EXPRESSION AND FUNCTIONAL EVALUATION OF EXENDIN 4 FUSED TO CHOLERA TOXIN B SUBUNIT IN TOBACCO CHLOROPLAST TO TREAT TYPE 2 DIABETES

by

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ABSTRACT

The prevalence of type 2 diabetes has been steadily increasing around the globe. Glucagon like peptide (GLP-1), a powerful incretin increases insulin secretion in a glucose dependent manner. But GLP-1 is subjected to rapid enzymatic degradation (half-life: 2 min in circulation). The commercially available GLP-1 analog, exenatide has a longer half life with potent insulinotropic effects (about 2.4 hr) which requires cold storage and daily subcutaneous injections. In this study, exendin 4 (EX4), lizard derived GLP-1R agonist, was expressed as cholera toxin B subunit (CTB)-fusion protein in chloroplasts of tobacco to facilitate transmucosal delivery in the gut by utilizing the ability of CTB pentamer to bind the GM1 receptors on the intestinal epithelium and to bioencapsulate EX4 within plant cells to confer protection in the digestive system. The LAMD tobacco leaves were bombarded with chloroplast vectors expressing modified EX4. The transgene integration was confirmed by PCR analysis and Southern blot analysis. Densitometric analysis revealed expression level of the protein varied from 9-13% of the total leaf protein depending on the developmental stage and time of harvest. The pentameric structure and functionality of CTB-EX4 fusion protein was confirmed by CTB-GM1 binding assay. The effect of transplastomic protein on insulin secretion was tested in β-TC6, a mouse pancreatic cell line. The plant derived CTB-EX4, partially purified with anti-CTB antibody conjugated protein A beads, showed the increase of insulin ~ 2.5 fold increase when compared to untreated cells. The transplastomic protein showed a linear increase in insulin secretion comparable to the commercially available EX4. The current cost of treatment with EX4 varies between $1800-$2200, annually. Production of functional EX4 in plants should facilitate low cost orally deliverable form of this drug for treatment of type 2 diabetes.

Dedicated to my parents, who have been with me in every stage of my life and been supportive in every step of my career

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LIST OF ACRONYMS/ABBREVIATIONS

*aad*A – Aminoglycoside 3’ adenyltransferase

BAP – Benzylaminopurine

BSA –Bovine serum albumin

CTB - Cholera Toxin Beta Subunit

CTB-EX4 – Cholera Toxin Beta Subunit fused with Exendin 4

DNA - Deoxyribonucleic Acid

dNTP - Deoxy nucleotide triphosphate

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic Acid

ELISA - Enzyme Linked Immunosorbent Assay

EX4 - Exendin 4

GM1 - monosialotetrahexosylganglioside

GPGP - Glycine Proline Glycine Proline

GLP-1 - Glucagon like peptide-1

HCl -Hydrochloric acid

H_2SO_4 - Sulfuric acid

Kb - Kilobase

kDa - Kilodalton

$MgCl_2$ - Magnesium chloride

MS - Murashige and Skoog

NaCl - Sodium chloride

NaOH - Sodium hydroxide

NAA - Naphthalene Acetic Acid

PBS - Phosphate Buffered Saline

PBST- Phosphate Buffered Saline-Tween 20

PCR - Polymerase Chain Reaction

PMSF -Phenylmethanesulfonylflouride

^{32}P - Radioactive phosphorus

psbA - Photosystem b/A

RMOP - Regeneration Media of Plants

RNA - Ribonucleic Acid

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SSC - Sodium Chloride and Sodium Citrate solution

TEMED - Tetramethylethylenediamine

T2D - Type 2 Diabetes

WT - Untransformed Plant

UTR - Untranslated Region

UV - Ultraviolet

CHAPTER ONE: INTRODUCTION

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2D), is a disease state marked by high levels of blood glucose. It is a non communicable disease and is predominantly diagnosed in adults and is the prevailing cause of diabetes in the world, which is responsible for 90-95% of the existing diabetic cases [1]. The factors contributing to the pathogenesis of T2D mellitus are multi factorial. It is caused by combination of beta cell dysfunction, insulin resistance and insulin insensitivity. The pre responsible factor among the three has been argumentative [2, 3]. The environmental factors and the lifestyle including changes in diet, reduced physical activity, aging can be included in the risk factors of T2D mellitus [2, 4]. Obesity is a major factor which predisposes for T2D mellitus and insulin insensitivity. Surplus production of hepatic glucose, reduced uptake of glucose also contribute to excess blood glucose levels [2]. The type 2 diabetic patients exhibit belittled incretin effect, which requires insulin secretion in response to high load of glucose in the blood [2]. There is a diminution in the beta cell mass of the pancreas. The insulin insensitivity precedes the hyperglycemic condition which exacerbates the prevailing condition and worsening the disease state [2]. T2D is characterized by a defective beta cell function rather than diminished number of beta cells [5].

