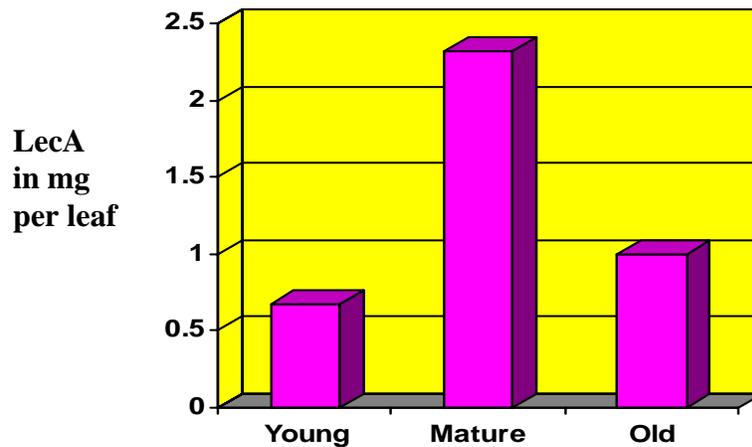
Fig. 5a

Fig. 5b

Figure 5. Quantification of LecA expression levels in transgenic plants (T_0 generation). A) Expression levels in % TSP of LecA expressed in Young, Mature and Old leaves under regular illumination conditions (16 hr light and 8 hr dark period). **B)** Amount of LecA (in mg) obtained from each of the Young, Mature and Old leaves based on the fresh weight.

DISCUSSION

The pLD-gene10-LecA vector was derived from the universal transformation vector, pLD-CtV (Daniell H. 2001a). The pLD-gene10-LecA chloroplast transformation vector containing the aadA gene, LecA coding region and 3' *psbA*, integrates the transgene cassette into the trnI–trnA region of the chloroplast genome via homologous recombination (Fig.6).

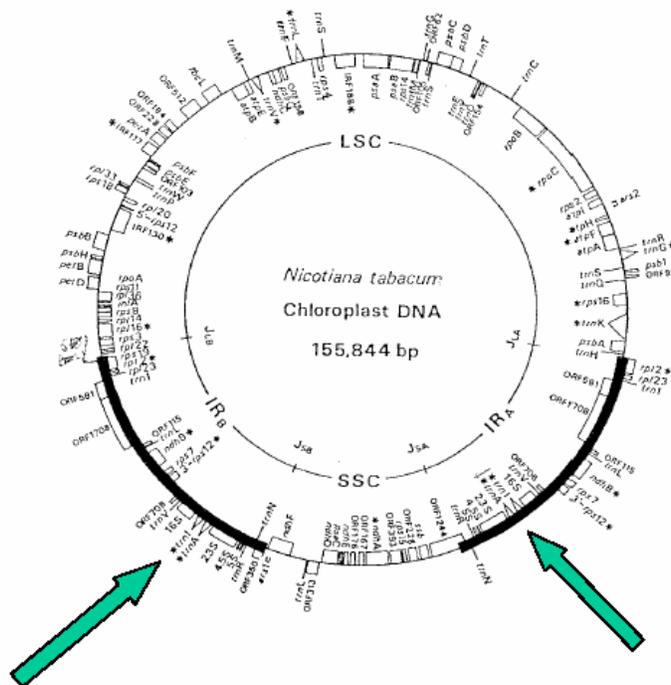**Figure 6. Chloroplast genome**

Arrows point to the trnI and trnA where homologous recombination occurs between the chloroplast and the pLD-gene10-LecA vector.

