2011

Stable Expression Of Tuberculosis Vaccine Antigen In Lettuce Chloroplasts

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STABLE EXPRESSION OF TUBERCULOSIS VACCINE ANTIGEN IN LETTUCE CHLOROPLASTS

by

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B.Tech Biotechnology, Anna University, India, 2009.

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 College of Medicine
at the University of Central Florida
Orlando, Florida

Fall Term

2011

Major Professor: Henry Daniell

ABSTRACT

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* and is one of the leading reasons of death by an infectious bacterial pathogen. The development of TB vaccines has been recognized as a major public health priority by the World Health Organization. In this study, a potential candidate antigen, ESAT-6 (6 kDa early secretory antigenic target) was fused with cholera toxin B subunit (CTB). Transplastomic lettuce plants were generated expressing these fusion proteins. Site-specific transgene integration into the chloroplast genome was confirmed by polymerase chain reaction and Southern blot analysis. In transplastomic leaves, expression levels of fusion protein (CTB-ESAT6) varied depending upon the developmental stage and time of leaf harvest with highest-level of accumulation in mature leaves harvested at 6PM. Transplastomic CTB-ESAT6 lettuce plants accumulated up to 0.75% of total leaf protein. Lyophilization increased CTB-ESAT6 protein content per gram of leaf material by 22 fold. Western blot analysis of lyophilized lettuce leaves showed that the CTB-ESAT6 fusion protein was stable and can be stored for prolonged period at RT. Hemolysis assay with purified CTB-ESAT6 protein showed partial hemolysis of red blood cells and confirmed functionality of ESAT-6 antigen. GM-1 binding assay demonstrated that the CTB-ESAT6 fusion protein formed pentamers to interact with GM1 ganglioside receptor. The expression of functional *Mycobacterium tuberculosis* antigens fused to CTB in transplastomic plants should facilitate development of a cost-effective and orally deliverable TB vaccine with potential for long term storage at room temperature.

ACKNOWLEDGMENTS

I would like to express my thanks to Dr. Daniell for giving me an opportunity to work in his lab and learn various aspects of scientific research. I would like to express my gratitude to my thesis committee members Dr. Annette Khaled, Dr. Saleh Naser for their valuable inputs and discussions. I am grateful to Dr. Dolendro Singh for his guidance and technical expertise in every stage of the project. Also, Dr. Kwang Chul Kwon, Dr. Dheeraj and Dr.jin have been kind enough to help me solve any scientific issues in my project. I would like to thank all my lab members Crystal, Ramya, Stew, Donevan, Neha, and Bindu who had given me valuable suggestions in my project. I would also like to thank all my friends in Orlando who have supported me. A special thanks to my friend Aarthi, who has been there for me through every stage of my life.

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LIST OF ACRONYMS/ABBREVIATIONS

aadA – Aminoglycoside 3’ adenosyltransferase

AIDS – Acquired Immuno DeficiencySyndrome

Ab – Antibody

ADP – Adenosine Di Phosphate

APS – Ammonium Persulfate

BAP – Benzyl amino purine

BCG – Bacillus Calmette Guerin

BME – Beta-mercaptoethanol

BSA – Bovine Serum Albumin

CT – Cholera Toxin

CTB – Cholera Toxin B Subunit

DOTS – Directly Observed Treatment, Short-course.

EDTA – Ethylene Diamine Tetra Acetic Acid

ELISA – Enzyme Linked ImmunoSorbent Assay

ESAT – 6 Early secreted antigenic target

GALT– gut associated lymphoid tissue

GFP – Green fluorescent Protein

MDR – Multidrug-resistant

NaCl – Sodium Chloride

NAA – α-Naphtalene Acetic Acid

PBS – Phosphate Buffered Saline

PBST – Phosphate Buffered Saline + (T – Tween 20)

PCR – Polymerase Chain Reaction

PPD – Purified protein derivative

psbA – Photosystem A

PTM – Phosphate Buffered Saline + Tween + Milk

RBC – Red Blood Cells

SDS – Sodium Dodecyl Sulfate

SDS-PAGE – Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

TNF-α – Tumor necrosis factor -alpha

TEMED – N,N,N,N’ Tetra-Methyl-Ethylene Diamine

TMB – 3,3’, 5,5’ Tetramethyl Benzidine

TSP – Total Soluble Protein

TLP – Total Leaf Protein

UTR – Untranslated Region

WHO – World Health Organization

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB), is one of the leading bacterial infections that is re-emerging due to drug resistant strains worldwide. The World Health Organization (WHO) estimates the global burden of TB in 2009 to be 9.8 million incident cases with multi drug resistant TB(MDR-TB), growing at a rate of half a million cases every year (WHO 2010). According to statistics, an estimated 250 000 (range, 230 000–270 000) had multi drug resistant TB in 2009. TB is also the leading cause of death in HIV-infected patients as Immuno suppression increases risk of reactivation of TB. The death toll caused by TB has steadily decreased in developed countries due to better health practices and antibiotic usage. But underdeveloped countries like Africa are still suffering from this punishing disease. The major reasons attributed to this rise are lack of health care, poverty, emergence of AIDS, malnutrition, spread of infection.

Pathogenesis & survival of Mycobacterium tuberculosis

Tuberculosis is triggered by infection with *Mycobacterium tuberculosis* (MTB), a bacillus bacterium. It is a small, gram positive rod shaped bacterium with high lipid content. It is spread by aerosol droplets by the coughing of a patient with pulmonary tuberculosis and through inhalation of small sputum particles by an uninfected individual. Its symptoms are mainly associated with bloody sputum, fever, coughing and weight loss. MTB can affect any part of the body as it can escape the lung and enter the bloodstream. This results in systemic disease affecting many organs including gastrointestinal tract, spleen, bones, joints, liver, and brain. Systemic form of the disease is fatal and can cause death shortly.(Anne DeGroot 1999)

Initially MTB on its entry in to the lungs reside in the macrophages. Initial development of TB brings about delayed type hypersensitivity response, characterized by the formation of small necrotic lesions comprising caseous centers. MTB multiplication is probably controlled while being encapsulated in these granulomatus lesions. T lymphocytes and mononuclear phagocytes of various levels of activation cause these lesions as a barrier to seal off the bacteria. This is the *latent infection* stage. It is effective until the immune system falters due to factors such as immunosuppression. The evasion mechanisms of tuberculosis have led to its survival in the human body. It has the ability to multiply in lung macrophages and also reduce the ability of macrophages to cause phagolysosome fusion that might lead to its death. It turns the immune system on self by causing inflammation of host tissue. It secretes virulent factors that release lysosomal enzymes and TNF-α, responsible for tissue damage. Other cases where proteases like cathepsin D are believed to play a role in the liquefaction of granulomas. Interference with macrophage activation has been observed by producing factors such as lipoarabinomannan, a glycolipid that represses proliferation of T-cells. This reactivation from latency period has confounded scientist as there has been research on how this bacteria can persist in the body. Two genes acr, isocitrate lyase have been identified which could play a role in the persistence of this bacteria.

Diagnosis and Treatment of Tuberculosis

The definition of infection of TB is limited to patients who convert from negative to positive on a skin TB test. PPD constituting mycobacterial proteins is injected in to the skin and tested for appearance of red rashes for positive skin test. No reaction in case of negative test. This test gives no information about active infection in case of patients who have been immunized with anti-TB vaccines and this is not specific to MTB, as other mycobacteria give a weak response. Sputum smear microscopy followed by

bacterial culture from patients, is employed for TB diagnosis. Early identification aids in prevention of lung damage and spread of infection. Of the people infected with TB, only 1% develop active TB infection. In developing countries, many people are already infected with MDR strains of TB. Drug combination therapy showed a success rate greater than 90% prior to MDR tuberculosis, even in AIDS patients. MDR tuberculosis is highly infectious and almost incurable with a mortality of 50%. TB is now becoming the leading cause of death among HIV infected patients killing with a greater rapidity. In the past, measures employed to control TB have involved the use of combinations of antibiotics. Currently, individually tailored combination of antibiotics is essential depending on the strain of MTB infection, due to many complications associated with multidrug-resistant strains. Surgical removal of the affected portion of the lung is necessary for severe cases of TB. A new effective control strategy by the WHO that is currently being implemented throughout the world is DOTS.

Development of tuberculosis Vaccine

Bacillus Calmette Guerin (BCG) is an attenuated strain of *Mycobacterium bovis* and is the only available licensed vaccine against TB. Lack of toxicity has enabled It to be distributed worldwide. Many trials that evaluated BCG on the basis of protective immunity and age of vaccination have been inconsistent and variable, ranging from 0 to 80 % efficacy. (Colditz et al. 1994; Brewer et al. 1995; Xing et al. 2006). BCG does not play a role in preventing the establishment of latent TB or reactivation of pulmonary disease in adults (Andersen 2007). BCG cannot be given in booster doses to establish protective immunity.

To remedy this, research groups are engaged in developing more efficient anti-TB vaccines which may have the potential to replace BCG as a primary TB vaccine or act as an effective boosting vaccine (Derrick et al. 2004; Andersen 2007; Doherty et al. 2007; Coler et al. 2009). Compared to attenuated live TB vaccines, subunit vaccines

offer several advantages including safety, efficacy and they are better suited for standardization (Agger et al. 2001; Tsenova et al. 2006). On the other hand, limitations include poor immunogenicity of purified antigens and restriction in the number of antigens exposed. This makes an immunostimulatory component all the more essential in an effective vaccine.

Many different fractions of MTB have been proposed as subunit vaccine candidates including surface components and secreted proteins. Some of the promising antigens are ESAT-6, Ag85B, MTB72F etc. The reasoning behind this has been that killed BCG vaccines has proven to be ineffective compared to attenuated vaccines. This led researchers to believe that active secretion of proteins can induce appropriate cell mediated response (Brodin et al. 2006). Any mycobacterial antigen which activates both CD4 & CD8 T - cells and imparts protective immunity is believed to be the ideal candidate for subunit vaccination against TB. Antigenic proteins actively secreted during the early phase of growth of MTB are best suited as potential candidates for subunit vaccines (Brodin et al. 2004).

Early secretory antigenic target - 6 (ESAT-6) vaccine candidate

ESAT-6 (6 kDa early secretory antigenic target), a protein comprising 95 amino acid (aa) residues, is one such promising vaccine antigen candidate that was identified based on its immunogenicity from the culture filtrate of mycobacterial antigens, that can strongly elicit a specific T-cell response (Brandt et al. 2000). ESAT-6 has been established to be present in the RD-1 region in all virulent strains of MTB but its also striking that its absent in the attenuated BCG vaccine strain (Andersen et al. 1995). Hence ESAT – 6 could prove to be one of the components essential to treat this complex disease.. ESAT-6 was found in a complex with another protein CFP-10, a

10 kDa protein and belongs to the ESAT-6 family (ESX-1). 23 ESAT-6 family members have been identified in *M. tuberculosis strain* H37Rv and has been shown to be located in 11 genomic loci.(Brodin et al. 2006). ESAT-6 and related mycobacterial proteins have shown to require a unique secretory apparatus encoded by genes flanking esxA and esxB, in its secretion from MTB.

