

Expression Of Lipase From Mycobacterium Tuberculosis In Nicotiana Tobacum And Lactuca Sativa Chloroplasts

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**EXPRESSION OF LIPASE FROM *MYCOBACTERIUM TUBERCULOSIS* IN
NICOTIANA TABACUM AND *LACTUCA SATIVA* CHLOROPLASTS**

by

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A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
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ABSTRACT

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* (*M. tuberculosis*), is a global threat and the leading cause of death among individuals infected with HIV. TB treatment requires multi-drug cocktails, due to the increasing rates of drug resistance of the bacterium. With multi-drug cocktails, strains have been documented to be resistant to all major drugs in the fight against TB. Since the strains are drug resistant, it calls for an increasing need for vaccine and treatment development for the purpose of preventing and managing the disease. The most widely distributed vaccine against TB is Bacillus Calmette-Gue´rin (BCG). Apart from being ineffective in certain individuals, BCG offers only a limited timeframe of protection, is unable to serve as a booster for extending this timeframe and due to the intradermal route of administration requires costly refrigeration and syringes.

LipY protein, a *M. tuberculosis* cell wall lipase, may play a potential role as not only a drug target but a potential vaccine antigen. LipY is known to be up-regulated during both active infection and dormancy. In a previous study, sera from TB patients had shown an IgG and IgM response against it.

In this study transplastomic *Lactuca sativa* and *Nicotiana tabacum* plants were generated by transforming the chloroplasts through the particle delivery system with pLsDv-LipY and pLD-LipY vectors respectively. The vectors were flanked by the native trnI and trnA gene sequence to facilitate homologous recombination into the chloroplast genome. The vector also contained the 16S rRNA promoter, the selectable marker gene, aadA for spectinomycin resistance, the rbcL untranslated region, the LsPpsbA (PpsbA in *N. tabacum*) promoter, and LsTpsbA (tpsba in *N. tabacum*) untranslated region.

Site specific integration of the LipY gene into the chloroplast genome was confirmed by PCR. Homoplasmy of transplastomic plants was confirmed by Southern blot analysis. These plants showed normal growth and were fertile, producing seeds. Once germinated, these seeds did not show Mendelian segregation of the transgene. Immunoblot analysis was performed to analyze the expression of the LipY protein. A 40kDa protein was produced in *E.coli*, and a 25kDa protein was produced in chloroplasts; a cleaved product in chloroplasts is still valuable as an antigen for vaccine production.

Future studies will include testing this chloroplast derived antigen in animal models for vaccine development.

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INTRODUCTION

Background and significance of *Mycobacterium tuberculosis*

Tuberculosis is a global threat, and current endeavors to eradicate the disease face the challenges of efficacy, cost-effectiveness, drug resistant organisms, and potential toxicity of available treatment (Cohen, Murray et al. 2011; Sharma and Yoder 2011). The causative agent of this threat is the bacteria, *Mycobacterium tuberculosis* (*M. tuberculosis*). *M. tuberculosis* enters the host through mucosal surfaces found in the digestive system and respiratory system. Once it enters through these surfaces it is eventually ingested by macrophages or other phagocytic cells.

In a typical immune response phagocytic cells play a major role in ridding the body of invading bacteria by ingesting them through phagocytosis and then eliminating them within lysosomes. They then act as antigen presenting cells (APC) in the primary immune response. While there is variety of pathways for an immune response, two that should be highlighted, as they pertain to *M. tuberculosis* infection and potential vaccine considerations, are the T_h1 and T_h2 immune response pathways. The T_h1 pathway participates in cell-mediated immunity is essential for controlling intracellular pathogens such as viruses and *M. tuberculosis*, and the main partner cells are macrophages. The T_h2 pathway provides help for B-cells and antibodies are required to control extracellular pathogens (humoral immune response) (Dietrich and Doherty 2009). The importance of these two pathways, how they relate to *M. tuberculosis*, and their relation to LipY will be addressed later in this paper.

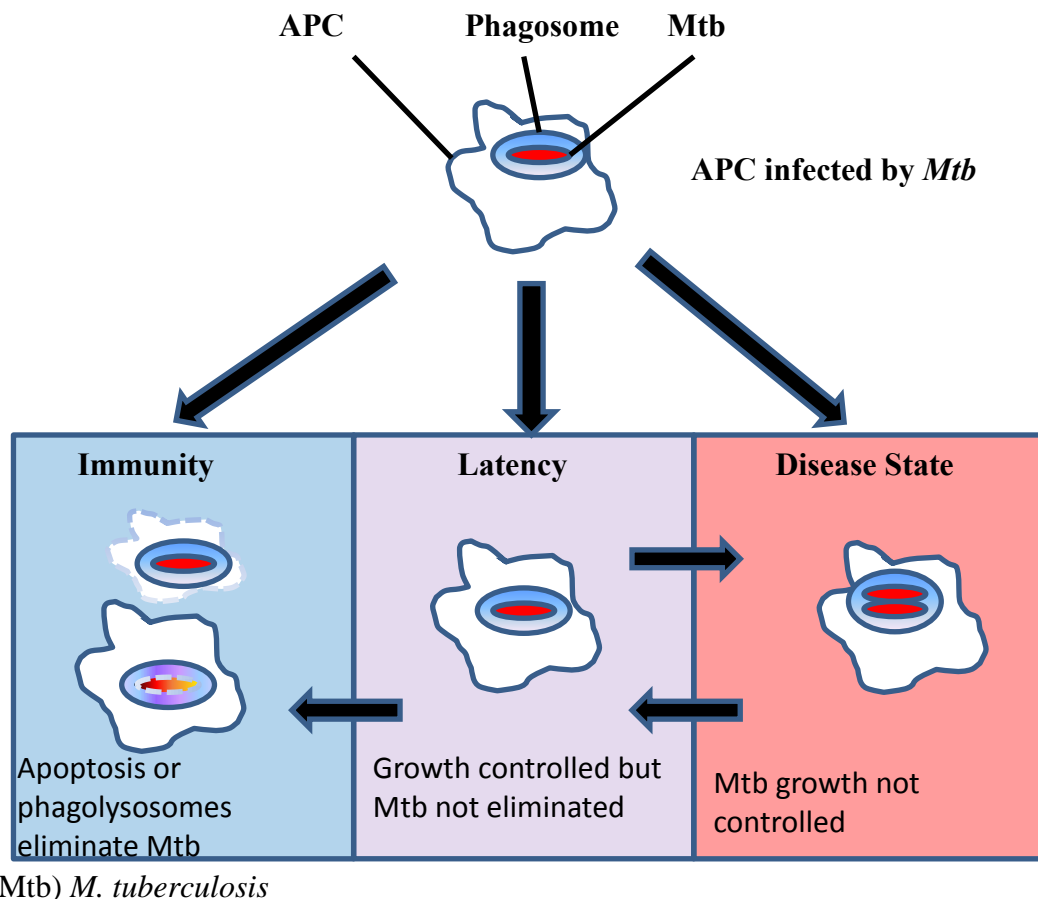


Figure 1: *M. tuberculosis* pathogenicity

In an immune response to *M. tuberculosis* in an individual with no immunity, phagocytic cells will ingest the bacterium and once ingested the bacterium disrupts lysosome maturation and in doing so prevents its own destruction. From here two outcomes may occur. First of which may be latency, the bacterium is prevented from growing but it is not eliminated. Second, the bacterium may cause the disease state, in which the bacterium grows unrestrained and spreading to neighboring cells through necrosis of the host cell. If the bacterium infects an individual who has acquired an immunity to the pathogen the bacterium will either be removed from the body

through apoptosis of the host cell or destroyed by a mature lysosome as shown in Figure 1 (Dietrich and Doherty 2009).

For infected individuals, drug cocktails are often necessary for the treatment of TB disease (latent infection may only require a single drug for treatment) and may be cured at relatively low costs. However, treatment may take many months (6-9 months) and costs do add up and may still be too great for those in socioeconomic constraints, while at the same time multidrug-resistant *M. tuberculosis* and extremely drug-resistant *M. tuberculosis* have developed. The combination time and cost requirements, as well as, drug resistance strains has stressed the importance for a greater availability, affordability and variety of preventative measures

Currently, Bacillus Calmette-Gue´rin (BCG) is the most widely distributed and used vaccination against *M. tuberculosis*. Deficiencies with BCG include a limited timeframe for protection, inability to serve as a booster to extend this timeframe, requirement for BCG replication within the host (prevented in sensitized individuals) (Prevention 2008; Dietrich and Doherty 2009; Prevention 2009). Due to the inadequacies of current prevention and treatment options a new vaccine is needed.

Efforts have been underway to create a new vaccine. Efforts that include, various types of weakened *M. tuberculosis* and *M. tuberculosis* fragments that show high immunogenicity (Cohen, Murray et al. 2011; Sharma and Yoder 2011) .

A new strategy to add to consider in the fight against *M. tuberculosis* is vaccine antigens expressed via the chloroplast genome

Chloroplast Genetic Engineering

Chloroplasts are plastids found in plants and eukaryotic algae. The belief is held that cyanobacteria are the ancestors of chloroplasts, and as such, chloroplasts' DNA has many similarities to cyanobacteria DNA (Martin, Rujan et al. 2002). Chloroplast DNA, since it is contained within the chloroplasts does not undergo recombination during reproduction, and thusly, genes within the DNA remain better intact through generations (Daniell, Khan et al. 2002).

Advantages of Chloroplast Expression Over *E. coli* Expression

E. coli have been historically used for transgene expression due to their ability to be grown in batches. The disadvantages of *E. coli* are that any product requires purification and refrigeration. Chloroplast transformation does not require purification, the product may be stored at room temperature, and the accumulation of 10,000 copies of the transgene per cell is possible (Daniell, Khan et al. 2002).

Advantages of Chloroplast Transformation over Nuclear Transformation

Eukaryotic cells each contain a single copy of their nuclear DNA with few exceptions (ex. Erythrocytes). In nuclear transformation a single gene is placed into the single nuclear genome. This results in the expression of only one copy of the desired gene per cell. Chloroplasts, which can number in the hundreds per plant cell, each contain hundreds of copies of their circular genome. Therefore, each plant cell has the potential to contain tens of thousands of copies of the transgene (Daniell, Khan et al. 2002). Apart from expression levels chloroplast transformation has the added benefits of not suffering from transgene silencing or

position effect; often observed in nuclear transformants. Lastly, chloroplast transgenes proteins are properly folded and receive lipid modifications that would not occur in nuclear transformants (Staub, Garcia et al. 2000; Daniell, Lee et al. 2001; Glenz, Bouchon et al. 2006).

Oral delivery of Bioencapsulated Vaccine Antigens will Confer Mucosal, Systemic Immunity and Protection of Immunogens in the Gut

Advantages for oral delivery of vaccines over injections include; mucosal immunity, avoidance of costly purification, cold storage and sterile delivery(Kamarajugadda and Daniell 2006). Antigen production via chloroplast genome expression has the potential advantages of high yield, proper folding of the protein, protection of crossing over events from generation to generation, and protection from gene silencing as previously described in chloroplast advantages(De Cosa, Moar et al. 2001). The high expression level and proper folding of the protein of the chloroplast expression system is essential for the creation of an economically viable and affective oral vaccine (Ruhlman, Ahangari et al. 2007; Singh, Ding et al. 2009).

Bacterial antigens that have already been expressed in the chloroplast genome include Cholera Toxin B (CTB, from *Vibrio cholera*) (Daniell, Lee et al. 2001; Ruhlman, Lee et al. 2006), Tetanus Toxin (from *Clostridium tetani*) (Tregoning, Nixon et al. 2003), Anthrax Protective Antigen (PA, from *Bacillus anthracis*) ((Verma, Samson et al. 2008)), Lyme disease (from *Borellia* genus of bacteria)(Daniell, Singh et al. 2009) and plague(Daniell, Chebolu et al. 2005). Non-bacterial antigens expressed include canine parvovirus (CPV), rotavirus, and amoeba (protozoan). The production of these antigens, within chloroplast, bioencapsulates the antigen allowing for the successful delivery to mucosal membranes in the gut by protecting them from digestion in the stomach.