The national diabetes fact sheet, 2011 states that diabetes is the seventh leading cause of death in United States and affects 8.3% of the population [1]. It is calculated that by 2030, 7.8% of adult world population would be affected by diabetes [6]. Diabetes is a global rising problem. Diabetic patients have high risk of incurring disease states as kidney disease, non traumatic limb impairment, blindness, heart disease, hypertension, nephropathy and stroke. These conditions

aggravate the diabetic state. According to the national diabetes fact sheet, the total cost associated annually for treatment and management of diabetes in United states is $116 billion whereas the indirect costs associated with work loss, disability and other factors was $58 billion adding up the total cost to $174 billion [1]. The treatment for diabetes includes either oral drugs (small molecules) with or without insulin injections. On an average, the diabetic person spends twice as much money more in medical costs compared to a non diabetic person [7]. The prevalence of diabetes is judged to escalate from 2.8% in 2000 to 4.4% 2030 which could double the associated costs. The diabetes will continue to grow like an epidemic and would be a burden on the society especially with increase in obesity [8]. Thus to control the glucose levels and thereby treat diabetes should be addressed to address the economic burden on the society.

Glucagon like Peptide-1

The Glucagon like peptide -1 (GLP-1) is a hormone secreted by the L cells of the intestine after ingestion of food in response to elevated blood glucose levels. It binds to the GLP-1 receptor on the pancreatic beta cells and increases the insulin secretion [9]. It has been established to play an important role in increasing the beta cell mass and has potent antidiabetic effects associated with weight loss [10]. The GLP-1 showed appropriate glycemic control in humans [11]. But GLP -1 has a very less half life of less than 2 min since it is degraded by the dipeptidyl peptidase IV (DPP IV) serum enzyme into biologically inactive form lowering the incretin action of the peptide [12]. Thus DPP IV resistant GLP-1 analogs are needed for the treatment of T2D. The agents which use incretin based therapy are currently being investigated more because they posses property of glucose control with preservation of beta cell mass [13].

Exendin 4

Exendin 4 (EX4), a peptide composed of 39 amino acids has been isolated from the saliva of the lizard *Heloderma suspectum* (Gila Monster) [14]. EX4 from the lizard saliva is 52% identical at the amino acid level to the mammalian GLP-1 and has been found to be encoded from a prohormone distinct from the proglucagon which encodes the GLP-1 in the intestinal cells [15]. EX4 binds to the mammalian GLP-1 receptor with the same efficacy as GLP-1 and functions as an effective agonist. EX4 has a nine amino acid sequence in the C terminal which increases the efficiency for binding the GLP-1 receptor and receptor binding was linked to increase in cAMP signal levels and glucose mediated insulin secretion [16]. EX4 is also known to stimulate the synthesis of glycogen in the liver and the muscles and as well as induces the glucose catabolism [17]. The advantage of EX4 is that, it is not subjected to rapid degradation by DPP IV enzyme and is extremely stable, almost 3 to 4 hr [18] thereby providing prolonged incretin effect and help increase the insulin secretion [19]. EX4 modulates the glucose level in a glucose dependent manner and the sensitivity to insulin is increased by EX4. It has shown promising biological activities in vivo for treating T2D [18]. Though EX4 and GLP-1 demonstrate similar insulinotropic effects, EX4 exhibited higher efficacy on insulin dependent glucose control [20]. EX4 provides better glycemic control than GLP-1 and so is considered a putative drug candidate for treatment of T2D showing potency in human volunteers to reduce glucose levels and calorie intake [21]. EX4 exerts a powerful and prolonged insulinotropic action in both diabetic and non diabetic patients [22]. EX4 has also shown to retard the development of T2D in type 2 diabetic mice. The mechanism involved is conceived to be by increase of the beta cell mass, neogenesis of beta cell and the control of apoptosis of beta cells [23, 24]. Thus, EX4 is

a potential candidate to be used in treatment for the T2D since it involves multiple mechanisms of action and remains the most favored drug choice of GLP-1 receptor agonist.