Role of ESAT-6 in mycobacterial infection is controversial. Based on study of ESX-1 mutants, their involvement in the spread of tubercle bacilli to neighboring cells has been established., ESAT–6 has been shown to be a virulent factor by inducing membrane pore formation in macrophages (Smith et al. 2008). Another report suggests that ESAT-6 can induce apoptosis in macrophages by activating caspase expression. Reports suggest that biological activity of ESAT-6 rests on the proper export of ESAT-6 by its secretion machinery.(Pym et al. 2003) As ESAT-6 has shown to be recognized by infected TB patients with higher sensitivity, a diagnostic potential for ESAT – 6 is also being explored. In mice, vital T cell epitopes of ESAT-6 antigen have been distinguished to be P1,at the N-terminus (residues 1–20)and another P6,at the C-terminus (residues 51–70) (Brandt et al. 1996). In animal models such as guinea pig, mice, ESAT-6 was used as a subunit vaccine and did not cause any toxicity.(Doherty et al. 2002). It was reported to induce production of gamma interferon (IFN-γ), a marker for protective immune response (Agger et al. 2001; Kumar et al. 2010) with protective immunity comparable to BCG (Brandt et al. 2000). Many vaccine trials in animal models such as mice The vaccines based on ESAT-6 antigen in combination with another mycobacterial antigen Ag85B have entered human clinical trials (van Dissel et al. 2010).

Mycobacterial antigens as subunit vaccines have been targeted by different delivery systems including recombinant viral vector system (Sereinig et al. 2006), recombinant bacterial vector system (Triccas 2010), lipoglycan - protein conjugate system (Hamasur et al. 2003). Here we plan to deliver tuberculosis antigens to the GALT – an integral part of the mucosal immune system. TB, being a respiratory disease, subunit vaccination targeting the mucosa is well disposed to initiate both mucosal and systemic immune response. In order to survive the extreme physiological

conditions of the gut, strategies have to be employed to protect the antigen and optimize dosage conditions to ensure antigen uptake.

Cholera Toxin B subunit as a mucosal adjuvant

Cholera Toxin triggers diarrhea by colonizing the small intestine. Cholera toxin is characterized to be a AB_5 toxin containing 2 subunits, one is a toxic 27 kDa A subunit exhibiting ADP ribosyl transferase activity and other is a nontoxic pentamer of 11.6 kDa B subunit, whose primary function is to binds to the A subunit and bring about the entry of the toxin into the intestinal epithelial cells (Daniell et al. 2001). Cholera toxin B subunit is a well researched mucosal adjuvant that has been reported as a carrier molecule for mucosal immune responses and oral tolerance (Langridge et al. 2010). It has been reported that adjuvanticity of CTB increases with coupling to antigens. This could be attributed to, one, better uptake of antigen across the mucosal linings. Two, efficient antigen presentation to dendritic cells and macrophages. Although there are some reports indicating that auto antigens coupling with CTB bring about oral tolerance, it is believed that the nature of the antigen fused to CTB plays an important role in the manner of immune response.(Langridge et al. 2010) ESAT-6 is an established immunodominant T-cell antigen. I believe CTB fused with ESAT-6,TB antigen could potentiate systemic and mucosal immune response. Plant vaccines are highly efficient delivery vehicles as they are capable of transporting large amount of antigen in an encapsulated form.

Chloroplast genetic engineering and its advantages

Expression of the TB antigens in plants through chloroplast transformation has several advantages over other production systems. Transplastomic plants have not

reported gene silencing, in spite of accumulating >100-fold higher transcripts compared to nuclear transgenic plants(Daniell et al. 2009). High levels of expression (up to 72%) of several biopharmaceutical proteins and vaccine antigens have been attained via the chloroplast system (Bally et al. 2009; Ruhlman et al. 2010). Hyper-expression of the biopharmaceuticals in chloroplast facilitates cost effective production without laborious purification methods and cold chain issues. In spite of their prokaryotic origin, plant chloroplasts are recognized to develop proper disulphide bonds (Ruhlman et al. 2007; Bally et al. 2008)and other post-translational modifications such as lipidation (Glenz et al. 2006).The functionality of chloroplast-derived vaccine antigens has been validated by several assays including the macrophage lysis assay (Koya et al. 2005), GM1-ganglioside binding assay (Daniell et al. 2001; Davoodi-Semiromi et al. 2010) and immune response to challenge by pathogens (Arlen et al. 2008) . In comparison to mammalian expression systems, there is minimal risk of animal or human pathogens contaminating the vaccines (Koya et al. 2005). Additionally, chloroplasts demonstrated transgene containment preventing escape through pollen because of maternal inheritance of the plastid genome (Daniell 2002; Daniell et al. 2009). Recombinant ESAT-6 has been expressed in transient plant production systems such as potato virus X vector based system (PVX), tobacco mosaic virus vector based system , agrobacterium mediated transformation (Rigano et al. 2004; Zelada et al. 2006; Dorokhov et al. 2007). But no stable integration of transgene for TB antigens in chloroplast has been obtained yet.

Oral delivery of vaccine antigens

In animal trials, many plant oral vaccines expressing foreign proteins fused to CTB have shown to be protected against degradation by stomach enzymes and offer protective immunity against disease states (Ruhlman et al. 2007; Arlen et al. 2008; Davoodi-Semiromi et al. 2010). Oral delivery of bioencapsulated coagulation factor IX

has shown to prevent inhibitor formation thereby avert anaphylaxis in hemophilia B mice (Verma et al. 2010). The most important advantage associated with oral delivery of plant antigens is bioencapsulation of antigens. Any antigen that is orally delivered undertakes risk of being degraded by the enzymes present in the stomach. This can be prevented by bioencapsulation via plant cells as they can prevent degradation of antigen by digestive enzymes. In a report of CTB-GFP oral delivery, transgenic leaf material has been administered to mice. The results observed the presence of CTB in the intestinal wall and fluorescence emitted by GFP in mouse intestinal sections, liver, and spleen (Limaye et al. 2006). Many edible crops have been reported to be developed for expressing vaccine antigens such as tomato, potato, carrot. But Lettuce is an ideal system to develop chloroplast engineered vaccines, as it is rich in chloroplasts. Lettuce (*Lactuca sativa)* was chosen as an alternative to tobacco for expression of TB vaccine antigens due to its many advantages such as it being an edible crop, its leafy nature and commercial importance. According to U.S department of agriculture statistics for 2009, about 32500 plants have been planted throughout. Yield of lettuce has b 345 cwt per acre of lettuce plants. Hence we can see how lettuce vaccine antigen could be feasible on a larger scale.

In this study, ESAT - 6 fused with CTB was expressed in lettuce chloroplasts to explore the possibility of chloroplast as an efficient bioreactor for oral delivery of vaccine antigens against TB. The expression levels of TB antigens were analyzed using western blot analysis. Lyophilization was performed on lettuce leaves for increasing antigen content. GM-1 binding ELISA assay confirmed binding affinity of CTB – ESAT6 to GM-1 receptor. Hemolysis assay demonstrated pore forming ability of CTB – ESAT6 in red blood cell membranes.

MATERIALS AND METHODS

Chloroplast Vector Construction

Lactuca sativa long flanking plastid transformation vector (provided by Dr.verma) (pLSLF) was constructed using primers derived from regions of known conservation in the *Nicotiana tabacum* plastome to amplify cognate sequences from the *Lactuca sativa* plastome. Lettuce flanking sequence vector (pLSLF) was designed to integrate the transgene into transcriptionally active spacer region between *trn*I and *trn*A genes as explained previously (Ruhlman et al. 2007; Verma et al. 2008).The gene cassette comprising promoter P*rrn* and *rbcl* from lettuce chloroplast genome and selectable marker *aad*A gene was built into pBSSK+ vector and cloned into pLSLF. pLD-5′-UTR-CTBPins(Ruhlman et al. 2007) vector was used as the template to amplify the CTB sequence. Further, the ESAT-6 sequence was amplified using sequence-specific restriction-site flanking primers and *Mycobacterium tuberculosis* genomic DNA as the template. The CTB- ESAT6 gene was ligated in to pLSLF to create pLS-CTB-ESAT6 vector along with *psb*A promoter and 5′ and 3′ UTR from lettuce regulatory regions. To facilitate proper folding of protein, both fusions (CTB-ESAT6 and CTB-MTB72F) had the GPGP hinge in between fusion proteins for reducing the steric hindrance.

The chloroplast transformation vector DNA was transformed in to competent cells Alpha-Select Gold Efficiency (Bioline) using CaCl2 method and *E.Coli* colonies were grown overnight on a pate containing ampicillin (100 mg/ml). The colonies were selected on the next day in LB broth containing ampicillin. These colonies were then screened by colony PCR to choose recombinant clones. Protein extraction ws performed on the positive clones by sonication and Immuno blot analysis using CTB antibody was performed as described later in detail.

Preparation of Gold Particles for coating plasmid DNA

Gold particles (150 µl) and 1 mL of 100% ethanol were mixed together to obtain even suspension in a 1.5 mL eppendorf tube. This mixture was centrifuged at 14,000 rpm for one minute. Follwing centrifugation, the pellet was retained and was washed in 1 mL of 70% ethanol. This gold particle suspension was left for twenty minutes in a vortexer at 4 °C. Centrifuging at 5,000 for three minutes was followed by removal of supernatant and the gold particles were vortexed with 1 mL of distilled water.This was allowed to incubate at room temperature for one minute and centrifuged again at 5,000 rpm for two minutes. This procedure was repeated three times to ensure complete washing (Kumar and Daniell 2004). After washing steps, the gold particles were finally resuspended in sterile 100% ethanol. This was stored in ice for final use during bombardment.

Based on earlier optimizations, 10 µg of plasmid DNA was coated on the 50 µL of gold particles in a 1.5 mL micro centrifuge tube. There were further additions of 100 µL of pre filtered 2.5 M CaCl2 and 40 µL of 0.1 M spermidine common stock for optimal binding of DNA to the gold particles. The supernatant was removed and complete removal of ethanol was ensured. The pellet was washed three times in 300 µL of absolute alcohol. The DNA-coated gold particles were resuspended in 100 µL of 100% ethanol. This mixture could result in ten shots of bombardment comprising of 10 µL of DNA-coated gold particles were loaded onto macro carriers and allowed to dry in the laminar flow hood. Then macrocarriers were loaded on to the bioliistic particle delivery system.

Bombardment of Lettuce leaf tissue

Chloroplast transformation including bombardment and regeneration was carried out as described previously (Kumar et al. 2004; Verma et al. 2008). Sterile fully expanded leaves of untransformed lettuce plants (wildtype) were placed on MS medium with adaxial side up for lettuce (Lactuca sativa). The leaves were bombarded with gold particles coated with plasmid DNA of pLS-CTB-ESAT6 using the biolistic device PDS1000/He (Bio-Rad). The macrocarrier with the DNA-gold coated particles were placed on the macrocarrier holders in the gene gun. The bombardment was performed using 900 psi rupture disks and at a target distance of 6cm between the leaves and the coated particle as optimized earlier. Then the leaves were incubated in 25°C in the dark.