Once delivered to the mucosal membranes the antigen may be delivered across the intestinal lumen and into systemic circulation where immunity is eventually acquired (Kamarajugadda and Daniell 2006; Singh, Ding et al. 2009)

Background and significance of LipY

LipY from *M. tuberculosis* is a cell wall protein that is up-regulated during dormancy and an active mycobacterium infection of macrophages. This allows LipY to play a potential role as not only a drug target but a potential antigen for *M. tuberculosis* vaccine production. Earlier studies have already shown serum from tuberculosis patients was shown to cause a humoral (IgG and IgM) response against LipY and that LipY may serve as a B-cell antigen. Also, due to lipase's part in the cell's latent phase, vaccines and drugs can be targeted to the latent phase cells, a limit of current drugs aimed against only actively replicating *M. tuberculosis* (Deb 2006; Mishra 2008). These aspects make LipY a desirable candidate for *M. tuberculosis* vaccine development.

Structurally, lipases vary in size from 20-60 kDa, with the LipY studied here being 42kDa in size. They also have varied amino acid sequences; with the exception that they all contain a serine residue in the active site containing a Ser-Asp/Glu-His motif, with a consensus sequence, Gly-x-Ser-x-Gly, near the active serine in most lipases and a characteristic α/β -hydrolase fold in the 3D structure (Jaeger, Ransac et al. 1994; Gupta, Gupta et al. 2004). The active site is covered by an α -helical structure resembling a lid that shifts to expose hydrophobic residues to allow contact to occur between lipase and its substrate when located at the lipid interface (Jaeger, Ransac et al. 1994).

Functionally, lipases are highly stable in organic solvents, and due to their substrate, triacylglycerol's low solubility in water, lipases' enzymatic activity occur at the lipid-water interface (Gupta, Gupta et al. 2004). Lipases are naturally produced in humans, plants, fungi and bacteria species, both gram-positive and gram-negative. Lipases catalyze the hydrolysis of ester bonds and long-chain triacylglycerols into free fatty acids and glycerol (Jaeger, Ransac et al. 1994; Beisson, Arondel et al. 2000; Gupta, Gupta et al. 2004). Lipases also have the ability to breaks down esters into alcohol and acid, or be esterolytic, making them ideal enzymatic candidates for processes requiring a high substrate variety (Gupta, Gupta et al. 2004).

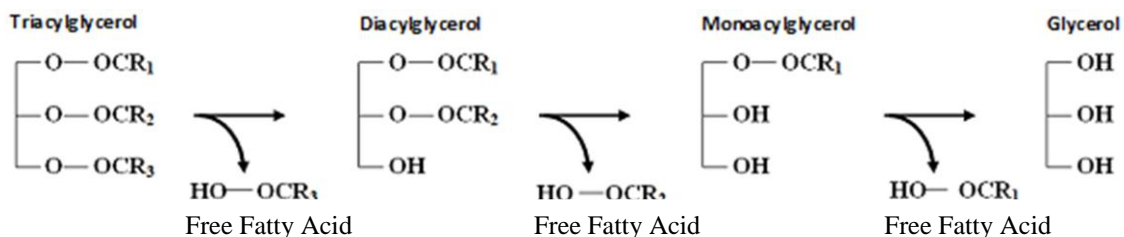


Figure 2. Triacylglycerol activity of LipY protein.

Research Methodology

The purpose of this project was to develop a bioencapsulated vaccine antigen for protection against *Mycobacterium tuberculosis* disease (TB Disease) using the chloroplast expression system. Chloroplasts were chosen due to their ability to produce desired proteins in abundance, providing a basis for low cost production of the desired proteins. Another advantage is the post-translational modifications the chloroplast expression system performs. Apart from mere plant expression, the chloroplast expression system has several unique advantages over the

more traditional nuclear transformation. Advantages include higher expression levels, decreased gene silencing, proper protein folding, and maternal inheritance of the LipY transgene. The high yield is possible due to the high expression levels allowed by a much larger copy number per cell over nuclear or *E. coli* expression systems, the absence of gene silencing, proper post translational modification, a lack of position effect. The low cost is possible due to the higher yield, elimination of purification required compared to other expression systems and the oral delivery system which requires neither costly syringes nor cold storage. The chloroplast expression systems chosen are the *N. tabacum* chloroplasts and *L. sativa* chloroplast. Both were chosen for their known chloroplast gene sequence and prior use in vaccine production.

To prepare the LipY gene for the chloroplast expression system genomic DNA was acquired from the Dr. Pappachan Kolattukudy Lab, and modified as described in Materials and Methods.

The LipY gene was cloned into the chloroplast expression vector and transformation of plant chloroplasts was performed by way of gene bombardment as described in Materials and Methods. Putative shoots were placed through three rounds of selection, as described also in Materials and Methods, on media containing spectinomycin to assure integration of gene cassette containing the selectable marker gene for spectinomycin resistance and PCR to cancel out false positives due to mutations in the wild type chloroplast genome and to assure integration of the LipY gene. Southern blot was performed to test for homoplasmy.

Immunoblot analysis ensured the presence of the protein product, as well as determining if cleavage or degradation of the protein occurred.

The chloroplast derived LipY will be assayed for its immunogenic response in an animal model. Once an immunogenic response is achieved the animal model will then be pathogen challenged with *Mycobacterium tuberculosis* to test for survival rates.

MATERIALS AND METHODS

Construction of pLD-LipY and pLsDV-LipY vectors for Chloroplast transformation

The LipY chloroplast vectors were constructed by Dr. Dheeraj Verma in Dr. Daniell's lab. The LipY gene was amplified using sequence specific primers and Mycobacterium tuberculosis genomic DNA (kind gift from Kolattukudy lab) as template. The amplified fragment was cloned into the pCRBluntII Topo vector (Invitrogen). The LipY coding sequence was excised from Topo vector using NdeI/XbaI and cloned into pLD UTR vector (Daniell, Lee et al. 2001; Verma, Samson et al. 2008) resulting in final tobacco chloroplast expression vector pLD-LipY. Also, the excised fragment was ligated into pLsDV-UTR vector to make final lettuce chloroplast expression vector pLsDV-LipY. The final vectors were confirmed by restriction digestion followed by sequencing to detect any errors.

Preparation of XL10-gold Ultracompetent Cells

Ultra Competent cells were prepared using the Inoue Method for Preparation of competent cells (Sambrook 1989). The following day a single colony was selected from this plate and transferred into a 250mL flask with 25mL of Lysogeny Broth (LB). This was allowed to incubate at 37°C with vigorous shaking for 6-8 hours. Before leaving the lab for the day this starter culture was used to inoculate a 1L flask containing 250mL SOB with 10mL starter culture, another with 4mL starter culture, and a third flask with 2 mL starter culture. These flasks were left to incubate over night at room temperature. The next day, the OD₆₀₀ of the 3 flasks was measured until they read 0.55. When any of the flasks reached 0.55 they were placed in an ice-water bath for 10 minutes, while the others were discarded. The cells were harvested

via centrifugation at 4°C, 2500g, for 10 min (IEC Centra CL3R). The medium was discarded and the centrifuge tubes placed upside-down on paper towels to dry. The cells were re-suspended in 80mL of Inoue transformation buffer pre-chilled on ice. The cells were once again harvested and the tubes dried in the same manner as before. The cells were suspended in 20mL of Inoue transformation buffer (ice-cold), 1.5mL DMSO is added, the mixture was swirled and then stored 10 minutes on ice. Working with a partner, the suspension was aliquoted into sterile micro-centrifuge tubes, chilled in a bath of liquid nitrogen and stored at -70°C.

Transformation of the Competent *E. coli* XL10-gold cells

For transformation with the plasmid DNA (either pLD-LipY or pLsDv-LipY) competent *E. coli* XL10-gold cells were removed from -80°C and placed on ice to thaw for 20 minutes. Previously chilled pipette tips were used to move 100 µL of cells to a pre-chilled polypropylene tube. 1-5 µL of DNA (50ng per 100 µL cells) was added to the cells, and mixed by flicking 4-5 times. These tubes were then let to rest on ice for 30 minutes without disruption. After this time the cells were placed in a 42°C water bath for a 90 second heat shock then immediately placed on ice for 5 minutes. 900µL of SOC was added to each tube then the culture was incubated at 37°C in a shaker for 90 minutes. The cells are then pelleted in a micro-centrifuge, 800 µL SOC was removed and the cells were re-suspended in the remaining SOC. The transformed competent cells were transferred and spread with a glass rod onto LB Agar medium with 25mg/L Ampicillin. The plates were incubated overnight at 37°C and any colonies appearing the next day were tested using colony PCR.

Colony PCR

The polymerase chain reaction, better known as PCR is a method of amplifying specific DNA sequences. This technique requires a reaction mix containing 11.8µl autoclaved ddH₂O, 5µl 5x buffer solution, 1.25µl 50mM MgCl₂ solution, 0.75µl 10mM dNTP solution, 0.5µl primer #1, 0.5µl primer #2, 5µl of the template DNA and at the last moment 0.2µl of the DNA polymerase, Mango Taq. Once the mix with the DNA is combined it is mixed gently by pipetting a small volume of the solution into and out of the PCR tube. The individual tubes with their corresponding reaction mix (and template DNA) are placed inside a PTC-100 Peltier Thermo Cycler from BioRad, and the steps (denaturing, annealing, elongation, etc.) adjusted according to the Template DNA size.

Denaturation is the stage where the hydrogen bonds of the double stranded DNA are disrupted and the strands are separated. Annealing is the stage where the primers anneal to the single-stranded DNA (template strand) and the polymerase binds to the primer/template-strand. Elongation is where the new complementary DNA strand is produced from the template strand. All three stages are repeated 30 times to increase the DNA concentration by 2³⁰ fold (though this number assumes 100% efficiency, which is not a likely occurrence)

Colonies were tested for the transgene by colony PCR. Each colony was touched with the end of a pipette tip, the tip then streaked a fresh agar plate labeled for the individual colonies (1,2,3, and so on), the plate incubated at 37°C and the tip placed into a PCR tube containing 10microL DNase free H₂O. The reaction mix consists of 5µl autoclaved ddH₂O, 5µl 5x buffer solution, 1.25µl 50mM MgCl₂ solution, 0.75µl 10mM deoxynucleoside triphosphates (dNTPs)

solution, 1µl primer #1, 1µl primer #2, and at the last moment 1µl of the DNA polymerase, Mango Taq is added. The tip used to streak the colony was swirled in the mixture to remove any cells. The tubes were then placed into a PTC-100 Peltier Thermo Cycler from BioRad and the stages set according to the length of DNA expected to be amplified (detailed set-up is found in the “Confirmation of transgene integration into the chloroplast genome by PCR analysis” portion of Materials and Methods). Once the PCR is complete the colonies on the fresh agar plate that correspond with the positive PCR results were used to inoculate a starter culture. This starter culture is then used to inoculate for midi prep.

Isolation of Plasmid DNA by Midi-prep

Midi prep is performed using the QIAgen Midi prep kit. A starter culture of pLD-LipY or pLsDv-LipY *E. coli* is used to inoculate 50mL LB broth (50mg/L Ampicillin) in a 250mL flask for overnight incubation at 37°C in a shaker. The next day, the culture is placed into 50mL centrifuge tubes and pelleted at 5,000xg for 10 minutes at 4degrees. Liquid is discarded, tubes drained and inverted on paper towels. The cells are vortexed to re-suspend in 3mL of re-suspension fluid. 3mL of cell lysis solution is then added to the culture, mixed by a gentle see-saw motion, and then incubated at room temperature for 3 minutes. 5mL neutralizing solution is then added to the lysed cells, the solution is then inverted 5 times to mix, and allowed to sit for 3 minute at room temperature before being centrifuged. The solution is centrifuged at 10,000 rpm for 10 minutes, the lysate added to a column stack consisting of a clearing column placed inside a 50mL Falcon tube, and allowed to rest 2 minutes. The column is then placed into a swinging bucket rotor and centrifuged at 1,500xg for 5 minutes. The filtered lysate is then added to a column stack consisting of a binding column and a new 50mL Falcon tube, then centrifuged at

1,500xg for 3 minutes, liquid is discarded. 5mL Endotoxin Removal Wash Solution is added to the binding column, centrifuged at 1,500xg for 3 minutes and the flow through discarded. 20mL of Column Wash Solution is added, the column centrifuged at 1,500xg for 5 minutes, the flow through discarded and column centrifuged at 1,500xg for 10 minutes. For DNA elution the column is placed into a fresh 50mL Falcon tube and 600microL nuclease-free water is added. The column is centrifuged at 1,500xg for 5 minutes and the filtrate is collected and stored at 4°C.