Integration takes place in one of the inverted repeats and is copied into the other inverted repeat via the copy correction mechanism (Devine and Daniell 2004). Expression of the LecA recombinant protein in the chloroplast depends on several factors. First, the pLD-gene10-LecA vector is designed to integrate into the inverted repeat region of the chloroplast genome via homologous recombination. The copy number of the transgene is thus doubled when integrated at this site. Increased copy number results in increased transcript levels resulting in higher protein accumulation (Guda *et al*. 2000, Daniell *et al*. 2001a). Second, the T7 bacteriophage gene10 5' UTR containing the ribosome binding site (rbs) and psbA 3'untranslated region (UTR) used for the regulation of transgene expression help in enhancing translation of the foreign protein (Kumar *et al*. 2004b). Third, homoplasmy of the transgene is a condition where all of the chloroplast genomes contain the transgene cassette. There are 100 to 1000 chloroplasts per cell and 100 to 1000 chloroplast genomes per chloroplast (Daniell *et al*. 2002, DeCosa *et al*. 2001, Devine and Daniell 2004); for optimal production of the recombinant protein and transgene stability, it is essential that homoplasmy be achieved through several rounds of selection on media containing spectinomycin. If homoplasmy is not achieved, heteroplasmy could result in 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' adenyl 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*. 2001a, 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' adenlyltransferase, which transfers the adenlyl moiety of ATP to spectinomycin and inactivating it. The pLD-gene10-LecA vector has a functional chloroplast origin of replication, which increases the number of templates for integration into the chloroplast genome (Daniell *et al*. 2004c). Fourth, expression can depend on source of the gene and its' relative AT/GC content. The prokaryotic-like chloroplast favors AT rich sequences, which reflects the respective tRNA abundance. Therefore, the LecA gene having 67% AT is expected to express well in the chloroplast. High expression of synthetic Human Somatotropin (HST), human serum albumin, human interferon-α2b, Human interferon-α, Insulin like growth factor shows that eukaryotic genes can also be expressed in the plastid (Staub *et al*. 2000, Fernandez *et al*. 2003, Daniell *et al*. 2005, Leelavathi and Reddy 2003, Daniell 2004) however; some eukaryotic genes need to be optimized for chloroplast expression. Genetic engineering of chloroplast genome to express LecA serves two purposes, high expression levels and gene containment. The use of T7 bacteriophage gene 10 5' UTR serves to enhance the translation in all tissues irrespective of the developmental stages (Kumar *et al*. 2004a). The 3'UTR region of the psbA gene present in the transgene cassette confers transcript stability.

PCR analysis was done to distinguish the chloroplast transformants from the nuclear transformants and the mutants. Southern blot analysis was utilized 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 and old leaves of up to 6.3 % of the total soluble protein which was quantified using the enzyme linked immunosorbent assay. Slight bleaching of mature and old leaves and delayed flowering were observed in the transgenic plants. The difference in LecA expression levels when calculated based on percentage TSP and fresh weight is due to the low TSP in old leaves when compared to mature leaves. This could possibly be due to degradation of soluble proteins when compared to LecA. Based on fresh weight, the mature leaves showed higher expression levels as the TSP is not taken into account. More number of chloroplasts in mature leaves, large size and more number of mature leaves per plant contribute to the higher expression. The calculation of LecA levels based on fresh weight helps in calculating the vaccine doses that could be obtained per acre of tobacco plants. This shows that using plants for the production of vaccine antigens could result in low cost vaccine as compared to bacterial expression system.

The present study reports the successful expression of the LecA protein for the first time in a plant expression system. This opens the doors for the expression of LecA in different crop plants so as to enable oral delivery of the vaccine antigen. The immunogenecity of the vaccine antigen needs to be tested in an animal model which is underway.

<u>Future Studies</u>

Assessing the immunogenecity of the plant derived vaccine antigen LecA

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 LecA. 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 LecA

Development of transgenic carrot expressing LecA will open the door for the oral delivery of the vaccine and develop mucosal immune response. An ideal vaccine for *Amebiasis* 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

This is the first time that the surface antigen of *Entamoeba histolytica* is expressed in plants. Since there is no vaccine for amebiasis as yet, this achievement could result in low cost vaccine because of high expression levels of vaccine antigens, 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 LecA was confirmed by PCR and homoplasmy was confirmed by Southern blot analysis. The expression of LecA, a 64 kDa protein in transgenic chloroplasts was confirmed by immunoblot analysis using anti-LecA antibody. Maximum accumulation of the LecA protein up to 6.3% of tsp was observed in the old leaves. The evaluation of the immune response of the vaccine antigen in animal model is underway.

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