Regeneration and Selection of Transplastomic Shoots

After incubation at 25°C in the dark for 2 days, the leaves were cut into 5x5 mm^2 pieces and transferred on to RMOP media – 1 litre, comprising of 1 packet of MS basal salt mixture, , 100 mg myoinositol, B5 vitamins (1mg pyridoxine,1 mg Nicotinic Acid,10mg of thiamine hydrochloride ,1 mg of glycine) , 30 g of sucrose, 200 µl of 1 mg/mL- 0.2 mg BAP, 100 µL of 1 mg/mL – 0.1 mg NAA. The pH was adjusted to 5.8 using 1NaOH. We also added 6g of phytagar (phytablend) for solidification before being autoclaved and cooled for pouring into plates containing 50 µg/mL of spectinomycin making sure the bombarded side faces the medium. The Petri dish was sealed using parafilm and placed in the culture room till appearance of putative transgenic shoots. This process is second round selection and the shoots are allowed to develop for 4-8 weeks. The third round of selection was performed when the shoots are

placed in half strength MS media (one packet of MS salt mixture along with 30 g of sucrose to 2 L of sterile distilled water; the pH was adjusted to 5.8 using 1N NaOH; 6 g of phytoblend) with 50 µg/mL spectinomycin. Once roots develop, they are ready to be transferred to incubator.

Rooted plants were transferred to Jiffy peat pots and placed in incubator for acclimatization. After considerable growth, plants were moved to green house for maturation, flowering and seed production.

Confirmation of site specific Transgene Integration

To confirm the transgene cassette(s) integration into the chloroplast genome, genomic DNA was extracted from leaf tissues of spectinomycin resistant and wild-type untransformed plants using Qiagen DNAeasy Plant Mini Kit (Qiagen,Valencia,CA) using the manufacturer's protocol. Leaf material from both incubator plants and *in vitro* greenhouse plants were ground with liquid nitrogen into fine powder. In the final step, the pellet was washed using 70% ethanol and centrifuged for ten minutes at 14,000 rpm. The pellet was air dried till we lost any scent of ethanol. Then the pellet was dissolved in 100 µL water or TE buffer depending on downstream purposes. In order to visualize the bands, the DNA samples were loaded on a 0.8% agarose gel. The concentration of the DNA was determined by a Nano drop 2000.

PCR analysis was performed using the primer pairs mentioned below

16SF (5'-CAGCAGCCGCGGTAATACAGAGGA-3') and 3M (5'-CCGCGTTGTTTCATCAAGCCTTACG -3'). Additionally, to validate the integration of transgene of interest, another primer pair 5P (5'-CTGTAGAAGTCACCATTGTTGTGC-3') and 2M (5'- TGACTGCCCAACCTGAGAGCGGACA -3') was used. :, 5 µL of 10X Buffer, 1 µL of DNA(depending on concentration), 2.5 µL of $MgCl_2$, 1 µL of dNTP's, 1 µL of forward primer, 1 µL of reverse primer, 1 µL *Taq* polymerase, and 37.5 µL of sterile

H_20. Initial denaturation cycle was set at 94°C for 5 minutes following amplification for thirty cycles of the following program: denaturation - 94°C for 30 seconds, annealing - 61°C for 30 seconds and extension - 72°C for 1 minute and 30 seconds.The last step was the fiinal extension of five minutes at 72°C at the end of PCR process.

Restriction Digestion of Genomic DNA

The transformed and untransformed samples containing equal concentration of DNA were digested with HindIII restriction enzyme in a reaction containing:(20 µL)1.5 µg of DNA, 4 µL of 10X Buffer 4 (New England Biolabs), 1 µL of HindIII (New England Biolabs), and 15 µL sterile distilled H_20 to make up the volume of 40 µL of total reaction volume. The digestion reaction was performed overnight at 37°C**.**

40 µL reaction volume digestion products were loaded on a 0.8% agarose gel and was run for 4-5 hours at 80 volts. After electrophoresis, the gel was visualized under UV light periodically to check the size of the products and if they were separated enough. After sufficient separation, the gel was depurinated with 0.3N HCl for 15 minutes (the color of the dye usually became yellow. This was followed by three washes with sterile H20. The gel was immersed with in transfer buffer (0.4N NaOH, 1M HCl) for 20 min. DNA was transferred from the agarose gel on to Nylon membrane. This was based on the Rapid Downward Transfer System, Schleicher & Schuell) protocol. Initially, one sheet of prewet filter paper in transfer buffer was placed in the stack tray. The gel was placed upside down upon the sheet. Nylon membrane (N+-Bond, Amersham Biosciences, USA) was pre soaked and placed on the gel. Followed by some application of transfer buffer, three sheets of prewet filter paper were placed on top. This was followed by placement of a stack of paper towels cut to fit the gel dimensions. In order to remove any air bubbles, we employed Rolling stick. A cover with a heavy weight on top was placed prevent evaporation, increase capillary action. The

transfer was performed overnight at room temperature. After transfer, it was rinsed with transfer buffer twice for five minutes each. This step was followed by air drying the membrane. UV Strata linker 2400 at the C3 setting semi wet membrane crosslink setup was used to crosslink the membrane.

Generation of Flanking Probe

The flanking probe was obtained by digesting Lettuce chloroplast flanking sequence pLS -CT DNA with BamH I and Bgl II enzyme and generating 1.3 kb fragment This product was eluted by loading it into a 0.8% agarose gel. The bands were excised after visualization using Bio Rad Gel Doc 2000. The gel fragments were extracted with the QIAquick Gel Extraction Kit (Qiagen) by following the manufacture's protocol.

Forty five micro liters of the eluted DNA fragment was denatured at 95°C for 5 min and immediately placed on ice for 2 min. Random primer labelling kit was used to produce high specificity probe as per manufacturer's protocol. The denatured probe was added to the DNA labeling beads and the tube was mixed gently by flicking the tube. Two or three microliter of radioactive ^{32}P (depending on the time and half life) was added to the probe mixture the mixture was incubated at 37°C for one hour. G50 (Probe Quant) microcolumns were used to purify the radiolabelled probe. The column was quickly vortexed for the resin mixing. The column was centrifuged at 3000 rpm to remove the solution in the resin. The probe DNA was added to the column and centrifuged at 3000 rpm for 3 min to purify the probe DNA.

Hybridization and Autoradiography

The membrane that was crosslinked and stored was placed in Quick Hyb solution (Stratagene). This was pre -hybridization process and was performed at 68□C

for 1 hour. 100 µL of salmon sperm DNA was added to labeled probe and then the probe was boiled for five minutes. After boiling, it was added to the hybridization bottle. This hybridization process was performed at 65°C. Next day, the membrane was washed twice with high stringency wash buffer I (2X SSC, 0.1% SDS) followed by washing with low stringency wash buffer II #2 (0.2X SSC, 0.1% SDS) for fifteen minutes each. The membrane was allowed to air dry behind the plastic shield. The radioactive membrane was wrapped with saran wrap. The membrane was exposed to X-ray film in a cassette in – 80°C for 2 days or more depending on the radioactivity counter. The exposed film was developed in a automated film developer.

Analysis of transgene Integration

In order to confirm maternal inheritance, seeds harvested from the CTB - ESAT6 plants transplastomic plants were germinated on ½ MS salt supplemented with spectinomycin (100mg/l for lettuce). Sterilization of seeds was performed with 1.5% bleach, followed by thorough rinsing in distilled water.. Seeds from untransformed plants were also grown in the same plate. The growth of the plants was observed after 10 days.

Extraction of Plant leaf protein

Transformed and untransformed leaves (～100 mg) were ground in liquid nitrogen with a mortar and pestle followed by extraction with a mechanical pestle in 200 µl of extraction buffer (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1%SDS, 400mM sucrose, 0.05% Tween 20, 2mM PMSF and proteinase inhibitor cocktail (roche)). The leaf extracts were then centrifuged for 5 min at 10,000 rpm to separate out the supernatant and pellet the insoluble plant material. The pellet was resuspended in

buffer and sonicated in ice for 10s pulse for a min. Bradford assay was performed to detect total protein concentration using Protein Assay Dye Reagent Concentrate (Bio-rad).Standard curve for this assay was generated with Bovine serum Albumin (BSA) with dilutions ranging from 0.8 mg/ml to 0.025 mg/ml. All samples were loaded in duplicate. Protein Assay dye was dilutes and absorbance was measured at 595 nm.

Detection of fusion protein using western blot analysis

To quantify the expression of the fusion protein ~one hundred milligram (mg) of the leaf samples at different developmental stages (young, mature and old) or at different time points (10 a.m., 12 p.m. and 6 p.m.) during the day were collected from plants exposed to regular lighting pattern (16h light and 8h dark). Homogenate, supernatant and pellet fractions were boiled for 5 min in sample buffer (0.5M Tris-HCl, 25% glycerol, 10% SDS, 0.5% Bromophenol blue and β-mercapto ethanol) and separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad). The separated proteins were then transferred on to a nitrocellulose membrane in a transfer cassette (Bio-Rad) at 85V for 1 hour. After blocking with phosphate-buffered saline (PBS),0.1% Tween 20, 3% milk powder (PTM), the membrane was incubated with anti-CTB primary antibody (1:4000, Sigma, St. Louis, MO, USA) diluted in PTM followed by 1:5000 horseradish peroxidase (HRP) - conjugated goat anti-rabbit secondary antibody (Southern biotech, Birmingham, AL, USA) for 1 hour 30 minutes. A Super Signal West Pico HRP Substrate Kit (Pierce, Rockford, IL, USA) was used for autoradiographic detection.

Densitometry analysis

To quantify the expression of fusion protein, in case of the lettuce plant, we performed densitometric analysis on the immunoblots with CTB antibody. Known concentrations of purified CTB (Sigma) were used as standards (25, 50, 75 and 100ng) to create a standard curve. Total protein concentration was measured using Bradford assay (Biorad). Fusion protein was loaded in different concentrations of total protein and their integrated Density values (IDV) was measured using Alphaimager 2000 and analyzed using Alphaease software. The percentage of fusion protein (%Total leaf protein) and amount of transgenic protein (µg/g) is calculated based on the formula published earlier (Verma et al. 2008).

GM1 - Ganglioside Receptor Binding Assay:

GM -1 ganglioside (Sigma G-7641) and BSA (control) was coated at a concentration 3 µg/ml in bicarbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6) on to a 96 well plate at 4ºC overnight. Washing was performed thrice with 1X PBST and water. The wells were then blocked with 200 µl of phosphate-buffered saline (PBS) - 0.1% Tween - 3% milk powder (PTM) for 2 hours at 37ºC followed by washing thrice with 1x PBST and water.CTB protein standards, Untransformed plant leaf TSP and transformed leaf TSP diluted in ELISA coating buffer were coated on to the plate in different concentrations and incubated for 2 hours at 37ºC. The plate was washed again as stated above and incubated with anti - CTB primary antibody (1:3000 dilution) for 1 hr at 37 ºC. Further, the plate was incubated with secondary HRP-conjugated goat anti-rabbit IgG in 1:4000 dilution. Following washing with 1X PBST and water thrice, 100 µl of 3, 3, 5, 5-tetramethylbenzidine substrate was added and incubated for 10 to 15 min at room temperature. The reaction was terminated by adding 50µl of 2 N H_2SO_4 per well and the absorbance was read on a plate reader at 450 nm.