Bombardment for the pLD-LipY

Preparation of Gold Particles

Preparation of gold particles for the purpose of *Nicotiana tabacum* (*N. tabacum*) chloroplast transformation 50 mg of 0.6 µm gold particles were added to a microcentrifuge tube containing 1ml of 70% ethanol. The mixture was vortexed for 3-5 minutes then incubated at room temperature for 15 minutes.

The particles were spun down by a brief centrifuge and the supernatant was discarded. 1ml of dH₂O was added to the particles, they were vortexed, allowed to settle for 1 minute and pulse centrifuged for 3 seconds. The supernatant was discarded and the process repeated three times. Gold particles were stored in 50% glycerol at -20°C (Verma, Samson et al. 2008).

***Nicotiana tabacum* tissue Culture Media Preparation**

Media was prepared in 1L dH₂O by adding 1 pack MS basal salt mixture, 100mg myoinositol, 1mg benzylaminopurine (BAP), 100µg β-naphthalene acetic acid (NAA) 1mg of

thiamine hydrochloride and 30g sucrose. pH was adjusted to 5.8 using KOH and 6g phytagar added followed by autoclaving.

Bombardment Protocol for *Nicotiana tabacum* Leaves

Prior to bombardment all equipment was sterilized; including stopping screens, rupture disks (1100 psi), macro carriers, macro carrier holders, and filter paper. Aseptic conditions were maintained during the bombardment procedure. The DNA coated gold particles were prepared by adding; 50µl of gold particles to a micro-centrifuge tube and vortexed while adding 50µl 2.5M CaCl₂, 5µl pLD-LipY DNA, 20µl 0.1M spermidine-free base in sequence. Mixture was vortexed for 20 minutes at 4°C. After vortexing, the particles were allowed to settle for 1 minute than vortexed at 8,000 rpm for 1 minute. The liquid was removed and 140µl 70% ethanol was added, the mixture was centrifuged at 5000 rpm for 60seconds, and the procedure repeated with 100% ethanol until the pellet of micro carriers easily dispersed in the ethanol with a slight tap of the tube. At this point the particles were re-suspended in 60µl of 100% ethanol, placed on ice until being placed onto the macro carriers (within 2 hours).

Young, healthy, aseptic *N. tabacum* plants grown in magenta boxes and their cut leaves were prepared for bombardment by placing their abaxial side up on selection free RMOP petri dishes with Whatman filter paper on top of the media.

The gene gun was prepared by sterilizing the inside chamber by spraying with 70% ethanol. Macro carriers were prepared by placing them inside macro carrier holders and placing 10µl of vortexed DNA coated gold particles. After setting up gene gun with a 1100psi rupture disk, stopping screen, macro carrier inside holder and a leaf, the gene gun, vacuum pump, and

helium tank (with pressure set at 1350psi) were turned on. The vacuum inside the gene gun was allowed to build up to 28psi, the machine placed in "hold" position and the "fire" button held until rupture disk broke. Vacuum was released by moving the "Hold" Switch to the "Vent" position. The petri dish removed, leaf covered and the process repeated for remaining samples. When finished, the plates were wrapped in plastic wrap and placed in the dark in a Sanyo Growth Cabinet for 72 hours at room temperature (Verma, Samson et al. 2008).

Tissue regeneration and selection following bombardment

After the 72 hour incubation period, the leaves were cut into 5mm² pieces and placed on RMOP media with spectinomycin (500µg/l for *Petit Havana*, 200µg/l for LAMD, 150µg/L for TN90 cultivars) 100 x 25 petri dishes. Regenerated spectinomycin resistant shoots appeared after 4-6 weeks (differed for the various cultivars). PCR analysis was performed using total DNA from the regenerated shoots to confirm transgene integration into chloroplasts. After PCR confirmation of transgene integration these shoots were cut into 5mm² pieces and placed onto fresh RMOP with spectinomycin for a second round of selection. After approximately 4 weeks on secondary selection regenerated shoots were transferred to magenta boxes with half strength MSO media containing spectinomycin for a third round of selection (Verma, Samson et al. 2008).

Bombardment for the pLsDV-LipY

Preparation of gold particles

Preparation of gold particles for the purpose of *L. sativa* chloroplast transformation was performed as described in the previous methods for *N. tabacum* chloroplasts.

***Lactuca sativa* tissue Culture Media Preparation**

Media was prepared in 1L dH₂O by adding 1 pack MS basal salt mixture, 100mg myoinositol, 200µg benzylaminopurine (BAP) 100µg β-naphthalene acetic acid (NAA) 10mg of thiamine hydrochloride, 1 mg Pyrodoxin , 1mg nicotinic acid, 1mg Glycine, 500mg polyvinylpyrrolidone (PVP, for *L. sativa*) and 30g sucrose. pH was adjusted to 5.8 using KOH and 5g phytagar added followed by autoclaving.

Bombardment protocol for *Lactuca sativa* leaves

The bombardment protocol for the purpose of *L. sativa* chloroplast transformation was performed as described in the previous methods for *N. tabacum* chloroplasts with a few adjustments. *L. sativa* plants were prepared for bombardment by placing the abaxial side down on selection free *L. sativa* regeneration media (LSRM) petri dishes, and a 900psi rupture disk was used.

Tissue regeneration and selection following bombardment

After the 72 hour incubation period, the leaves were cut into 5mm² pieces and placed on LSRM media with spectinomycin (50µg/L) in 100 x 25 petri dishes. Shoots appeared after 4-12 weeks. Before secondary selection, PCR analysis was performed using total DNA from the regenerated shoots to confirm transgene integration into chloroplasts. These shoots were cut into 5mm² pieces and placed onto fresh LSRM with spectinomycin for a second round of selection. After approximately 4 weeks on secondary selection shoots were transferred to magenta boxes with half strength MSO media containing spectinomycin for a third round of selection.

Total genomic DNA extraction

Whole genomic DNA was extracted from regenerated shoots using the DNeasy Plant Mini Kit by Qiagen. 100mg of plant leaf material was ground in a 1.7mL micro centrifuge tube using a micro pestle. To the ground leaf material 400µl of buffer AP1 and 4µl RNase A were added. The mixture was incubated at 60°C for 10 minutes, while mixing twice during the incubation. 130µl of buffer AP2 is added to the sample; the sample was vortexed and allowed to incubate on ice for 5 minutes. After the incubation period, the samples were vortexed at 14,000 rpm for 5 minutes and the supernatant transferred to a Qias shredder Spin Column placed inside a 2mL collection tube. The set-up was centrifuged at 14,000 rpm for 2 minutes. The flow through was carefully removed (so as not to disturb the pellet that formed on the bottom of the 2mL collection tube), placed in a fresh 2mL tube and 1.5 parts buffer AP3/E added and mixed by pipette. 650µl of the sample was moved to a DNeasy Mini Spin Column placed inside a 2ml collection tube and centrifuged at 8,000 rpm for 1 minute. The flow through was discarded and the remaining sample was added to the column and centrifuged for a second time. The flow through was again discarded and the column placed into a fresh 2mL collection tube. 500µl buffer AW (wash buffer) was added to the column and the column centrifuged at 8,000 rpm for 1 minute. The column was again washed using 500ul wash buffer. The column was placed into a fresh 1.7mL tube, the column allowed to dry (for approximately 5 minutes) and 60µl of buffer AE added directly to the DNeasy membrane. The column is left at room temperature for 5 minutes and then centrifuged at 8,000 rpm for 2 minutes. The column was then discarded and the eluted DNA stored at 4°C.

Confirmation of transgene integration into the chloroplast genome by PCR Analysis

The transgene cassette integration into the *N. tabacum* chloroplast genome was confirmed using the primer pairs 3P (5'-AAAACCCGTCCTCGTTCGGATTGC-3') and 3M (5'-CCGCGTTGTTTCATCAAGCCTTACG-3'). Since the cassette integration using the primers 3P and 3M does not depend on the gene size, a known transformant for *N. tabacum* chloroplasts was used as a positive control, wild type (WT) was used for the negative control. The PCR protocol was set as follows- Initialization was set as 94°C for 5 minutes, denaturation was set to 94° for 1 minute, annealing was set to 55°C for 1 minute, and elongation was set to 72°C for 2 minutes. All three steps were repeated for a total of thirty cycles and a final elongation was set at 72°C for 10 minutes.

LipY gene integration was confirmed using primer pairs 5P (5'-CTGTAGAAGTCACCATTGTTGTGC-3') and 2M (5'-GACTGCCCACCTGAGAGCGGACA-3'). PCR parameters were set as follows-Initialization was set as 94°C for 5 minutes, denaturation was set to 94° for 1 minute, annealing was set to 55°C for 1 minute, and elongation was set to 72°C for 1.5 minutes. All three steps were repeated for a total of thirty cycles and a final elongation was set at 72°C for 10 minutes.

The transgene cassette integration into the *L. sativa* chloroplast genome was confirmed by the polymerase chain reaction using the primer pairs 16SF (5'-CAGCAGCCGCGGTAATACAGAGGA-3') and 3M (5'-CCGCGTTGTTTCATCAAGCCTTACG-3'). Since the cassette integration using the primers 16SF and 3M does not depend on the gene size, a known transformant for *L. sativa* chloroplasts

was used as a positive control, wild type was used for the negative control. Initialization was set as 94°C for 10 minutes, denaturation was set to 94° for 30 seconds, annealing was set to 60°C for 45 seconds, and elongation was set to 72°C for 1.5 minutes. All three steps were repeated for a total of thirty cycles and a final elongation was set at 72°C for 10 minutes (Verma, Samson et al. 2008).

LipY gene integration using primer pairs DV209 (5'-AGAATTCCATATGGTGTCTTATGTTGTTGCGTTGCC-3') and DV210 (5'-TGGCTAGTCTAGATCAGGCGGCGATAACCGAGTTGCT-3') was used for analysis of both the *L sativa* and *N. tabacum* chloroplasts. Known transplastomic plant DNA was used as the positive control and WT plant DNA used as the negative control to monitor the PCR reaction. Initialization was set at 94°C for 5 minutes, denaturation was set to 94°C for 1 minute, annealing was set to 58°C for 1 minute, and elongation was set to 72°C for 3 minutes. All three steps repeated for a total of thirty cycles and a final elongation was set at 72°C for 10 minutes .

Southern blot analysis of transplastomic plants

Restriction Digestion of plant genomic DNA

Total plant DNA was extracted from transplastomic and wild type leaf material using the DNA Extraction protocol described above. The total plant DNA was digested with SmaI (chosen because it had no restriction sites within the gene) overnight at 25°C.

Generation of Probes

To prepare the probe, 10µl pUC-CT DNA was digested with 1µl BamHI, 1µl BglII, 2µl BamHI, and 6µl water (Figure 12A). The digestion was allowed to occur for 3 hours at 37°C.

The digest was run on a 0.8% agarose gel, the 0.8kb fragment was cut out from the gel using a scalpel, and a gel elution was performed using the Qiaquick gel extraction kit to isolate the probe DNA.

Probe labeling

To label the probe; 45µl of probe DNA was denatured at 94°C for 5 minutes (water bath) and placed on ice for 3 minutes. Any droplets were brought down by pulse centrifugation. Probe DNA was then added to the ready mix (Amersham Ready-To-Go DNA Labeling Beads (-dCTP)) and mixed by gently flicking the tube. 5µl of P³² was then added to the tube, any droplets were brought down by pulse centrifugation and the mix incubated at 37°C for 1 hour.

The probe DNA was then purified with an Illustra Microspin G-25 Columns. The column was prepared by vortexing to re-suspend the resin, the cap loosened (1/4 turn), the bottom plug removed, the column placed into a micro centrifuge tube and centrifuged at 3,000 rpm for 1 minute. The micro centrifuge tube was discarded along with the flow through and the column placed into a fresh collection tube. The probe DNA was added to the center of the resin and the column centrifuged at 3,000 rpm for 2 minutes. The column was disposed of in radioactive waste container stored in radioactive fridge.

The amount of labeled DNA was determining by adding 1µl labeled probe to 98µl STE buffer. 50µl of this solution was added to two separate containers containing 3mL of Opti-Fluor with one blank containing 3mL Opti-Fluor and no probe solution. The activity read in a Beckman LS 5000TD.

Agarose gel electrophoresis and DNA transfer

A 0.8% agarose gel was prepared and the 24 μ l of digestion reaction plus 6x loading dye was loaded into the wells. The wild type *N. tabacum* was used as the negative control.