Antibody Affinity purification:

Total leaf protein of CTB – ESAT6 was extracted with 2X PBS, 0.1% Tween – 20,pH 8, proteinase inhibitors and sonicated (Sonicator 3000 Misonix) for 1 min in ice. Supernatant was obtained after centrifuging for 5 min at 2000 rpm. Pre- clearing of the supernatant was performed with 100mg of Protein A Sepharose CL-4B (GE Healthcare) beads along with protease inhibitors (Roche) overnight at 4ºC. Rabbit anti-cholera toxin B subunit polyclonal antibody (Abcam, ab34992) was coated on to washed protein A beads in a ratio of 50µg: 200µl in 1X PBS overnight at 4ºC for antibody binding to beads. Washes were performed with 1X PBS followed by 0.1M sodium borate buffer (pH 9). 20mM Dimethyl pimelimidate DMP (sigma, D8388) crosslinker in sodium borate buffer was added to the bead- antibody mixture and incubated for 30 min at room temperature twice. Washes were continued with 50mM glycine pH 2.5 followed by 1X PBS. Cross linked antibody was added to the precleared supernatant and incubated overnight at 4 ºC. Elutions were performed with 100 mM glycine buffer, pH 2.5 in 100 µl volume with 10µl of 1M Tris-HCl pH 9 for neutralization at room temperature. All Elution fractions were analyzed by Biorad Bradford assay and nanodrop spectrophotometer via absorbance at 280nm for total protein concentration in mg/ml. Silver staining was performed to detect purity %

Silver staining:

SDS-PAGE gel (12%) was fixed using a fixative (50% methanol, 12% acetic acid) overnight at 4ºC and washed twice with 50% ethanol. The gel was pretreated with 0.02% sodium thiosulphate solution ($Na_2S_2O_3$) for 1 min and then washed thrice in distilled water for 1 min each. Gel was stained with Silver nitrate solution (0.2% silver

nitrate in formalin) for 20 min. The gel was rinsed twice in distilled water for 1 min and developer solution (2% Na_2CO_3, 0.0004% $Na_2S_2O_3$, formalin) was added. Gel was shaken to observe bands in developer solution. Developer solution was replaced after 5 min and reaction was stopped with 1% acetic acid as soon as protein bands were observed.

Lyophilization:

CTB – ESAT-6 lettuce leaves were frozen in liquid nitrogen and then lyophilized in Freezone Benchtop Freeze Dry Systems(Labconco) in vacuum for 2 days at -50ºC at 0.036 mBar. After lyophilization, they were stored at room temperature in vacuum for few days. The samples were ground to fine powder in waring blender and further testing was performed.

Hemolysis Assay:

To test the hemolytic activity of the ESAT-6 protein, hemolysis assay was performed with affinity purified CTB – ESAT6 fusion protein. Pore formation in red blood cell membranes can be measured by hemolysis assay as shown previously (Smith et al. 2008). Affinity purified CTB – ESAT-6 was solubilized with 0.1M KCl – HCl for 1 min followed by neutralizing with 50mM Tris - HCl, pH 8 as described earlier (Ruddock et al. 1996; Tinker et al. 2003). After solubilization, CTB-ESAT6 fusion protein was mixed with sheep red blood cells (1×10^9 cells) in a micro centrifuge tube in the ratio 1:2 and allowed to incubate at 32ºC for 2 hours. As controls, non solubilized CTB-ESAT6, distilled water and PBS were incubated with sheep red blood cells in the same ratio and under same conditions. The cells were then resuspended and centrifuged for about 7

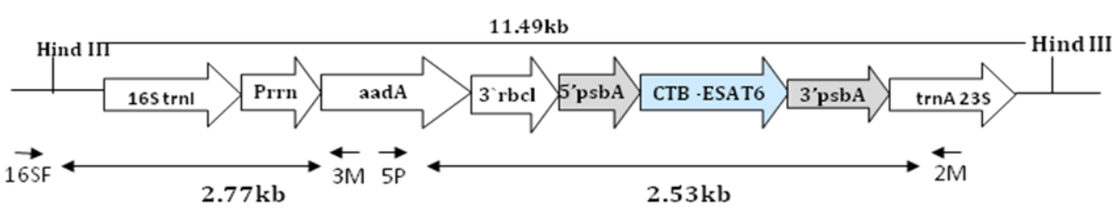
min at 4000 rpm. Supernatants were loaded on to a 96 well plate and absorbance of the supernatants was read at 405 nm.

RESULTS

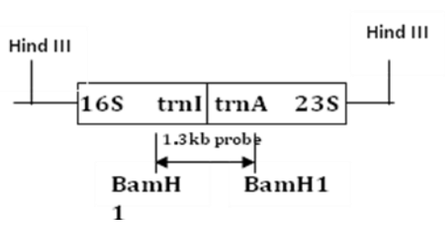
Construction of lettuce chloroplast transformation vectors containing TB antigen

The lettuce chloroplast vector pLS-CTB-ESAT6 harboring CTB-ESAT6 was created (Fig. 1a). CTB-ESAT6 contained GPGP (Gly Pro Gly Pro) hinge in the middle of fusion proteins to assist in correct folding of each protein by lowering the steric hindrance. The lettuce vectors contained flanking sequences 16S/*trn*I and *trn*A/23S (Fig. 1a) to facilitate recombination into the native chloroplast genome. The fusion gene cassettes were regulated by endogenous *psb*A promoter and 5' untranslated region (UTR) to achieve higher levels of expression due to the presence of multiple ribosome binding sites (Fernandez-San Millan et al. 2003; Ruhlman et al. 2010). The *psb*A 3' UTR located at the 3' end of the introduced gene cassette conferred transcript stability (Stern et al. 1987). The endogenous constitutive 16S rRNA promoter (Prrn) was employed to regulate expression of the *aad*A (aminoglycoside 3' adenyltransferase) gene with a GGAG ribosome binding site upstream of the start codon AUG (Dhingra et al. 2006) to confer spectinomycin resistance. The final chloroplast transformation vector pLS-CTB-ESAT6 was sequenced and used for transformation studies.

(a)



(b)



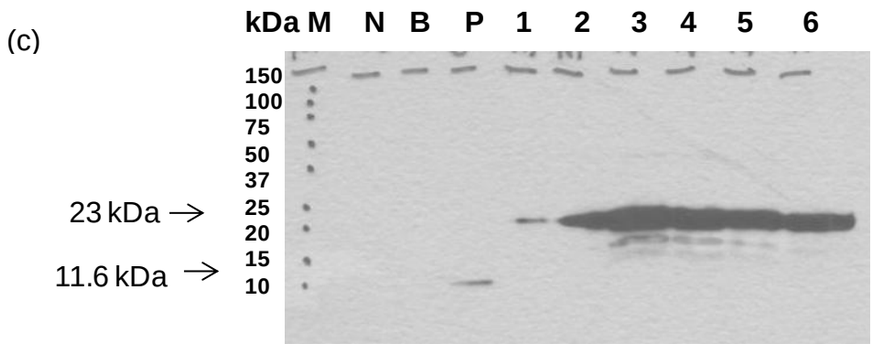


Figure 1: Vector construction for chloroplast transformation.

(a) pLS - CTB - ESAT6 vector. (b) Schematic diagram of expected products from digestion of the untransformed lettuce chloroplast genome respectively for the flanking sequence hybridization probe. (c) Immunoblot analysis of CTB-ESAT6 transformed *E.coli* cells for expression of CTB – ESAT6.(N – untransformed e.coli, P – CTB standard(50 ng); 1-6 – transformed CTB – ESAT6 expressing E.coli; B – Blank; M- Marker.

Confirmation of site specific transgene integration by PCR analysis

Four spectinomycin resistant lettuce shoots per forty bombardments were recovered with pLS-CTB-ESAT6 vector coated on gold particles. Site specific transgene integration of independent spectinomycin resistant shoots was verified by polymerase chain reaction (PCR) using 16SF/3M and 5P/2M primer pairs in lettuce. The 16SF primer anneals to the native chloroplast genome within the 16S rRNA gene whereas 3M primer anneals to the *aad*A gene (Fig.1a). PCR reaction with 16SF/3M primers generated a 2.77 kb fragment in lettuce CTB-ESAT6 transplastomic lines (Figure 2a, Lanes: 1-4), which should be obtained only if site specific integration had occurred. Nuclear transformants, mutants and untransformed plants did not show any PCR product (Figure 2a, Lane: N). Nuclear transformants could be distinguished because 16SF will not anneal and mutants were identified because 3M will not anneal and thus eliminates shoots that have nuclear integration or spontaneous mutation of the

16S rRNA gene. The integration of transgene cassette was further tested by using 5P/2M primer pair. The 5P primer anneals to the *aad*A gene upstream of CTB-TB antigen fusion gene cassette whereas 2M anneals to the *trn*A gene (Fig.1a). The 5P/2M primer pair generated a 2.53 kb PCR product in CTB-ESAT6 lettuce transplastomic lines respectively while no amplification was observed in untransformed plants as expected (Fig. 2b).

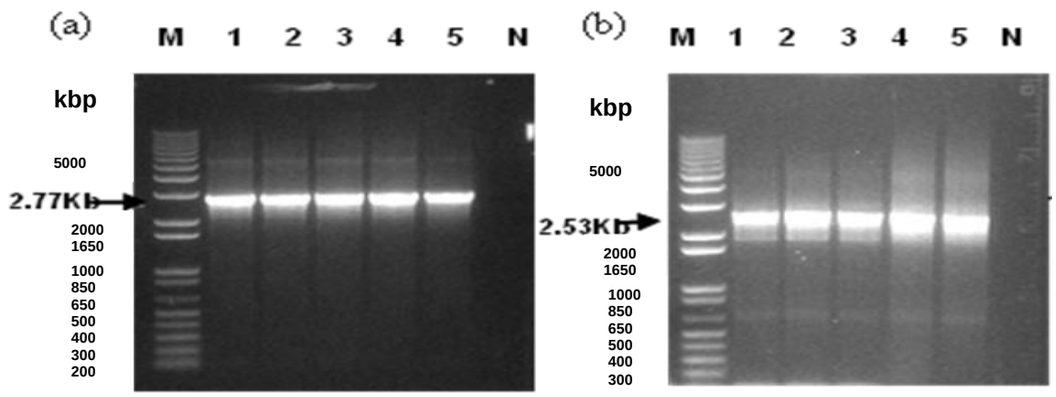


Figure 2 : PCR analysis of lettuce transplastomic plants using (a) 16SF/3M primer (b) 5P/2M primers

(WT, untransformed plant; Number 1 to 4, transformed plants; 5, positive control; M, DNA ladder)

Evaluation of homoplasmy in transplastomic plants by Southern blot analysis

Following PCR analysis, transplastomic plants were subjected to two additional rounds of selection (second and third) to promote homoplasmy. Southern blot analysis was performed to determine homoplasmy or heteroplasmy and to supplement the PCR confirmation of site specific transgene integration. The flanking sequence probe (1.13 kb in lettuce Fig. 1b) allowed detection of site-specific integration of the gene cassette into the chloroplast genome as it hybridizes with the *trn*I and *trn*A genes. The

transformed chloroplast genome digested with HindIII produced fragments of 11.49 kb for pLS-CTB-ESAT6 (Fig.1a) when hybridized with flanking sequence probe. The untransformed lettuce chloroplast genome digested with *Hind*III produced a 9.11 kb fragment respectively confirming that these plants lacked foreign genes (Fig.1a). The flanking sequence probe helps us identify if homoplasmy of the chloroplast genome was achieved through more rounds of selection. Absence of 9.11 kb fragment in lettuce CTB-ESAT6 transplastomic lines respectively confirmed homoplasmy (within the detection limits of Southern blot) and stable integration of foreign genes into the chloroplast genome (Fig 3). Plants confirmed by Southern analysis were transferred to jiffy pellets and placed in 16hr light/8hr dark cycle incubator until they were acclimatized.

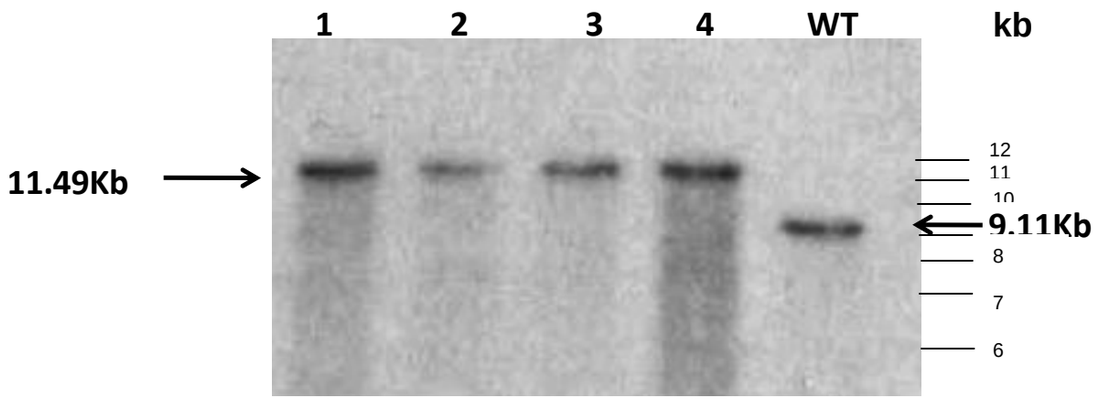


Figure 3: Southern blot analysis of lettuce CTB-ESAT6 homoplasmy transplastomic plants

(WT, untransformed plant; Number 1 to 4, transformed plants).

Selection and Generation of Transplastomic Plants

Transplastomic shoots were selected and generated by the use of RMOP selection media for lettuce with spectinomycin (Figure 4 A). Upon determination of positive transformants by PCR analysis, further selection and generation of transplastomic shoots was continued (Figure 4 B). For rooting of the plants, ½ MS

selection media containing spectinomycin was used (Figure 4 C). The *in vitro* plants were confirmed homoplasmic by Southern Blot analysis. Later they were transferred to green house where they matured and seeds were collected

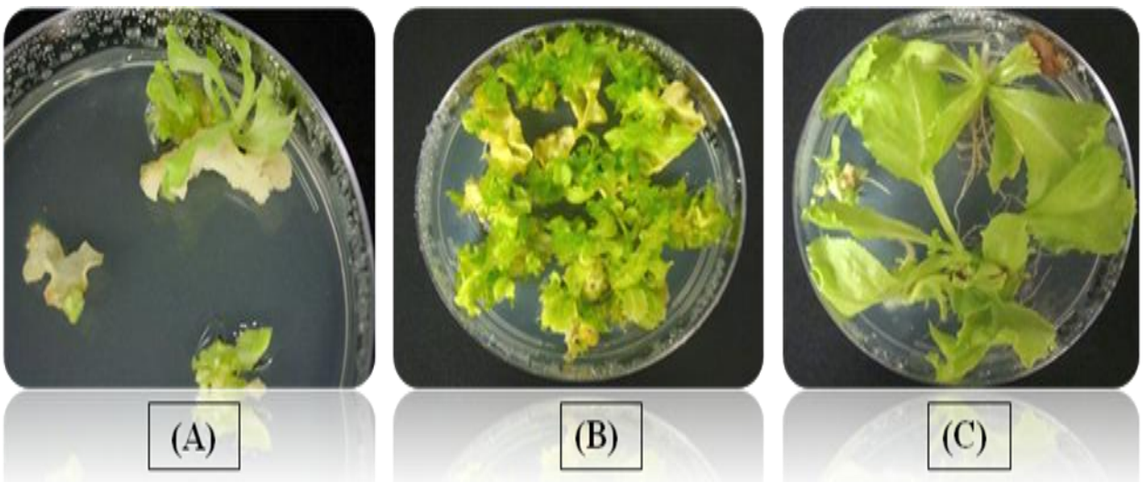


Figure 4: Selection of transplastomic plants

(A) Four to eight weeks after bombardment, transplastomic shoots can be seen on RMOP selection medium. **(B)** The second round of selection leaves from PCR positive transformants are transferred on to spectinomycin containing RMOP selection medium. **(C)** The third round of selection, shoots that were regenerated are transferred to ½ MS selection medium and rooting takes place in ten days.

Analysis of transgene segregation

Seeds collected from only T_0 CTB - ESAT6 transplastomic and untransformed lettuce plants were germinated on ½ MS medium with spectinomycin in the same plate. All T_1 CTB-ESAT6 lettuce seeds germinated and developed into uniformly green plants. This lack of Mendelian segregation of genes confirmed maternal inheritance of transgenes. None of the untransformed seeds germinated on the selection media.

Further, after transfer to green house, all T1 plants flowered, set seeds and showed similar phenotype when compared to T_0 and untransformed plants.

(a) (b)

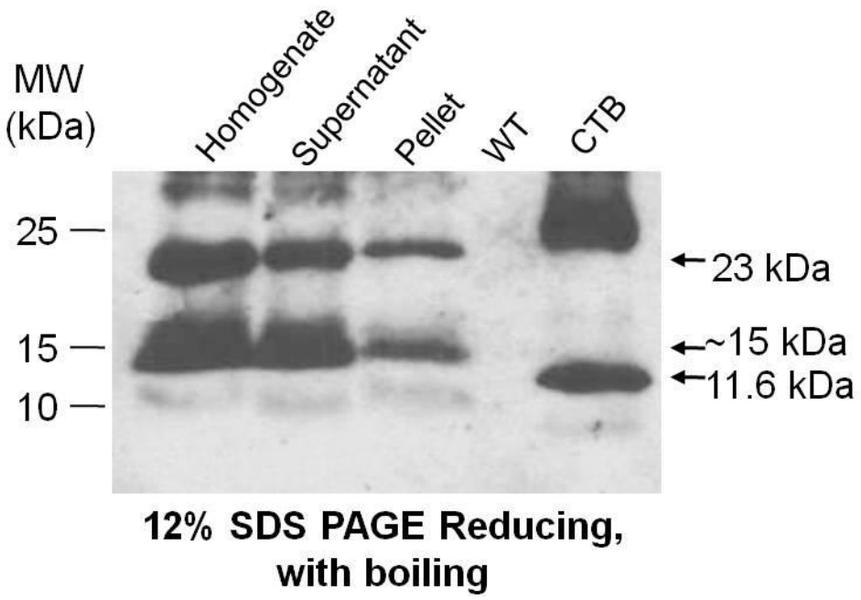
Figure 5: Analysis of transgene integration

(a) Maternal inheritance was confirmed with Wild Type (WT) vs. T1 seedlings. (b) Normal phenotype of transplastomic plants

Characterization of the Chloroplast-Derived CTB- ESAT6 protein

Expression of the fusion proteins ESAT-6 were analyzed by immunoblots with different extraction fractions of leaf extracts using anti-CTB polyclonal antibody. Under reducing conditions, blots probed with anti CTB polyclonal antibody revealed CTB-ESAT6 (CTB-ESAT6-Ls), the monomer form of fusion protein corresponding to 23 kDa was observed (Fig. 6a). Lettuce CTB-ESAT6 plants showed an additional lower band of about 15 kDa with anti–CTB antibody (Fig.6a).

(a)



(b)

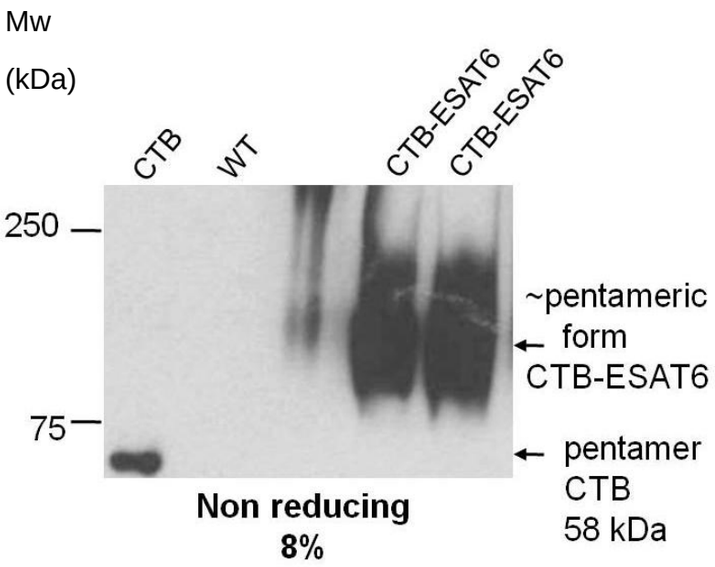


Figure 6: Immunoblot analysis under reducing and nonreducing conditions.

(c)

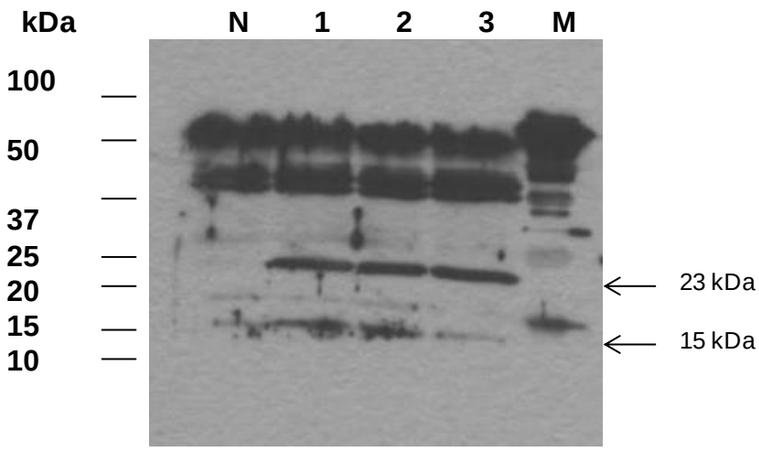


Figure 7: Immunoblot analysis under reducing and nonreducing conditions

Different leaf extraction fractions analyzed using CTB antibody (a) reducing conditions (b) :pentameric form of CTB-ESAT6 is shown along with pentameric form of CTB standard.(WT, – wildtype lettuce leaf protein, CTB- CTB purified protein (c) Immunoblot analysis with ESAT6 antibody (WT, N – wildtype lettuce leaf protein; 1,2,3 - CTB-ESAT6 plant protein).

This band might have been formed by proteolytic cleavage of fusion protein due to the presence of putative protease-sensitive sites. Since, the band is larger than CTB protein size (11.6 kDa), cleavage should be in ESAT-6. Analysis of different extraction fractions of fusion protein CTB-ESAT6 from lettuce indicated that most of the fusion protein existed in soluble form. Some of the protein was also detected in pellet under our experimental conditions (Fig. 6a).

We performed Peptide Cutter analysis the ExPASy server on ESAT-6 sequence to predict protein cleavage sites (Gasteiger E. et al. 2005). Peptide Cutter identified several cleavage sites closer to N-terminus of ESAT-6 (~25 amino acid) indicating a higher probability of cleavage in ESAT-6 protein.

Table 1: Possible cleavage sites of CTB - ESAT6 using peptide cutter software.