The gel was run at 50V for approximately 3 hours. At the end of the run the gel was taken to the gel doc (BioRad Gel Doc 2000) and imaged beside a fluorescent ruler. This image was later used to approximate the band size. Once gel doc image was taken, the gel was removed from the tray with the excess gel above the wells removed and the bottom right corner cut to serve as a guide. The gel was placed in a glass dish and rocked for 15 minutes with de-purination solution (0.25N HCl). Afterwards, the gel was washed with dH₂O for 5 minutes twice and soaked in transfer buffer (1M NaCl, 0.4N NaOH) for 20 minutes.

For the transfer, all materials were pre-soaked in transfer buffer and stacked so as not to have any air bubbles between the layers. A counter top was covered with plastic wrap, two gel sized pieces of Whatman paper were stacked and the gel inverted and placed on top. A nylon membrane of the same dimensions as the gel was placed above the gel, two more Whatman papers of the same size as the gel were stacked above that and a stack of dry paper towels above that (again, the same size as the gel). Finally, the stack was weighted down and left for transfer overnight. The next day, the membrane was washed in 2X SSC (0.3M sodium chloride and 0.03M sodium citrate solution) for 5 minutes, allowed to dry and cross-linked using the C3 setting (150 m joules) of the Bio-Rad GS gene Cross Linker. Afterwards, the membrane was wrapped in plastic wrap and stored until use.

Prehybridization, hybridization and washing of membrane

The membranes were prepared for hybridization by first being placed into the hybridization bottle with the bottom placed against the bottle. 5mL of pre-hybridization solution (Stratagene Quick-Hyb solution) was added and the membrane incubated in a Fisher Biotech Hybridization incubator at 68°C for 1 hour.

100µl of salmon sperm (10mg/ml) was added to the necessary amount of probe DNA (as determined by the method described earlier) and the solution heated at 94°C for 5 minutes. 1 mL of pre-hybridization solution was removed from membrane bottle, added to the salmon sperm and probe, then the mixture was returned to the bottle and incubated at 68°C for 1 hour.

To wash; the hybridization solution was removed and stored for later use and the membrane washed with 50mL of wash buffer I (2X SSC and 0.1% SDS) at 25°C for 15 minutes two times. Then the membrane was washed with 50 mL pre-warmed wash buffer II (0.1X SSC and 0.1% SDS) at 60°C. All radioactive liquid waste was discarded into the appropriate container for liquid radioactive waste in the radioactive hood.

Autoradiography

The membrane was then dried, wrapped in plastic wrap, placed into a film-cassette top-side-up and brought to the dark room. Once in the dark room, with the red light on, x-ray film was placed above the membrane, the cassette closed, placed into black bag, and stored at -80°C overnight. The next day, the blot was removed from the freezer and allowed to warm to room temperature before being returned to the dark room and developed .

Characterization of chloroplast and *E.coli* derived LipY

Bradford assay for protein quantification

For the Bradford assay, to determine protein concentration, samples were ground using liquid nitrogen and 200 µl of Plant Extraction Buffer (PEB, 200µl 5M NaCl, 200 µl 0.5M EDTA, 2mL Tris-HCl, 5µl Tween-20, 100µl 10% SDS, 10µl Beta-mercapto-ethanol (BME), 4µl 1M sucrose, 3.285mL autoclaved ddH₂O, 200µl 100mM PMSF, and 154mg DTT) was added. These samples were vortexed for 20 minutes at 4° and centrifuged for 5 minutes at 8,000rpm. The protein quantification was performed using of 5, 10 and 20 times dilution and BSA was used as standard.

Extraction of Protein from Transformed *E. coli* Cells for SDS-PAGE

E. coli protein extracts were prepared by taking 800µl of *E. coli* culture and centrifuging, at 8,000 for 2 minutes. The supernatant was discarded and the pellet was washed with 1mL PBS, and resuspended in 50 µl PBS. The samples were then mixed with 50 µl 2x loading buffer and placed in a boiling water bath for 4 minutes.

Extraction of Protein from Transformed *Nicotiana tabacum* and *Lactuca sativa* Leaves for SDS-PAGE

Plant samples were prepared by grinding leaf material in liquid nitrogen aliquoting 100mg into separate 1.7mL tubes. 200 µl PEB was added to each sample on ice and mixed with a mechanical pestle. The samples were then centrifuged at 14,000 rpm for 5 minutes and supernatant placed in fresh tube. A Bradford assay was performed, as described earlier, to determine protein concentration and the necessary volumes of sample were taken to obtain the

chosen protein amount (30µg -75µg). These samples were then mixed with equal volumes 2x loading buffer and placed in a boiling water bath for 4 minutes.

SDS-PAGE Buffers and Gels

To test the LipY protein expression of transgenic *E. coli* and *N. tabacum* or *L. sativa* total protein extracts were run on an SDS-PAGE and stained with coommasse blue, SyproRed or transferred to a membrane and used for an immunoblot. SDS-PAGE gels were made utilizing the buffer solutions: 30% Acrylamide/Bis solution (BioRad), the resolving buffer (1.5M Tris-HCl, pH 8.8. Made by adding 27.23g Tris base to 80mL water, adjusting pH to 8.8 with 6N HCl, added water to bring volume to 150mL, autoclaved and stored at 4°C), the stacking buffer (0.5M Tris-HCl, pH 6.8 made by adding 6g Tris base to 60mL water, adjusting pH to 6.8 with 6N HCl, added water to bring volume to 100mL, autoclaved and stores at 4°C), 10x Electrode buffer (30.3g Tris base, 144.0g glycine and 10.0g SDS brought to a volume of 1L with dH₂O. Stored at 4 °C), 2x loading buffer (3.55mL water, 1.25mL 0.5M Tris-HCl pH 6.8, 2.5mL glycerol, 2.0mL 10% SDS and 0.2mL 0.5% Bromophenol Blue. 50 µl beta-Mercapto-ethanol is added to 950 µl of this mix before use.), 10% (w/v) Sodium Dodecyl Sulfate (SDS: 10g SDS in 90mL water, water added to make final volume of 100mL. Stored at room temperature), N,N,N,N'-Tetra-methyl-ethylene diamine (TEMED, from BioRad), 20% Ammonium Persulfate (APS: 20 mg of APS in 1ml dH₂O, stored at 4 ° C).

The SDS-PAGE cassette was prepared by first cleaning the glass plates (1.0 mm size) with 70% ethanol, drying them and then locking them into the green apparatus. The apparatus was then placed in to the clear holder and water added to test for leaks. When no leaks were

detected the water was poured out and between the plates was dried with Whatman paper. The green comb was placed inside the plates to mark depth and removed.

The 12% resolving gel was made by mixing 3.4 mL water, 4 mL 30% Acrylamide/Bis Solution, 2.5mL resolving buffer and 100 μ l 10% SDS in a 50mL Falcon tube. Right before being added to the cassette 50 μ l 20% APS and 10 μ l TEMED were added and the tube swirled. The solution was then carefully poured in between the plates to just below the mark for the comb depth, water slowly added on top and allowed to polymerize for 20 minutes. Once set, the water is poured off and the stacking gel added.

The 5% stacking gel was made by mixing 5.7 mL water, 1.3 mL 30% Acrylamide/Bis Solution, 2.5mL resolving buffer and 100 μ l 10% SDS in a 50mL Falcon tube. Right before being added to the cassette 50 μ l 20% APS and 10 μ l TEMED were added and the tube swirled. The solution was then poured atop the resolving gel, until slightly overflowing from the plates, the comb placed inside with care not to create any bubbles and allowed to polymerize for 20 minutes.

After polymerization was complete, the comb was removed, the wells rinsed with water and plates placed into the PAGE apparatus with short place facing inward, and filled with 1X electrode buffer (brought 100mL volume of 10X electrode buffer to 1000 mL with water). The wells were loaded with 20 μ l *E.coli* protein extract or between 30 μ g and 70 μ g total plant protein and run at 85V for 15 minutes (or until the samples passed from the stacking gel into the resolving gel), then run at 150V for approximately 1 hour or until the sample front was

approximately 5mm from the bottom. At this point the gel was used for a coommassee stain, SyproRed stain or immunoblot.

Transfer of protein and Immunoblot analysis

After the SDS-PAGE was complete the glass plates were separated, the stacking gel was carefully cut away and discarded, and the resolving gel is carefully removed and placed into the transfer set-up .cassette with black side down, sponge, filter paper, gel, nitrocellulose membrane, filter paper, sponge, cassette closed). The transfer was performed at 85V for 60 minutes. The membrane was removed and rinsed with water 3 times.

The membrane was then blocked at room temperature for 1 hour on a rocker with a solution containing 100mL 1X PBS, 100µl tween 20 and 3g dry milk (PTM). After 1 hour the solution was poured off and 15mL of PTM containing primary antibody, Rat-anti-LipY(Δ PE) at a concentration of 1/2500 was added and the membrane was incubated for 2 hours at room temperature with gentle rocking. After the 2 hours, the primary antibody solution was poured off and the membrane rinsed twice with water. 20mL of PTM containing the secondary antibody, Goat-anti-rat-HRP conjugated (HRP: Horseradish peroxidase) at a concentration of 1/7000 was added and the membrane incubated for 90 minutes, at room temperature with gently rocking. The secondary antibody solution was poured off and the membrane washed with a solution of 100ml PBS and 50µl Tween-20 (PBS-T) 3 times for 15minutes, and then PBS for 10 minutes.

The membrane was brought to the dark room along with the film cassette, plastic wrap, a 1mL Pipette with tip, tape, x-ray film and colorimetric solution (SuperSignal West Pico

Chemiluminescent Substrate for detection of HRP) The solution was washed over the membrane with the pipette approximately 100X, the membrane was covered with the plastic wrap, taped into the film cassette and the x-ray film placed on it for 1 minute. The film is then run through the film processor (AFP Mini-Medical 90 Film Processor) and this run was used to determine if more or less time was needed for exposure. Once the correct exposure time was determined the final run through the film processor was performed.

Lipid Profile

Leaf tissue from wild type *N. tabacum*, white transformants, mosaic transformants and green transformants was cut into small pieces and placed inside individual Erlenmeyer flasks containing a 2:1 solution of chloroform/methanol and stirred for 4 hours. The mixtures were then strained through Whatman filter paper and emptied into a separating funnel. 6N HCL was added for acidification and the mixture was shaken vigorously. After allowing the phases to separate the organic layer was emptied into a clean Erlenmeyer flask. The remaining aqueous layer was treated with chloroform and the organic layer collected two times. Any organic solvents were removed using a vacuum rotary evaporator. A small volume of the chloroform/methanol solution was used to re-suspend the dry lipids before being separated by thin layer chromatography with a solvent system of chloroform/methanol/water in a ratio of 65:25:2 (v/v/v). Iodine vapor was used to visualize lipid bands and analysis was performed through comparison of MGDG and DGDG in wild type leaf material versus transformed materials (Miège, Maréchal et al. 1999; Nishiyama, Hardré-Liénard et al. 2003).

Lipase activity assays

Extraction of Protein from Transformed *E. coli* Cells for activity assays

Glycerol stocks of the XL10-gold cells transformed with the pLD-LipY vector were used to inoculate 50mL of LB broth containing 25mg/L ampicillin in a 250mL flask incubated at 37°C for 16-18 hours. A separate flask was inoculated and incubated under the same conditions; with glycerol stocks of the XL10-gold cells transformed with the pLD-UTR vector as a negative control. The 50mL cultures were centrifuged at 8,000rpm for 10 minutes at 4°C. The supernatant was discarded; the tubes inverted on paper towels to dry and then re-suspended in 50mM Tris-HCl pH 8. Sonication was used to lyse the cells. The samples were then centrifuged at 10,000 rpm for 8 minutes at 4°C. The supernatant removed and saved, the pellet discarded. The supernatant was then used for activity assays.

Extraction of Protein from Transformed *Nicotiana tabacum* Leaves for activity assay

N. tabacum leaf material (with mid-rib removed) was ground with a mortar and pestle in liquid nitrogen and placed into a micro centrifuge tube in aliquots of approximately 100mg. 200µl of extraction buffer, with 1 protease inhibitor tablet per 10mL, was added and the samples were mixed with a micro pestle for approximately 2 minutes. The samples were centrifuged at 14,000 rpm for 5min and the supernatant placed into fresh 1.7mL micro centrifuge tubes.