No.	Name of enzyme	# Cleavages	Position of Cleavage sites
1.	Arg-C proteinase	1	74
2.	Asp-N endopeptidase	2	29 58
3.	Asp-N endopeptidase + N-terminal Glu	9	2 11 29 30 48 58 63 77 86
4.	BNPS-Skatole	3	6 43 58
5.	CNBr	3	1 83 93
6.	Chymotrypsin-high specificity (C-term to [FYW], not before P)	6	6 8 43 51 58 94
7.	Chymotrypsin-low specificity (C-term to [FYWML], not before P)	17	1 6 8 26 28 29 36 39 43 51 58 65 69 72 83 93 94
8.	Clostripain	1	74
9.	Formic acid	2	30 59
10.	Glutamyl endopeptidase	7	3 12 31 49 64 78 87
11.	Iodosobenzoic acid	3	6 43 58
12.	LysC	3	33 38 57
13.	LysN	3	32 37 56
14.	Pepsin (pH1.3)	23	5 6 7 8 27 29 36 38 39 42 43 50 51 57 8 64 65 68 69 71 72 93 94
15.	Pepsin (pH>2)	15	7 8 27 29 36 38 39 64 65 68 69 71 72 93 94
16.	Proteinase K	45	2 6 8 9 11 13 14 15 17 18 22 23 25 28 29 36 37 39 40 41 42 43 50 51 54 58 60 61 62 63 65 68 69 72 73 75 76 79 82 84 86 90 91 94 95
17.	Staphylococcal peptidase I	7	3 12 31 49 64 78 87
18.	Thermolysin	30	7 8 10 13 14 16 17 21 24 27 28 35 38 39 40 41 53 61 67 68 71 72 75 81 82 83 89 92 93 94
19.	Trypsin	4	33 38 57 74

Quantification of CTB–ESAT6 fusion protein in transplastomic lettuce plants by densitometry

Immunoblots of different extraction fractions of CTB–ESAT6 lettuce transplastomic plants indicated the presence of fusion protein in both supernatant and pellet (Fig.6b). Quantification of CTB-ESAT6-Ls fusion protein by ELISA couldn't be correlated with western analysis. Therefore, CTB-ESAT6-Ls expression levels were determined on western blots by comparing homogenate fraction with known quantities of purified CTB and analyzing them by spot densitometry. Linearity of the standard curve was established using 25, 50 and 75 ng of purified CTB, enabling the estimation of CTB-ESAT6 expression (Fig. 7b). In lettuce, CTB–ESAT6 accumulated up to 0.75% of total leaf protein (Fig 7a). Mature leaves showed highest expression followed by young leaves (Fig. 7c). Old leaves showed lowest level of expression probably due to senescence and proteolytic activity. Similarly different time points of harvest were analyzed using densitometry. We observed an increasing trend from morning to evening with highest expression levels of fusion protein at 6 PM (Fig.7d). One of the limitations in oral delivery studies is the low level of expression of protein.

(a)

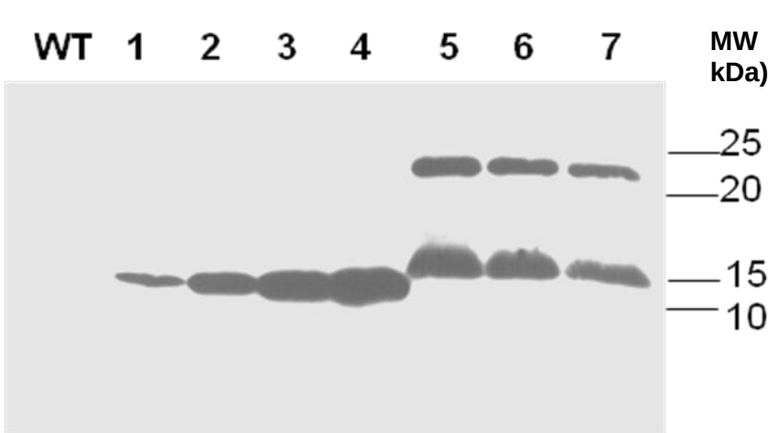
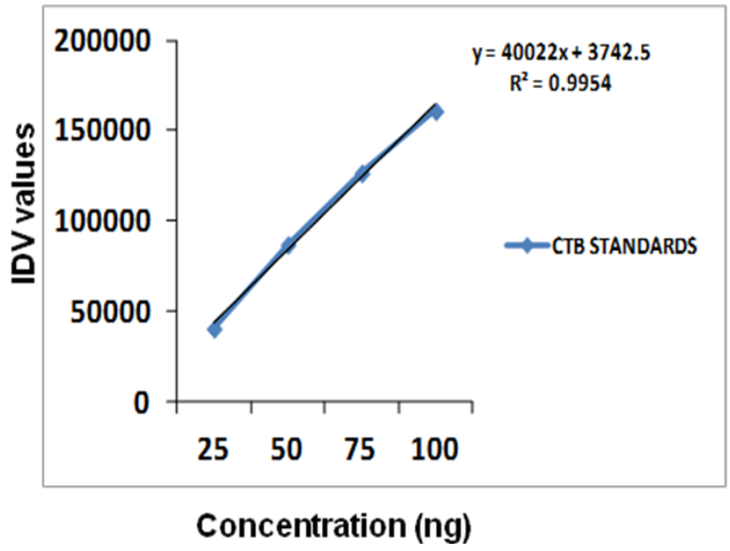
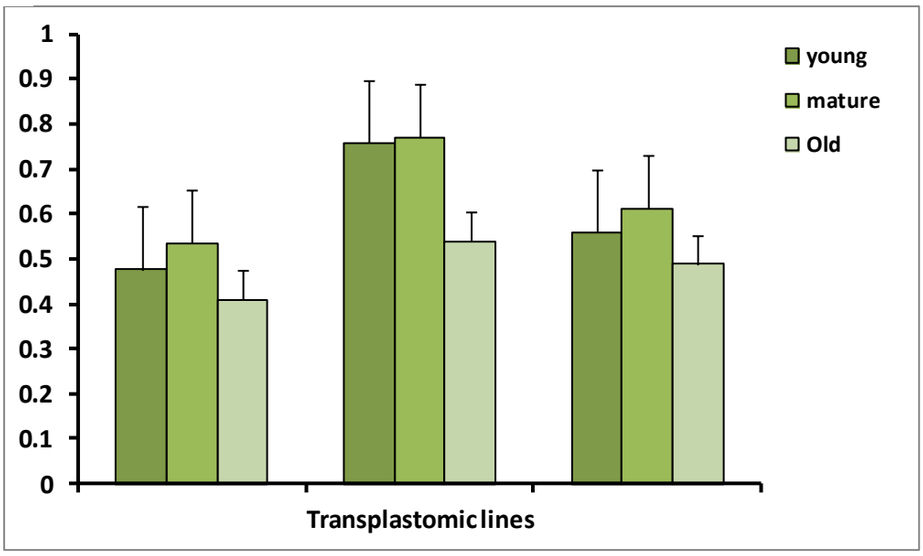


Figure 8: Quantification of Chloroplast-Derived CTB-ESAT6 expression

(b)



(c)



(d)

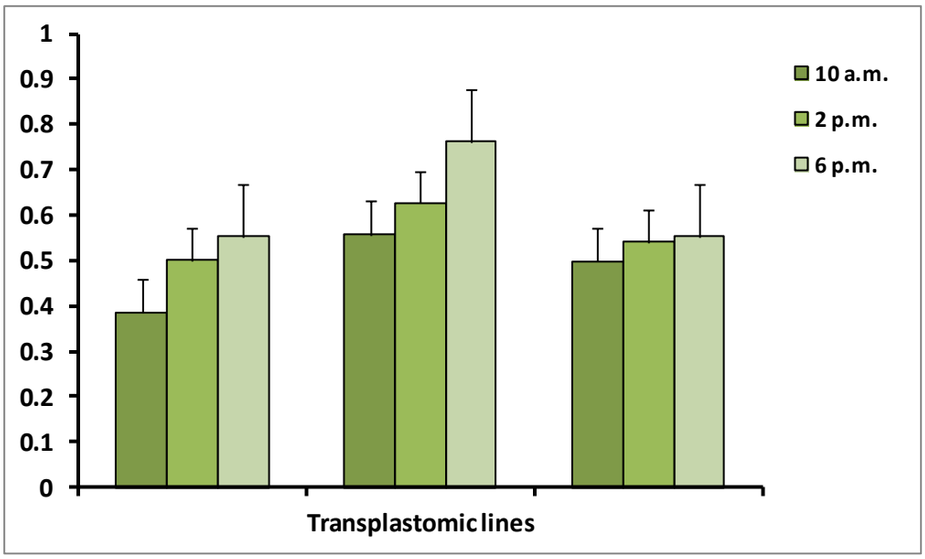


Figure 7: Quantification of Chloroplast-Derived CTB-ESAT6 expression

(a) Bacterial CTB protein of known concentrations were loaded as standards and different concentrations of transgenic protein were evaluated with anti - CTB antibody Lanes 1,2,3,4 : CTB standards 25 ng, 50 ng, 75 ng,100 ng lane 5,6,7 young, mature, old. (b) Standard curve was plotted with alpha ease software. Quantification of CTB-ESAT6 (Ls) in T0 lettuce transplastomic plants at different developmental stages and harvesting time by densitometry. (c) Expression levels of lettuce CTB-ESAT6 (Ls) in percent TP at different developmental stages. (d) Expression levels at different harvesting time points. (1, 2, 3 – Transplastomic lines)

Affinity of plant-derived CTB-ESAT6 for GM1-ganglioside receptor

To evaluate whether CTB-ESAT6 fusion protein produced in lettuce retained its biological function of binding to the GM1 receptor, we performed GM1-binding ELISA assay. A pentameric structure of CTB protein is required for binding to its receptor GM1-ganglioside *invivo* (Tsuji et al. 1995; de Haan et al. 1998). CTB- ESAT6 plants along with purified CTB protein showed strong binding affinity to GM-1 (Fig. 8). Untransformed plants and bovine serum albumin (BSA) didn't show binding to the GM1 receptor(Fig 8).

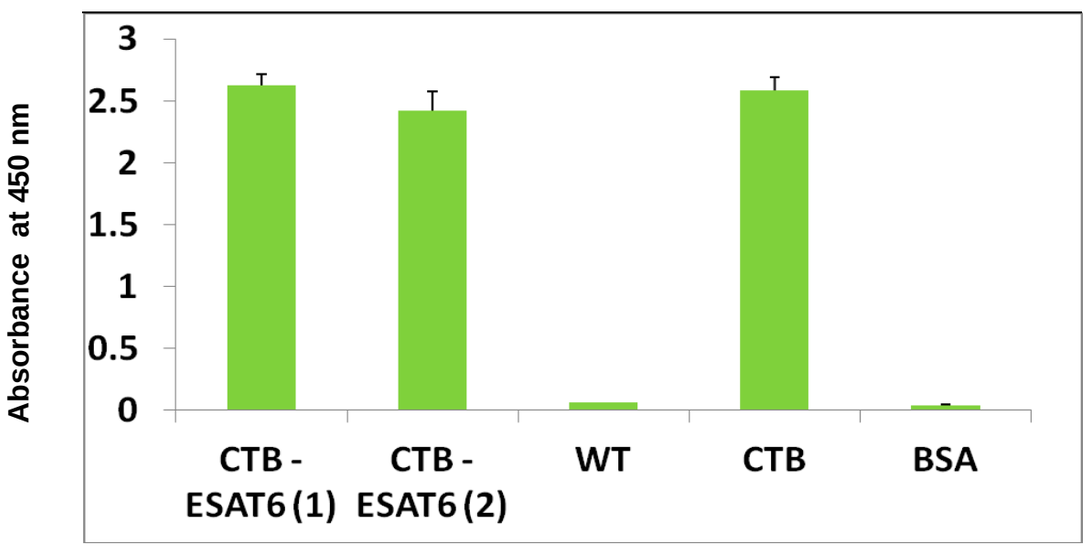


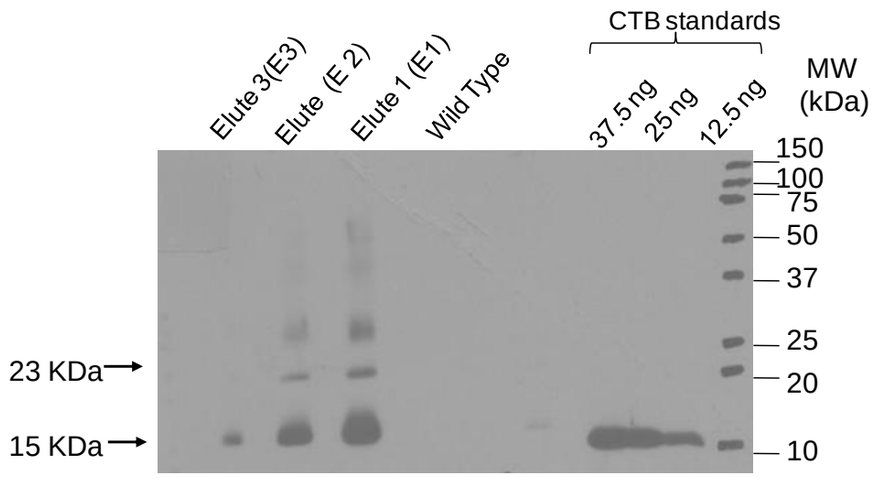
Figure 9: Ganglioside GM1 ELISA binding assay

Functionality of CTB was confirmed based on its binding affinity to Ganglioside GM1 (E1,E2 - CTB–ESAT6 (Ls) total protein (15µg); WT - Wild type lettuce leaf protein (15µg); CTB – purified CTB standard - 50 ng; BSA - 50ng).

Detection of pore formation in red blood cell membranes by purified CTB-ESAT6 protein using Hemolysis assay

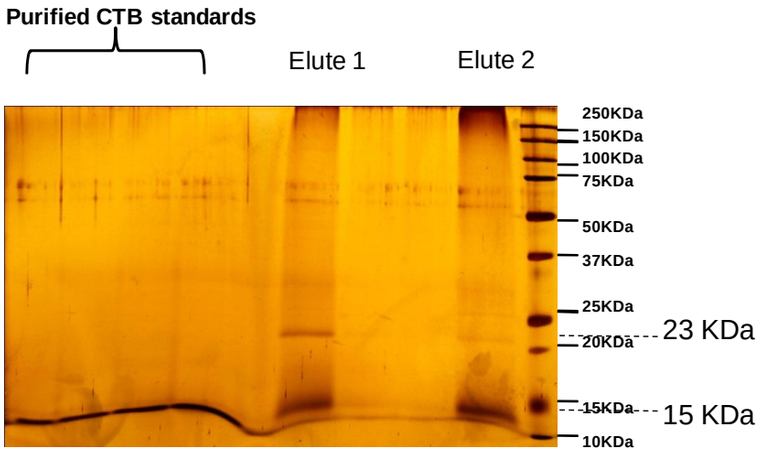
Lettuce expressing CTB-ESAT6 protein is an oral edible vaccine for TB; therefore there is no need to perform purification of antigens. Hence, a tag such as histidine was not incorporated in the coding sequence. To perform hemolysis assay purification of CTB-ESAT6 fusion protein is necessary. Therefore, we purified CTB–ESAT6 fusion protein from lettuce plants using immunoaffinity purification with CTB antibody. Western blot analysis of purified protein detected multiple bands corresponding to monomeric 23 kDa, cleaved 15 kDa and aggregates or multimers of >23 kDa molecular weight (Fig. 9a). Based on densitometry analysis, upto 40 µg/ml of 80% pure CTB–ESAT6 was obtained and this concentration was further confirmed by ELISA. Silver staining of purified protein showed two protein bands of ~23 kDa and ~15 kDa (Fig. 9b). Presence of ~15 kDa protein band showed that the cleavage occurred in the ESAT6 protein. Purified CTB protein showed band at their corresponding size (Fig. 9b). Protein band corresponding to CTB-antibody was not detected in silver staining as CTB-antibody was cross linked on to the protein A bead support to prevent antibody being co eluted with the antigen. The purified CTB-ESAT6 protein was used for hemolysis assay.

(a)



Immunoblot of 12% SDS – PAGE gel using anti – CTB Antibody

(b)



Silver staining of 12% SDS - PAGE gel

Figure 10: Antibody affinity purification of CTB-ESAT6

(a) Immunoblot analysis to confirm the presence of antigen with CTB antibody S1, S2, S3: CTB standard samples (12.5, 25, 37.5 ng); E1, E2, E3: elution fractions (100, 50, 25 ng) respectively; B4IP – CTB-ESAT6 protein before purification (10 µg); WT- wild type lettuce total leaf protein (10µg). (b) Silver staining after affinity purification of CTB – ESAT6. C1, C2, C3, C4: purified CTB standards (100,200,300,400 ng); after affinity

purification, elution fractions of CTB-ESAT6 (LS) - E1 (400 ng), E2 (200 ng). M – Protein standard marker 0.4µl.

MTB uses ESX-1 secretion system to export virulence proteins during infection. ESAT-6 is one of the secreted proteins in ESX-1 system and has been reported to play a role in the escape of *Mycobacterium* from the phagolysosome (van der Wel et al. 2007) by membrane pore formation (Smith et al. 2008). Purified ESAT-6 has been proven to cause dose dependent hemolysis in red blood cells by membrane pore formation (Smith et al. 2008). The red blood cell lysis by pore forming proteins occurs by osmotic shock. The hemolytic effect of plant-derived partially purified ESAT6 was investigated on red blood cell membranes. Hemolysis was measured by the absorbance (O.D.) of the red blood cell supernatant which contains lysed cell hemoglobin. Partially purified CTB-ESAT6 when solubilized resulted in dose dependent hemolysis. CTB–ESAT6 formed aggregates in its native form and hence was solubilized to dissociate the oligomeric protein into its monomer form. Purified protein without solubilisation did not cause hemolysis at a concentration of 40 µg/ml (Fig. 10). After solubilisation, CTB–ESAT6 at 40 µg/ml protein concentration caused partial hemolysis (45%) of red blood cells with an absorbance of 0.85. Decrease in absorbance to 0.4 (21%) was observed when the protein was diluted two fold (Fig.10). This indicated that the fusion did not modify ESAT-6 protein ability to form pores and lysed red blood cell membranes in a dose-dependent manner. Red blood cells incubated with water resulted in complete lysis (absorbance of 1.9) as the cells swell and burst due to movement of water into cells. Red blood cells incubated in PBS solution were intact and therefore no absorbance was detected (Fig. 10). Complete hemolysis of red blood cell membranes was not accomplished with solubilized purified CTB-ESAT6 protein as higher concentrations (>40 µg/ml) could not be achieved by affinity purification.

$$\%\ \text{Hemolysis} = \frac{\text{absorbance of sample}}{\text{absorbance of positive control(H2O)}} \times 100$$

Percent hemolysis was calculated using the above formula.