Gel/Plate Assay

For the plate assay Tween agar plates were prepared by first mixing 5 g NaCl, 0.01g CaCl₂, 20g agar and 1L ddH₂O which was then set to pH 7. This mixture along with a separate bottle containing tween-20 or tween-80 was autoclaved. Before pouring into plates 10mL of the

tween was added to the agar mixture. Once solidified, 10mm wells were bored into the plates. The wells were filled with culture filtrate and allowed to incubate for 16 hours at 37°C. Lipase activity was determined by the formation of a clear halo on the turbid media.

A chromogenic plate assay prepared with 8g nutrient broth, 4g NaCl, and 10g agar. The medium was adjusted to pH 7 and autoclaved along with a separate bottle of tween-80. A 0.001% rhodamine B solution was prepared and filter sterilized. Once the mixture had cooled to approximately 60°C, 31.25mL of tween-80 and 10mL of the rhodamine B solution were added and the plates poured. Once solidified, 10mm wells were bored into the plates. The wells were filled with culture filtrate and allowed to incubate 24hours and 48 hours at 37°C. With this assay, activity was seen as orange halos in the pink media under UV light (Kouker 1987) .

Assay measuring the release of p-nitrophenol

Lipase activity was assayed using *p*-nitrophenylstearate. To prepare the reaction mixture; stock solutions of 10mM *p*-nitrophenylstearate in isopropanol were made. These stock solutions were mixed with a solution containing 50mM Sorenson Phosphate buffer (pH 8.0), 2.3mg/mL sodium deoxycholate and 1mg/mL gum arabic in a 1:9 ratio. 240µl of this solution was added to 10µl of protein extract (all protein samples were adjusted to the same concentration using the Sorenson buffer) and incubated at 37°C for 60 minutes in a 96-well micro well plate and measured using a BioRad Model-680 Microplate Reader at 405nm using p-nitrophenol standard (Mishra 128, West 1695).

Tween Assay

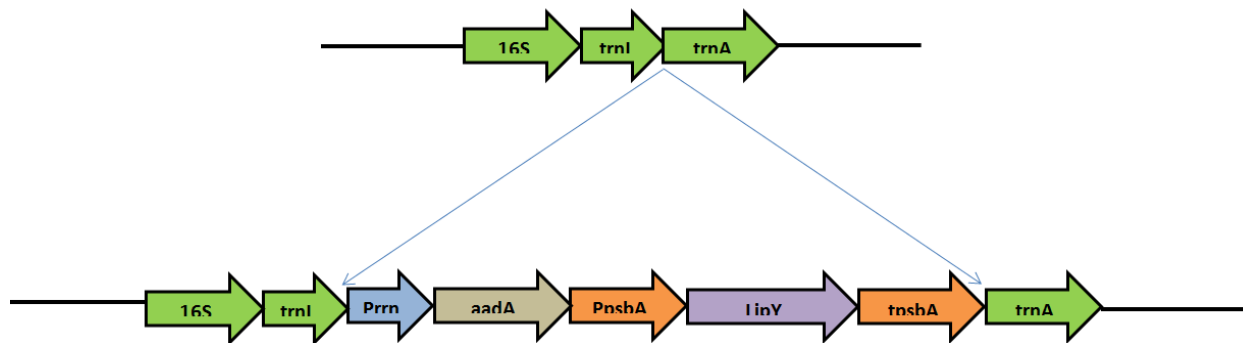
Lipase activity was assayed using Tween 80 as substrate. A reaction mixture was prepared containing 33mM calcium chloride, 0.33% Tween 80, in a 50mM Tris HCl buffer at pH 8.0. In 5mL culture tubes, the reaction mix was added to the protein extract (all protein samples were adjusted to the same concentration using 50mM Tris-HCl buffer) in a 5:1 ratio with ddH₂O used in place of protein extract for the blanks and incubated at 37°C for 30 and 60 minutes in a water bath. Turbidity was measured at 405nm. This absorbance change was due to the cleavage of Tween-80 to alcohol and a fatty acid that forms an insoluble fatty acid salt in the presence of calcium (Pratt J 2000).

RESULTS AND DISCUSSION

Construction of pLD-LipY and pLsDV-LipY vectors for Chloroplast transformation

The chloroplast expression vector harboring LipY gene was created to achieve high expression levels in tobacco and lettuce plants (Figure 3, Figure 4). The chloroplast expression vector includes a promoter (psbA for tobacco, LsPpsbA for lettuce), regulatory elements (utr), aminoglycoside 3 adenylyl transferase gene selectable marker (aadA), chloroplast flanking sequence used for homologous recombination (trnI and trnA), and the lipase gene (LipY) (Verma and Daniell 2007).

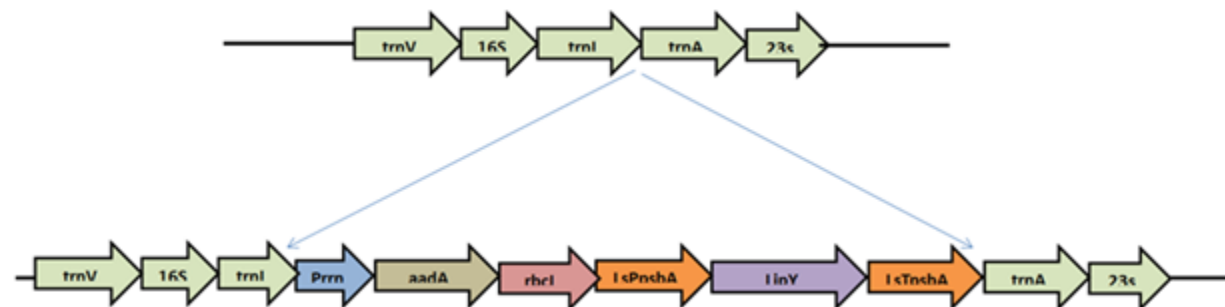
The LipY genomic DNA was initially cloned into the Topo vector (Figure 5B) for amplification of the DNA fragment. Once amplification was confirmed, showing the expected 1.31kb band, the Topo-LipY vectors were digested and sequenced. Upon confirmation of sequence the final chloroplast vector was constructed and digestion confirmed proper cassette construction for both tobacco and lettuce (Figure 5D, Figure 6B).



A) Schematic representation of cassette for *N. tabacum* chloroplast transformation.

16S) codes for the 16S ribosomal rRNA , **trnI)** chloroplast flanking sequence used for homologous recombination, **Prn)** rRNA operon promoter, **aadA)** aminoglycoside 3 adenylyl transferase gene, selectable marker, **PpsbA)** promoter, and 5' untranslated region of the psbA gene, **LipY)** Lipase gene, **tshA)** 3' untranslated region of the psbA gene, **trnA)** chloroplast flanking sequence used for homologous recombination, The blue arrows represent the location of integration into the chloroplast genome. Vector Constructed by Dr. Dheeraj Verma.

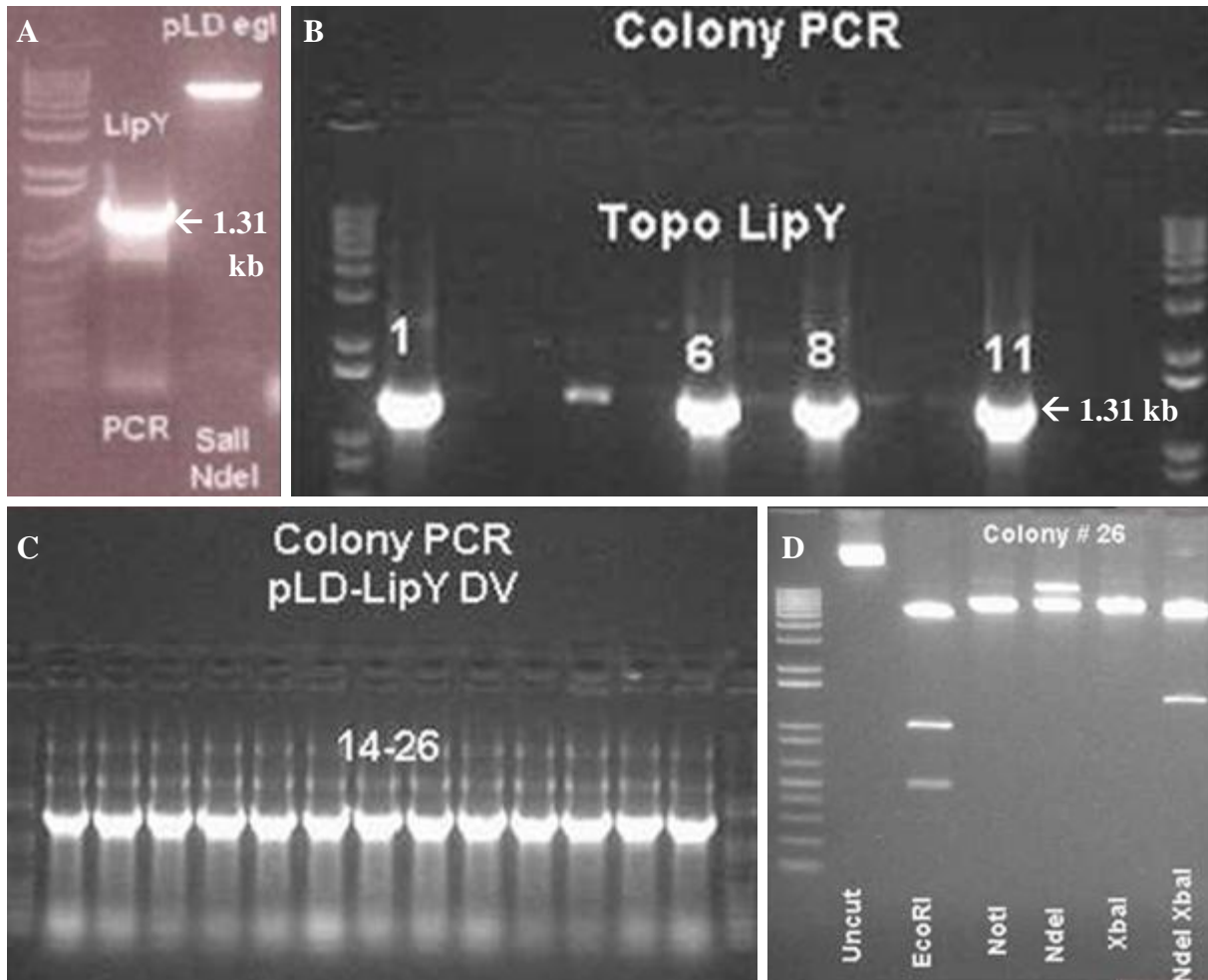
Figure 3: pLD-LipY Vector



Schematic representation of cassette for *L. sativa* chloroplast transformation.

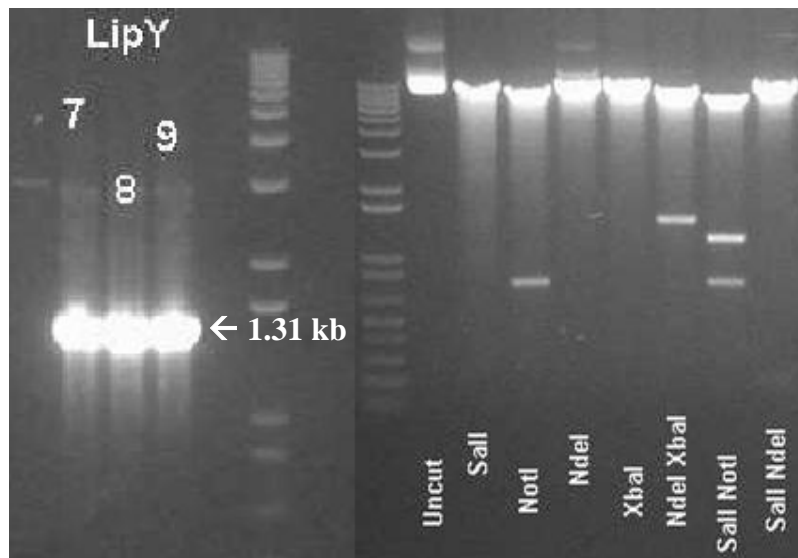
16S) codes for the 16S ribosomal rRNA , **trnI)** chloroplast flanking sequence used for homologous recombination, **Prn)** rRNA operon promoter, **aadA)** aminoglycoside 3 adenylyl transferase gene, selectable marker, **LsPpsbA)** promoter, and 5' untranslated region of the psbA gene, **LipY)** Lipase gene, **LsTshA)** 3' untranslated region of the psbA gene, **trnA)** chloroplast flanking sequence used for homologous recombination,. The blue arrows represent the location of integration into the chloroplast genome. Vector Constructed by Dr. Dheeraj Verma

Figure 4: pLsDV-LipY Vector



Gel electrophoresis of PCR products performed on 0.8% agarose gel. **A) LipY gene.** Showing expected 1.31kb band was cloned into the **B) Topo vector.** Colony PCR confirms amplification of 1.31 kb DNA fragment in four *E. coli* colonies expressing the transgene. **C) Colony PCR for pLD-LipY for 13 colonies.** All showing the proper 1.31kb band size. **D) Digestion results for pLD-LipY.** All expected size

Figure 5: Chloroplast Vector Construction Confirmation



Gel electrophoresis of PCR products performed on 0.8% agarose gel **A) Colony PCR for pLsDV-LipY for 3 colonies.** All showing the proper 1.31kb band size., **B) Digestion results for pLsDV-LipY.** All expected size

Figure 6: pLsDV-LipY Vector Construction Confirmation

PCR Analysis of *N. tabacum* Chloroplast Integration of Transgenes

Following the leaf bombardment of plasmid DNA (pLD-LipY) coated gold particles. Approximately 30 shoots appeared in the three cultivars, *Petite havana* (PH), TN-90, and LAMD (Figure 9). Each shoot was tested for chloroplast integration of transgenes via PCR analysis. The need for the confirmation arises from the three possibilities for the shoots to develop on medium containing spectinomycin. PCR allows for distinguishing between mutations providing spectinomycin resistance, nuclear transformation and chloroplast integration of the transgene.