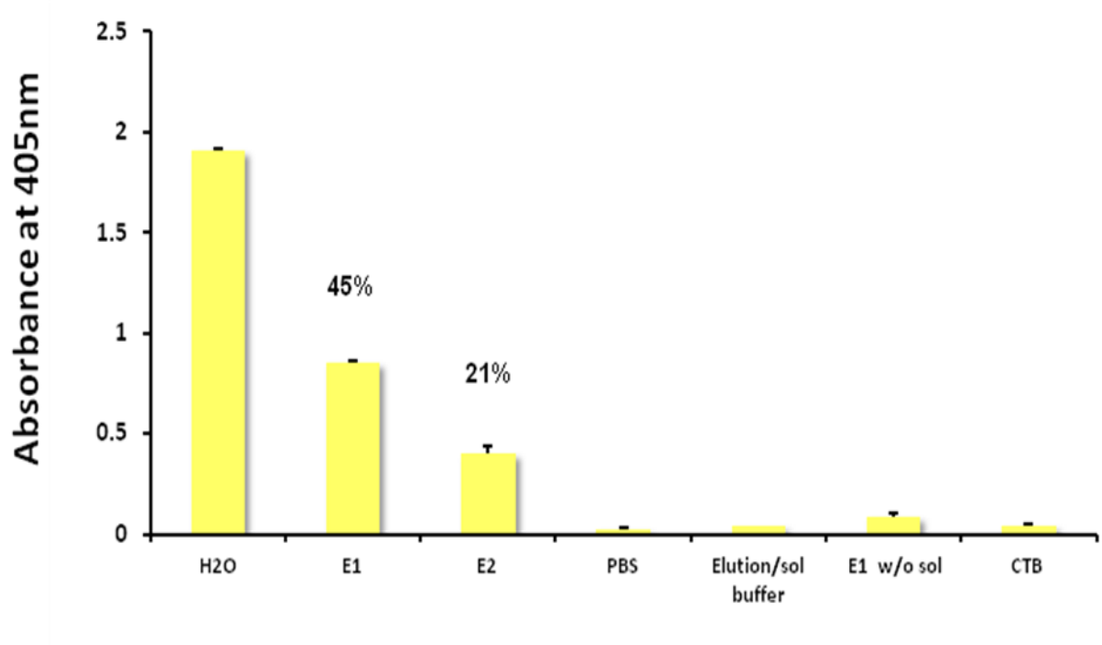


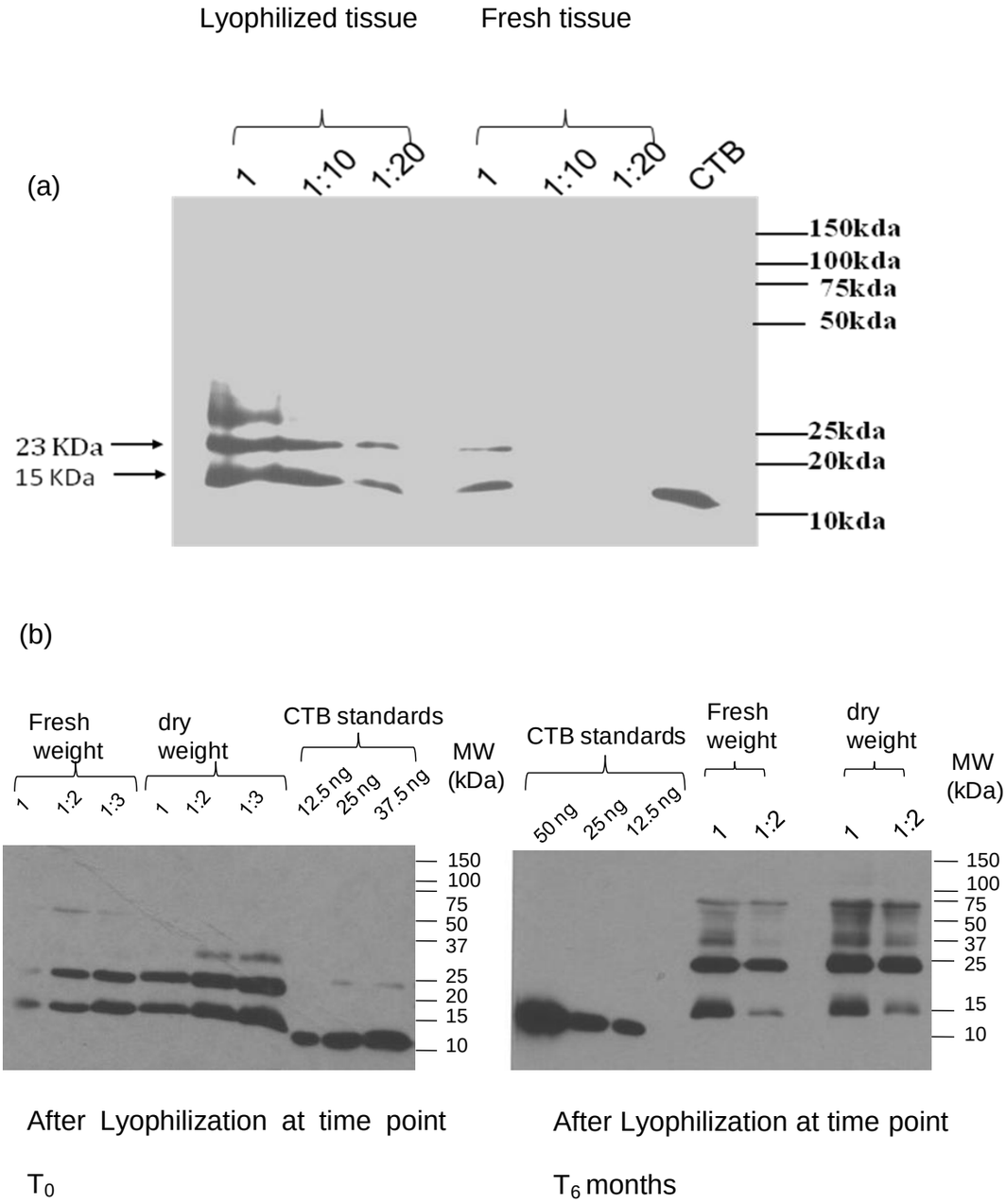
Figure 11 : Hemolysis assay to determine functionality of ESAT-6 in partially purified fusion protein CTB-ESAT6

H_20 – distilled water; E1- CTB-ESAT6 – partially purified protein 40 µg/ml ; E2 – CTB-ESAT6 – partially purified protein 20 µg/ml; PBS – control; Elution/sol buffer – elution buffer used for protein elution/solubilisation; Nonsol E1 – CTB – ESAT6 (Ls) before solubilisation. CTB – purified protein 40 µg/ml;

Lyophilization studies for storage of CTB- ESAT6 oral vaccine

We performed lyophilization of CTB–ESAT6 expressing lettuce leaves for stable storage and discovered that the amount of antigen per gram of leaf material increased. After lyophilization, leaves were reduced to 5-8% of their fresh weight thereby increasing the CTB-ESAT6 antigenic content per gram of leaf material. Analysis of lyophilized material on immunoblots with anti-CTB antibody revealed same protein

bands as obtained with fresh leaf material (Fig. 11). There was a 22 fold increase in antigenic content per gram in lyophilized leaves when compared to fresh leaves.



After Lyophilization at time point T_0

After Lyophilization at time point T_6 months

Figure 12 : Lyophilization studies - Immunoblot analysis of comparison of fresh weight and lyophilized material.

(a) CTB – ESAT6 Protein loaded in equal concentration as both fresh weight and lyophilized tissue material. (b) Effect of lyophilized tissue for storage: Immunoblot analysis at time point T0 immediately after lyophilization and 6 months after lyophilization at time point T6 months.

Effect of lyophilization on the binding ability of CTB-ESAT6 fusion protein was also tested with GM-1 ELISA binding assay. An extract from CTB-ESAT6 lyophilized lettuce leaves effectively bound to the GM1-ganglioside receptors (Fig. 7). Further, serial dilution of extract from fresh, lyophilized leaves and purified CTB showed decrease in absorbance accordingly (Fig. 7). GM1 binding of CTB-ESAT6 fusion protein from lyophilized and fresh lettuce leaves indicates that the CTB in the fusion protein has retained its native pentameric structure and has not been disrupted by its fusion to ESAT-6 or by lyophilization process. Binding to GM1 receptor is essential for antigen uptake in the gut.

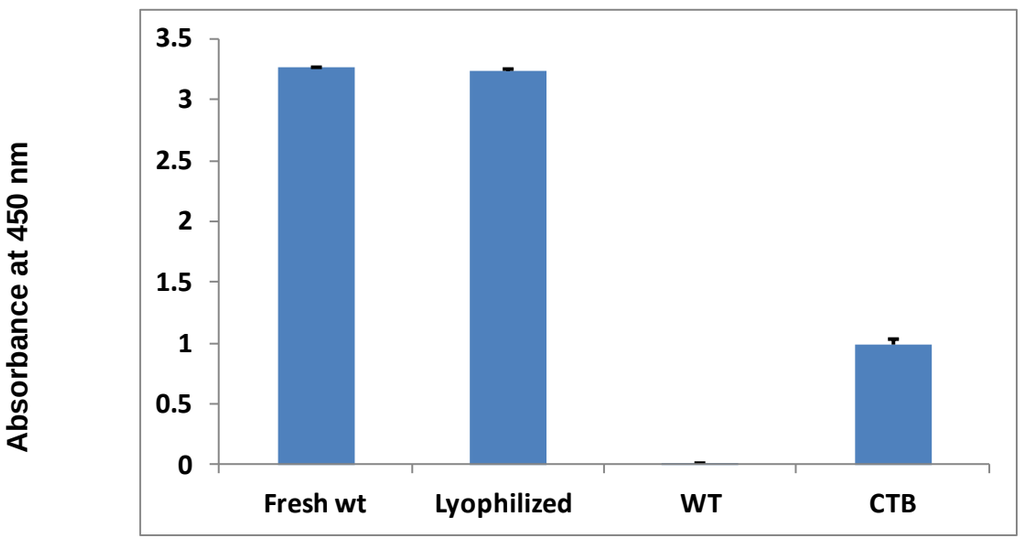


Figure 13: GM1 ELISA comparison between lyophilized and fresh tissue

Equal volume loaded at equal concentrations of TSP (15µg) with decreasing dilutions gave similar trend. Purified CTB – 10 ng. WT – untransformed TSP (15µg)

DISCUSSION

Recent developments in genetic engineering have revealed enormous potential in plant chloroplasts such as high expression, cost-effectiveness, scalability and safety in production systems for recombinant biopharmaceuticals and vaccines ((Ma et al. 2003; Daniell 2006; Yusibov et al. 2008; Daniell et al. 2009). To my knowledge, this is the first report of expression of TB vaccine antigens in chloroplasts. In this study, we expressed ESAT-6 ,an attractive candidate antigens for TB vaccine development in lettuce chloroplasts. Results exhibit an efficient and stable expression of the recombinant fusion protein CTB-ESAT6 and CTB-MTB72F in chloroplasts. The main factors that are essential for practical development of oral plant vaccines are sufficient expression level of antigens in the system used, adequate amount of dosage (leaf material) required for immunization, stability and storage of vaccines apart from efficacy. Chloroplast expression system is very well suited for production of TB vaccine antigens to achieve all the parameters mentioned above.

The CTB–ESAT6 lettuce transplastomic plants have modest expression levels of 0.75% of total leaf protein (TP). In comparison to established tobacco system, lettuce has shown lower expression levels with other antigens (Ruhlman et al. 2010). The variation in expression levels of recombinant proteins can be due to many factors including nature of protein, plant system, environmental conditions, protein stability in chloroplasts and regulatory elements present in expression cassette (Scotti et al. 2011). Since lower expression levels of CTB–ESAT6 in lettuce, variation could be due to protein stability and production in chloroplasts of lettuce.

Our results indicated that CTB–ESAT6 lettuce plants expressed 11.2 µg/g (fresh weight mature leaf) of antigen whereas lyophilization increased the yield 22 fold as the antigenic content built up to 249 µg/g of lyophilized leaves. Hence large amount of transgenic protein is available for oral delivery. In human trials for TB subunit vaccine ESAT-6, 50 µg of vaccine antigen was injected intramuscularly (van Dissel et al. 2010).

So if 50 µg were to be orally fed, based on quantification of antigenic content, only 200 mg of lyophilized material would be needed. Lyophilized lettuce expressing hepatitis B surface antigen has been successfully used in orally delivered plant vaccine animal studies (Pniewski et al. 2011). Therefore, all *in vitro* functional studies were carried out with lettuce plants.

Lettuce (*Lactuca sativa)* was chosen as an alternative to tobacco for expression of TB vaccine antigens due to its many advantages such as it being an edible crop, its leafy nature and commercial importance. The lettuce CTB-ESAT6 plants showed modest expression levels which can be remedied with lyophilization, a process of freeze drying. Since lettuce has high water content (95%), it can be freeze dried to a greater magnitude than tobacco, potato etc. Lettuce is a relatively easy plant to grow with no special conditions except cooler environment and can be grown in a few months from sowing. Since it is a leafy vegetable, more antigen could be concentrated by lyophilization of leaf tissue. The CTB–ESAT6 protein was stable in lyophilized material stored at room temperature for six months. This stability of antigens in plant tissue could help in eliminating cold chain during storage and distribution required for conventional vaccines.

Functional analysis of CTB-ESAT6 fusion protein was performed. The ability of CTB to form pentamers allows it to bind to GM-1 Ganglioside receptors and gives it the advantage of increased antigen uptake. GM-1 ELISA binding assay revealed the ability of CTB–ESAT6 fusion protein to form pentamers and bind to the GM-1 receptors. ESAT-6 is a secreted protein that is observed in early mycobacterial infections. Its activity has been characterized as a cytolysin that can disrupt lipid bilayers (Hsu et al. 2003). Hemolysis assay established the ability of partially purified CTB-ESAT6 to create partial lysis of red blood cell membranes. Plant derived CTB-ESAT6 has been shown to retain its biologically activity and has potential to be an effective oral vaccine.

Production of an oral subunit vaccine against tuberculosis in chloroplast is a promising strategy to overcome the cost constraints such as production, purification,

processing, cold storage, transportation and delivery linked to large vaccination campaigns, especially with the increasing TB incidence in some developing countries where there is a dire need of such vaccines. Lyophilization can help improve the stability of the vaccine for prolonged storage. This could help in improving shelf life of antigens. It is estimated that the costs associated with production and delivery of the recombinant proteins in bacterial, insects or mammalian cells is much higher when compared to plants (Chebolu et al. 2009). Easy and unlimited scalability of production and absence of the viral contamination can make plant-derived biologics economical and safer for large-scale production (Goldstein et al. 2004; Yusibov et al. 2008).

For oral delivery analysis of this TB vaccine antigen expressed in chloroplast, animal studies will be performed in future to test its immunogenicity. Lettuce, an edible crop plant is an ideal system for oral delivery of CTB-ESAT6 vaccine antigen. Multistage vaccines will be significant against a complex disease such as tuberculosis. As chloroplasts have polycistronic mRNA, we have the added advantage of expressing multiple TB genes in the chloroplasts. So constructing a vaccine with multiple antigens that can target early and late stage tuberculosis can pave the way for future oral TB vaccine.

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