Resistance to spectinomycin is acquired by the presence of the *aadA* gene in the gene cassette. When performing PCR with the two primers, 3P and 3M; the 3P primer anneals to the

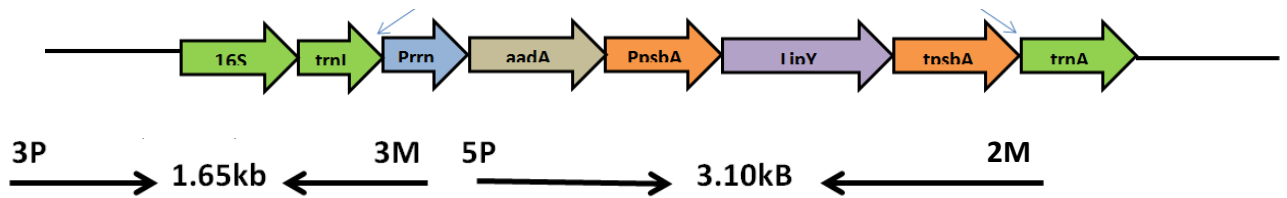
16S rRNA gene of the native chloroplast DNA, while the 3M anneals to the aadA gene. Through PCR analysis using 3P and 3M primers mutants were eliminated because the 3M primer will not anneal to a mutated 16S rRNA gene as seen in Figure 8A. Nuclear transformants may be ruled out by performing PCR with the 3P and 3M primers. Since the 3P primer only anneals to the 16S rRNA gene (in the chloroplast genome) and 3M anneals to the aadA gene (found in the cassette), nuclear integration will not allow for the 3P primer to attach (Verma, Samson et al. 2008; Jin, Kanagaraj et al. 2011)

The final possibility was chloroplast integration of the transgene. If this occurred, the 3P and 3M primers will yield a fragment that is 1.65kb in size (Figure 8A). Integration is then further confirmed by performing PCR analysis with the 5P and 2M primers (Figure 8B). The 5P primer anneals to the inner region of the aadA gene while the 2M primer anneals to the internal region of the trnA gene yielding a band 3.10kb in size as shown in Figure 8A (Jin, Kanagaraj et al. 2011).

Once PCR confirmed, the plants were transferred through various rounds of selection in order to produce mature homoplasmic plants as shown in Figure 9. Then finally moved to the greenhouse for increase biomass production. Once there the transformants showed a decrease in pigmentation with age. This may be due to either a toxicity associated with the accumulation of the LipY protein or as an adaptation to the increased foreign protein synthesis in the recombinant chloroplasts (Bally, Nadai et al. 2009).

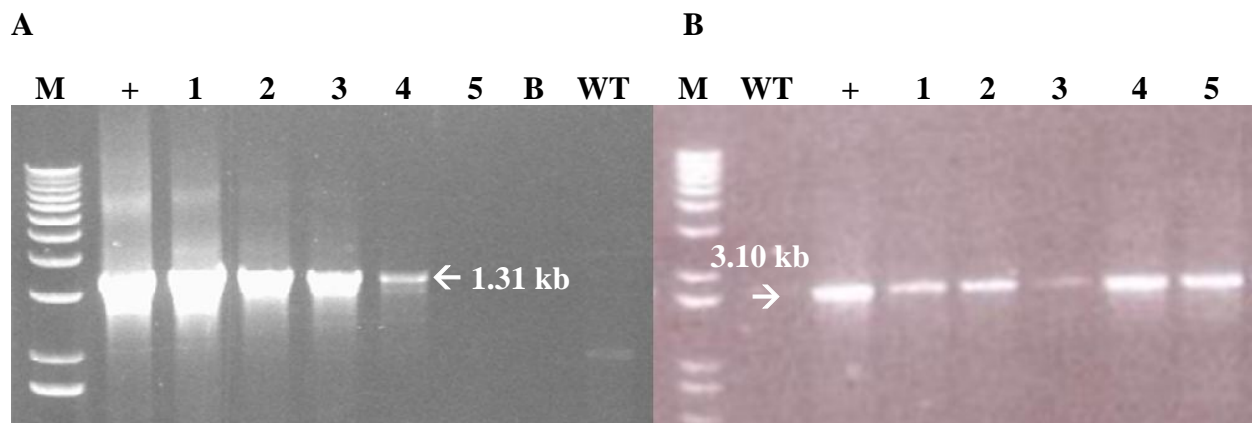
Transformants did produce seeds and the seeds when germinated confirmed maternal inheritance by having 100% of the germinated seeds produce seedlings with spectinomycin

resistance (showing green pigmentation) when grown on media containing spectinomycin as shown in Figure 9. If the inheritance had not been maternal there would have been crossing over resulting in at least a small number of seedlings without spectinomycin resistance.



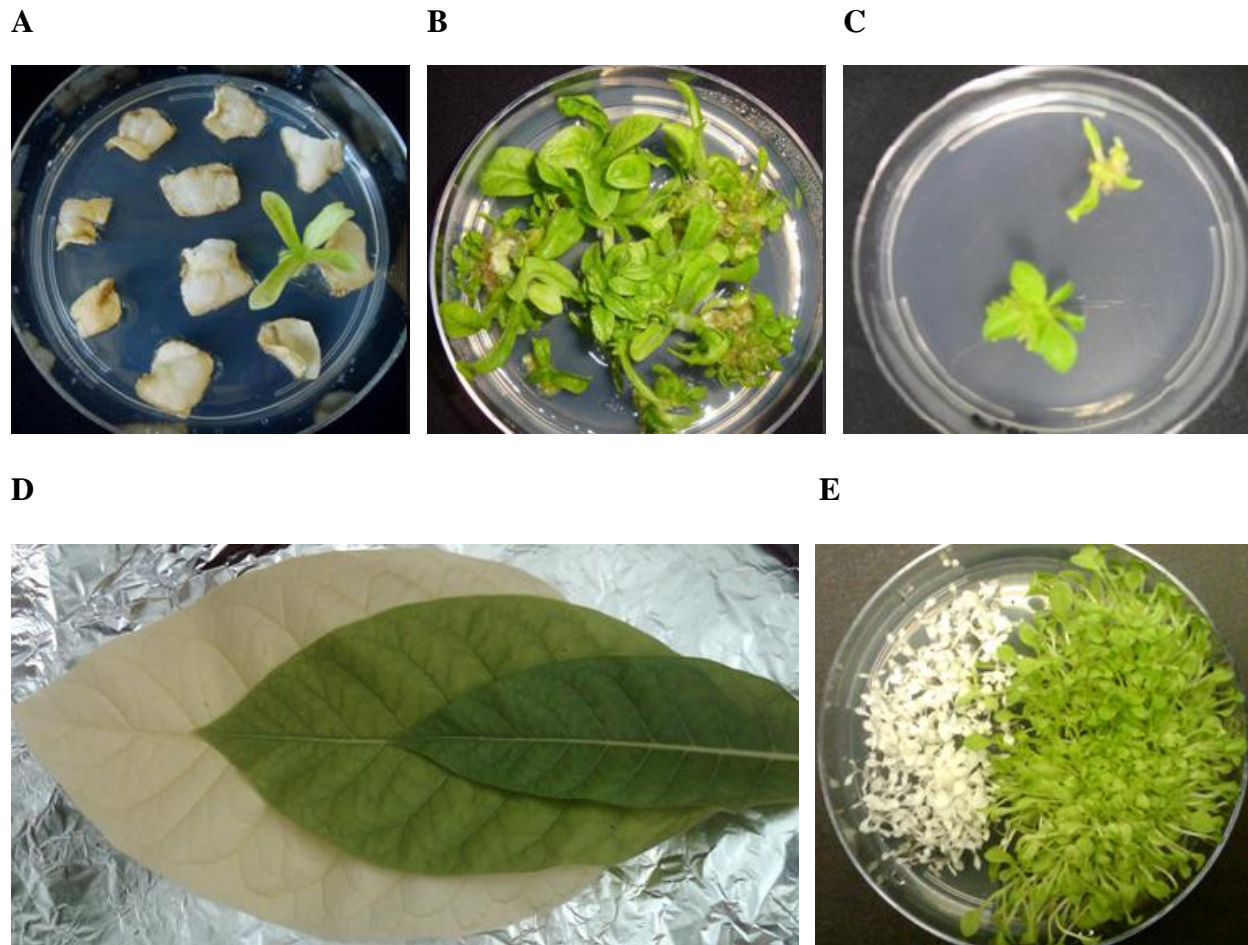
Schematic representing the transgenic cassette with primer landing sites, with respective band sizes produced from the primers pairs for PCR confirmation

Figure 7: *N. tabacum* chloroplast transgene schematic.



Gel electrophoresis of PCR products performed on 0.8% agarose gel. **A) 3P/3M primers:** Marker, 1kb+ DNA ladder (M), Positive control (+), Shoots #1-#5, Blank (B), Wild type/negative control (WT). **B) 5P/2M primers:** Marker, 1kb+ DNA ladder (M), Positive control (+), Shoots #1-#5, Wild type/negative control (WT).

Figure 8: PCR analysis of *N. tabacum* chloroplast integration of transgene.



A) 1st round transformants on RMOP media containing 200mg/L spectinomycin, **B)** 2nd round transformants on fresh RMOP media containing 200mg/L spectinomycin, **C)** 3rd round transformants on MSO rooting media containing 200mg/L spectinomycin, **D)** Left to right, Old, Mature, and Young leaves from greenhouse grown plants **E)** T₁ Generation of LAMD Transformants on the right and the WT-LAMD, negative control to the left

Figure 9: Selection of putative LAMD transformants

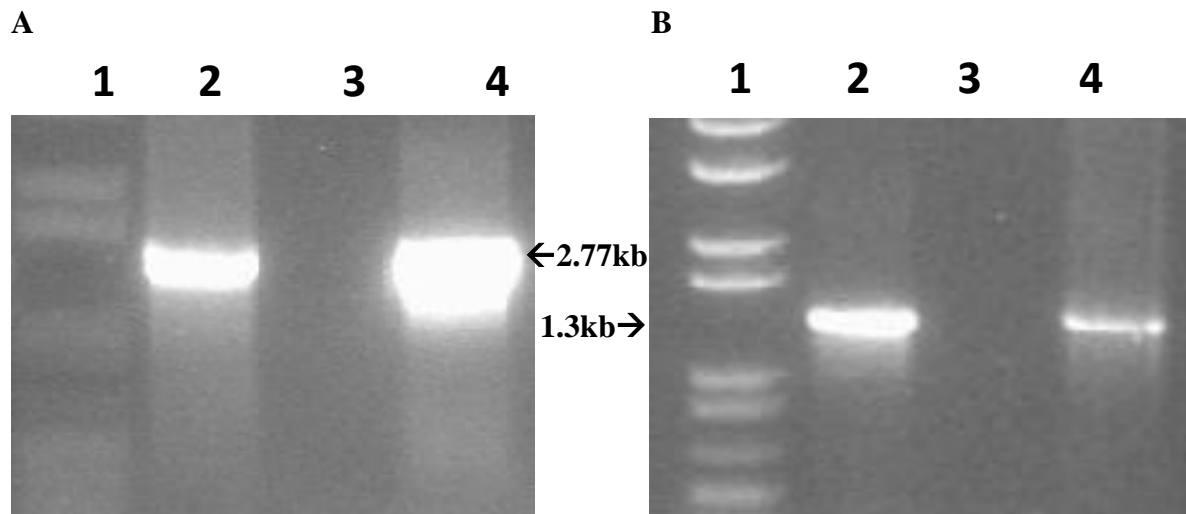
PCR Analysis of *L. sativa* Chloroplast Integration of Transgenes

Following the leaf bombardment of plasmid DNA (pLsDv-LipY, Figure 10) coated gold particles. Approximately 20 shoots appeared. Each shoot was tested for chloroplast integration of transgenes via PCR analysis using the 16SF/3M primers with the 2.77kb expected band (Figure 11A) and for gene integration using the gene specific primers with a 1.3kb expected band (Figure 11B).



Schematic representing the transgene cassette with gene specific primer landing sites with bands produced from the various primers pairs for PCR confirmation

Figure 10: *L. sativa* chloroplast transgene schematic.



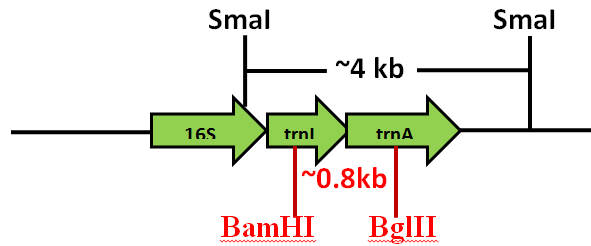
Gel electrophoresis of PCR products performed on 0.8% agarose gel **A) 16SF/3M Primers:** 1) Marker, 2) Positive control, 3) Wild type/negative control, 4) Putative shoot. **B) Gene specific Primers:** 1) Marker, 2) Putative shoot, 3) Wild type/negative control, 4) Positive control..

Figure 11: PCR analysis of *L. sativa* chloroplast integration of transgene.

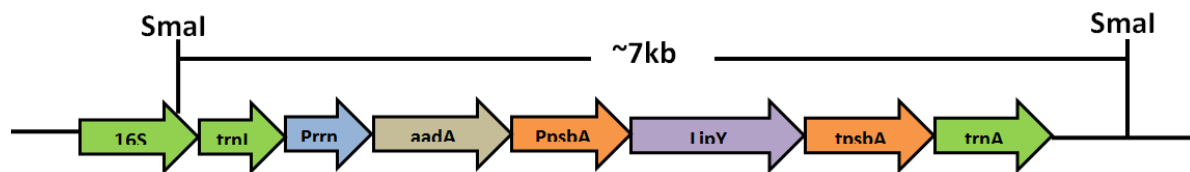
Southern blot Analysis of transplastomic *N. tabacum* plants

The plants that successfully completed three rounds of selection following PCR analysis were tested for site specific integration of the transgene and homoplasmy by Southern analysis. Only the DNA from plants grown in jars was used for this purpose. After various exposure times the following blot in Figure 12 was created. Lane 1 was loaded with the wild type and was used as a 4kb reference in determining the size of the transformant bands (~7kb). Both homoplasmy and heteroplasmy were achieved (Figure 12C).

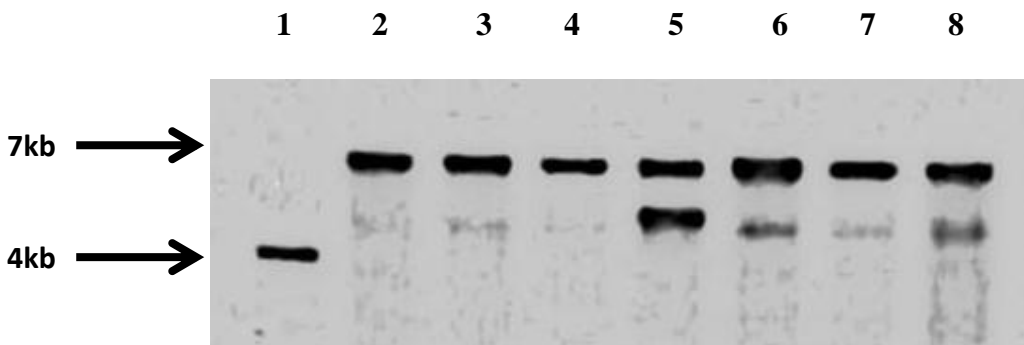
A



B



C



A) **Schematic diagram showing probe generation.** Probe generation (in red) using pUC-CT DNA digested with BamHI and BglII to produce the 0.8kb probe that will recognize the wild type chloroplast DNA (approximately 4kb) and the transformant DNA (approximately 7kb). B) **Schematic diagram showing transformant restriction site for SmaI.** SmaI located outside of the gene of interest and still including the complementary sequences for probe labeling, producing a 7kb band. C) Southern blot hybridized with generated probe. Lane 1; wild type, 4kb reference mark. Lanes 2 to 8, transformants.

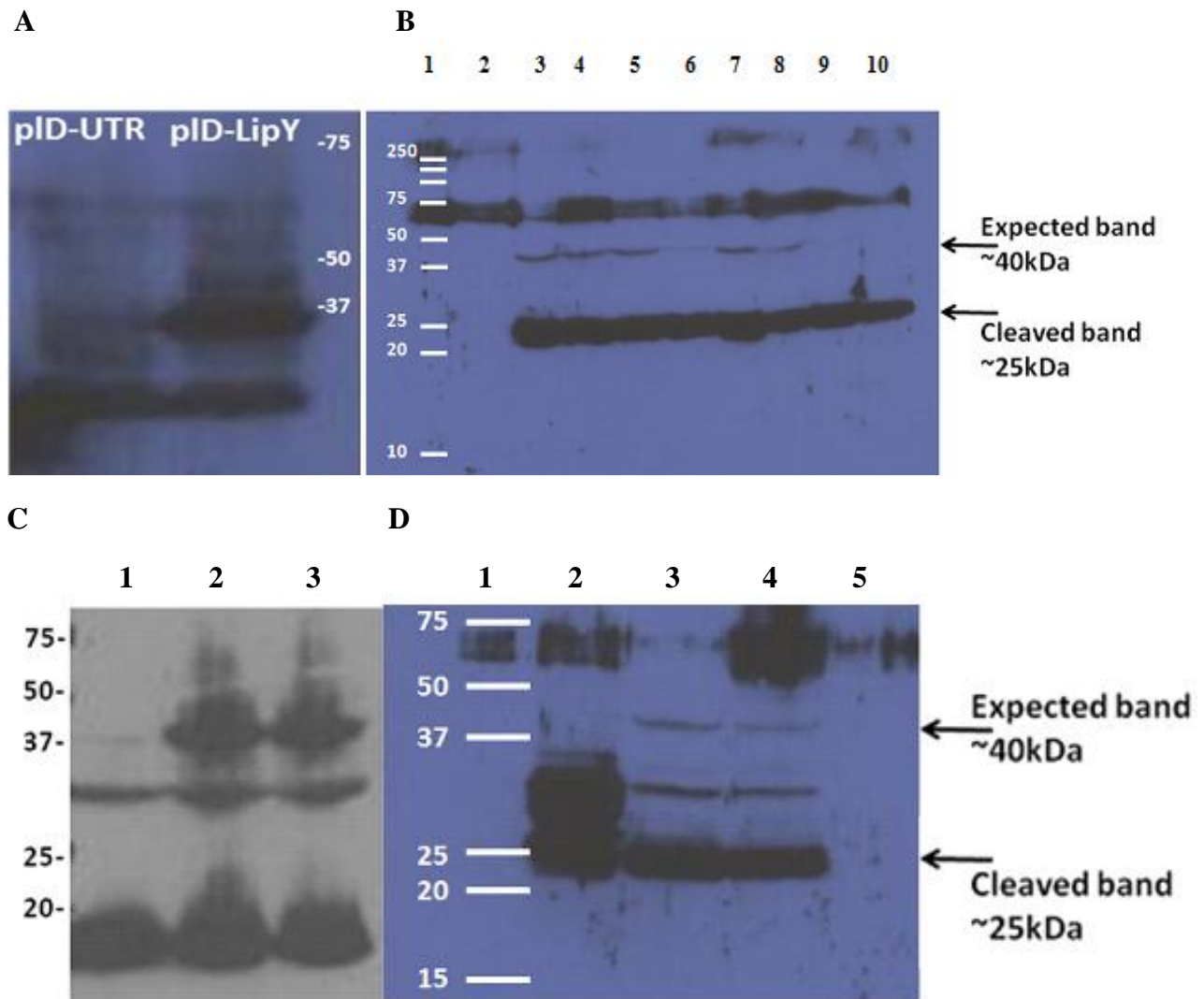
Figure 12: Southern blot analysis of PCR positive transformants.

Immunoblot analysis

Following immunoblot with rat-anti- LipY(Δ PE) primary and goat-anti-rat-HRP secondary antibodies Figure 13 resulted. While awaiting putative shoot

For the immunoblot analysis of transplastomic *N. tabacum*, as shown in Figure 13B: the marker was placed in lane #1, the wild type in lane #2, transformants in lanes #3 to #10. All lanes showed non-specific binding around 60kDa, and only the transformant lanes showed a faint band at the expected 40kDa size, and a very strong 25kDa band. This result suggested that the protein was produced; however, without the tell-tale smear of degradation, it was determined to be cleaved to a 25kDa peptide.

For the immunoblot analysis of transplastomic *L. sativa*, as shown in Figure 13D: the marker was placed in lane #1, LipY standard in lane #2, transformants Supernatant in lane #3, transformant homogenate in lane #4, and wild type in lane #5. All lanes showed non-specific binding around 60kDa, and only the transformant lanes showed a faint band at the expected 40kDa size, and a very strong 25kDa band. There is an additional 32kDa band present in the transformants and standard that was not present in the wild type. This band was previously observed in an immunoblot for the protein produced in *E. coli*. This result suggested that the protein was produced; however, it was determined to be cleaved.



rat-anti-LipY(Δ PE) primary and goat-anti-rat-HRP secondary antibodies. **A)** Immunoblot of pLD-LipY and pLD-UTR expressing *E. coli*. Expected band shown at 37kDa mark. **B)** Immunoblot of pLD-LipY *N. tabacum chloroplast transformants*. Lane 1: Marker, Lane2: Wild type, Lanes 3 to 10: Transformants. ~40kDa expected band shown, cleaved ~25kDa band appearing as well. **C)** Immunoblot of pLsDv-LipY and pLsDv-UTR expressing *E. coli* Lane1: pLsDv-UTR, Lane 2 -3: pLsDv-LipY transformants. **D)** Immunoblot of pLsDv-LipY *N. tabacum chloroplast transformants*. Lane 1: Marker, Lane2: LipY standard, Lanes 3: Transformant Supernatant, Lane 4: Transformant Homogenate, Lane 5: Wild type.

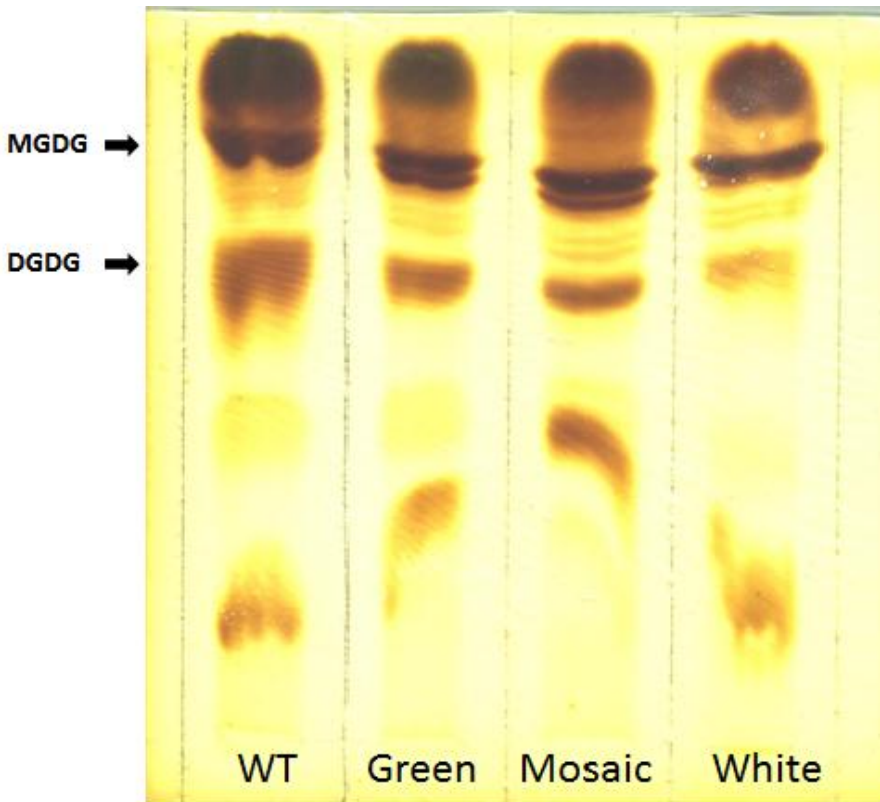
Figure 13. Immunoblot analysis of crude extracts expressing LipY.

Lipid Profile

Mature *N. tabacum* plants were showing a loss in green color as the leaf material aged from young to mature to old (Figure 9D). To determine the possible cause for this loss in color, a lipid profile was performed to test for the presence of the two most common chloroplast membrane lipids

Monogalactosyldiacylglycerol (MGDG), the major chloroplast membrane lipid, constitutes 50% of chloroplast membrane galactolipids and digalactosyldiacylglycerol (DGDG) constitutes 25% of chloroplast membrane galactolipids (Miège, Maréchal et al. 1999; Benning 2008).

MGDG and DGDG galactolipids were analyzed by a lipid profile assay. When comparing the MGDG profile young, green and mature/mosaic transplastomic leaf material showed a similar profile to that of the mature/green wildtype leaf material. The old/white transgenic leaf material showed an approximately 50% reduction in MGDG as compared to the wild-type. IN comparison



TLC of leaf lipid extracts from untransformed plant, and lipY transplastomic plants. Lipids bands were visualized using iodine vapors and were compared to standard MGDG and DGDG to identify the position of MGDG and DGDG in the samples. **MGDG** (Monogalactosyldiacylglycerol), **DGDG** (digalactosyldiacylglycerol) **WT**) Wild Type green leaf tissue, **Green**) young, green transplastomic leaf tissue, **Mosaic**) mature, mosaic transplastomic leaf tissue, **White**) old, white transplastomic leaf tissue.

Figure 14. Lipid Profile.

Gel/plate assay

No activity was observed in either plate assay. Since the protein is cleaved, it is possible that no activity will be observed outside of what the wild type is already capable of performing.

Assay measuring the release of p-nitrophenol

To determine the lipase activity of the plant material, the substrate pNP- was assayed with crude extracts from *E. coli* and *N. tabacum* using the assays outlined in West et al (West, Chow et al. 2009).

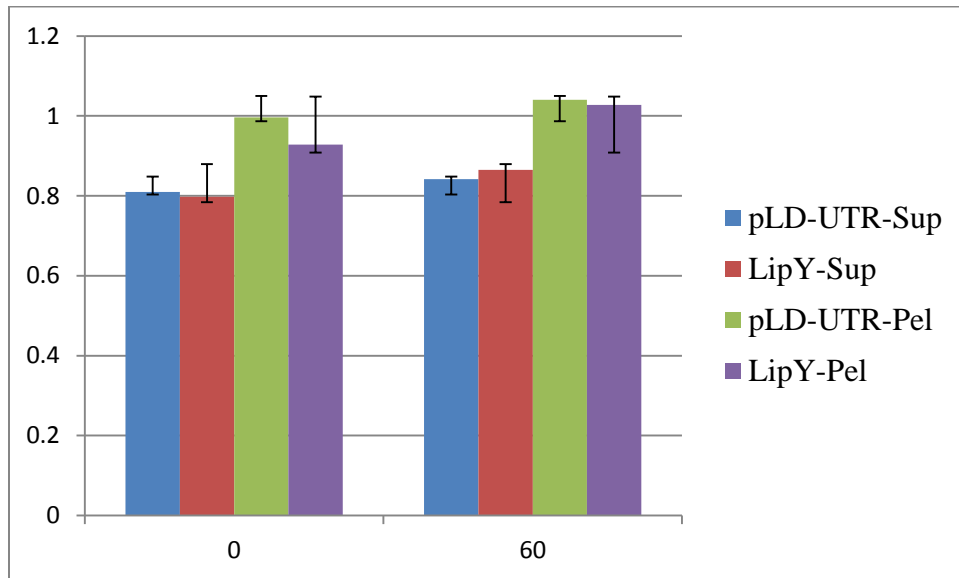
pNP-stearate analysis showed no valuable difference in activity between pLD-UTR and pLD-LipY *E. coli* extracts (Figure 15A). There was a greater change in absorbance for the wild type *N. tabacum* over the transformant extracts (Figure 15B).

Tween assays

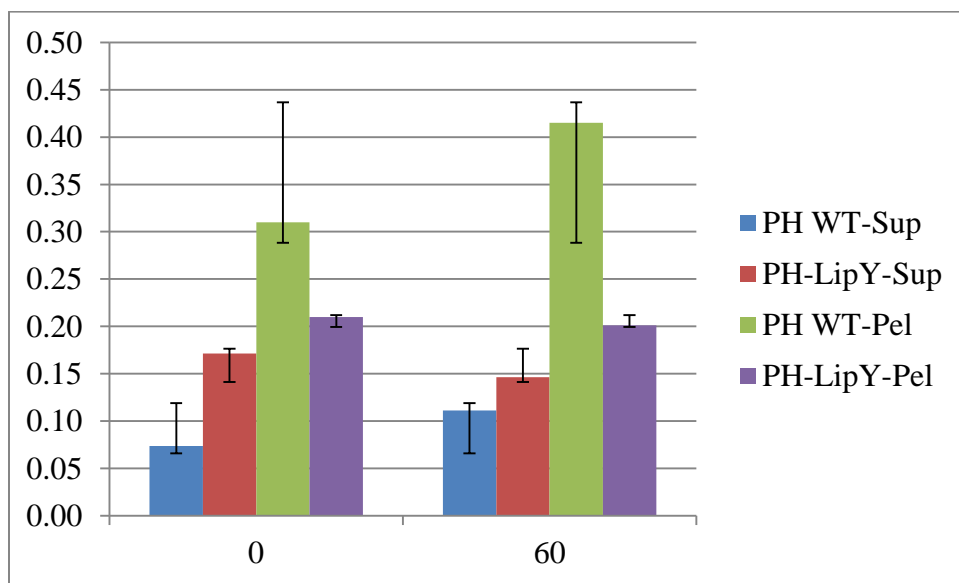
To determine the lipase activity of the plant material, the substrate, tween-80 (Figure 16) was assayed with crude extracts from *E. coli* and *N. tabacum* using the assays outlined in West et al (West, Chow et al. 2009).

Tween-80 analysis showed that in *e. coli*, the pLD-LipY pellet and supernatant formed below the corresponding pLD-UTR pellet and supernatant (Figure 16A). Tween-80 analysis of *N. tabacum*, like in the *e. coli*, showed an overall decrease in absorbance; with the Wild-type showing the highest absorbance over-all (Figure 16B).

A



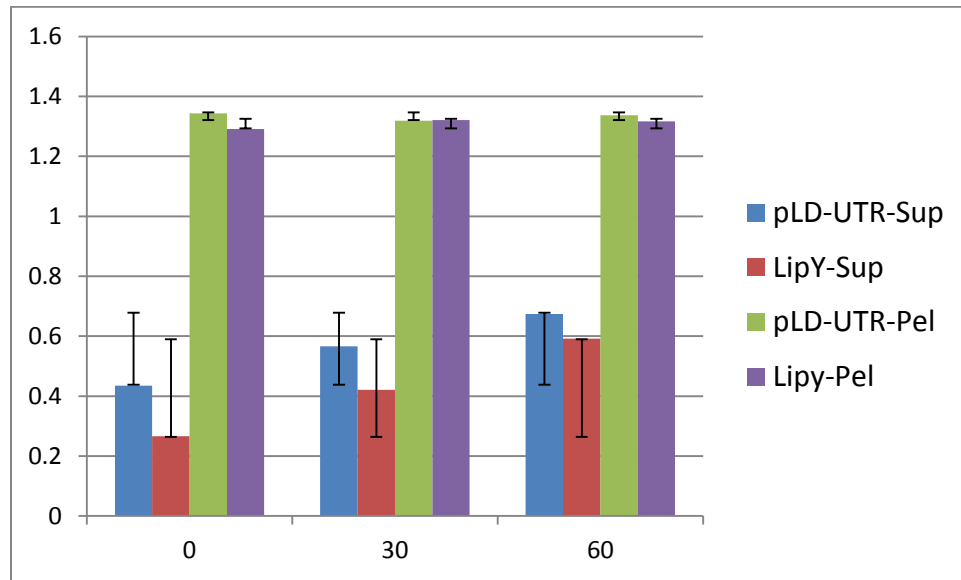
B



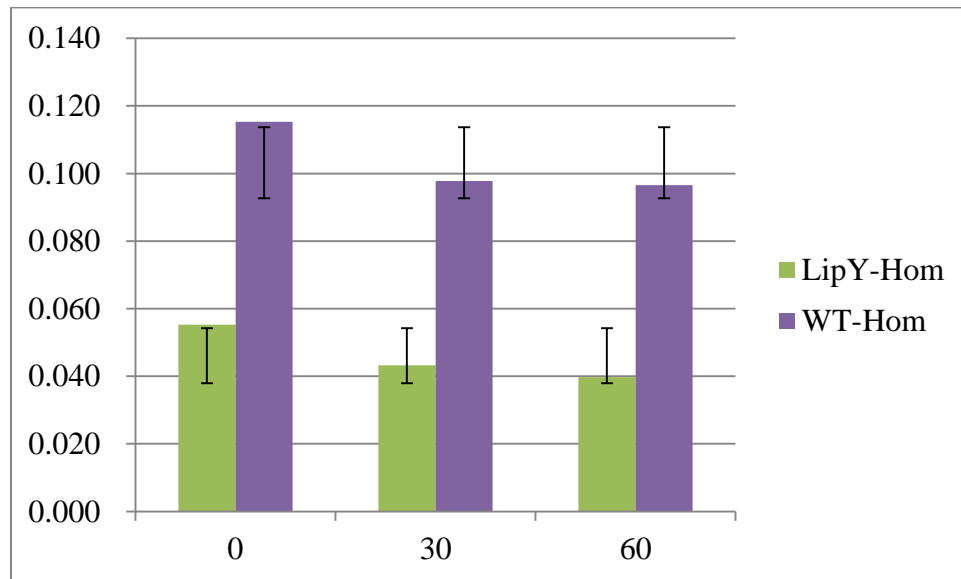
A) *E. coli*: pNP-stearate assay. **B)** *N. tabacum*: pNP-stearate Assay. In both instances the wild-type shows a greater change in absorbance. Error Bars Representing the standard deviation, n=3.

Figure 15. Lipase activity assay with pNP-stearate substrate.

A



B



A) *E. coli*: Tween-80 Assay. In both instances the corresponding pLD-UTR performs better than or as well as the pLD-LipY cells. **B)** *N. tabacum*: Tween-80 Assay. In this assay most samples actually showed a decrease in absorbance. Samples for figure B were homogenates. Error Bars Representing the standard deviation, n=3.

Figure 16. Lipase activity assay with Tween-20 and Tween-80 substrates.

Future Studies

The cleaved protein may still serve as epitope in the production of vaccines against *Mycobacterium tuberculosis* and successful shoots have been developed from the LAMD (low nicotine) cultivar. In this respect, immunization experiments will need to be taken *in vivo* to test for the production of antibodies against the cleaved LipY antigen.

CONCLUSION

PCR confirmed the integration of LipY into the chloroplast genome of both *L. sativa* and *N. tabacum*. Southern blot analysis confirmed homoplasmy in *N. tabacum* chloroplasts and immunoblot analysis confirmed the presence of a 22-25kDa cleaved protein product in both *N. tabacum* and *L. sativa* chloroplast transformants. Though the full 40-45kDa protein would be favorable functional evaluation, a cleaved protein still contains possible epitopes for the stimulation of an immune response to generate immunity. Further immunological studies will be necessary in confirming this as a potential vaccine antigen.

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