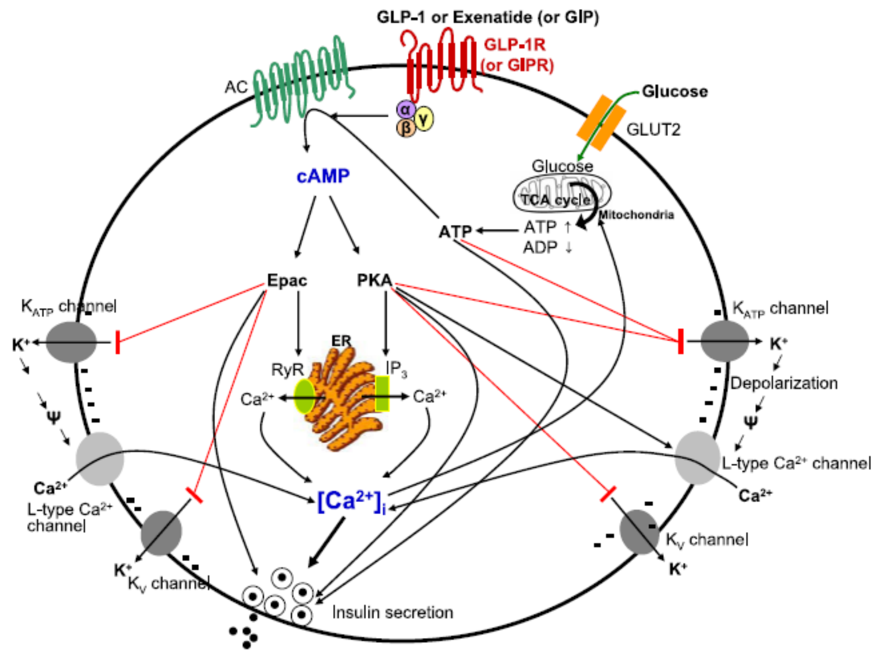

Figure 1: Action of GLP-1 agonist on insulin secretion

Figure adapted from [25].

Exenatide

Exenatide is a synthetic EX4 which is the first drug that carries out the function of incretin approved by the United States FDA to be used for glycemic control in addition to an oral anti diabetic medication. The amino acid sequence of the exenatide is identical to the EX4 sequence. Studies pertaining to check the functionality of exenatide has shown glycemic control with reduction in the glycated hemoglobin levels when used along with existing therapy [26, 27]. The human trials carried out with exenatide long acting release form showed its action on

glycemic control and weight loss is dose dependent with meager hypoglycemic effects [28]. The prominent side effects associated with exenatide is nausea, vomiting and diarrhea. The exenatide is available in subcutaneous injectable form commercially. The manufacturer states that exenatide needs to be stored between 2°C and 8°C when used for first time, after which they can be stored anywhere between 2°C and 25°C, requiring cold storage and sterility. In addition the use of needles decreases patient compliance. Thus, the incretin based therapy to treat T2D requires alternative methods of production and delivery of EX4 to improvise the executability of the treatment [29].

Plant derived production of proteins

The use of plants to produce therapeutic proteins, molecular farming is emerging as an alternative new technology and expected to replace the current fermentation system. The cost of producing purified immunoglobulin A is 20 times higher in the mammalian cell culture technique than the plant derived production [30]. The purification costs are less expensive if the protein or the vaccine antigen candidate is being consumed as food which is the fundamental concept behind oral delivery. The plant derived production of proteins includes two main categories: nuclear derived transformation and plastid derived transformation. The nuclear transformation utilizes prominently agro bacterium mediated transfer of transgenic DNA to the plants. The initial research on plant derived production used nuclear mediated transfer to produce proteins. A number of proteins including human growth hormone [31], human epidermal growth factor [32] and vaccine antigen candidates including Cholera Toxin B subunit [33], Hepatitis B surface antigen [34], Griffithsin [35] have been produced through nuclear transformation

technology in plants like tobacco, rice & potato. The main disadvantage of nuclear mediated transformation is the very meager levels of protein expression, most of them less than 1% of the total soluble protein [36]. The nuclear transgene technology has also problems like transgene silencing, position effects, risk of cross pollination with the non transplastomic plants [36, 37] making the technology less favorable.

Chloroplast derived transformation

The chloroplast technology integrates the transgene into chloroplast genome through homologous recombination [38]. The technique offers several advantages including very high copy number of up to 10,000 copies of the plastid genome per plant cell allowing the very high expression level of the transgene in this burgeoning technology [30]. The maternal inheritance pattern of chloroplast is a very important characteristic feature of the method which provides biological containment of the transgene and prevents genetically modified pollen contamination which is a major concern in the agro biotechnology field [37, 39]. The evaluation of chloroplast genome for maternal inheritance showed that only 6 out of 2.1 million seedlings showed paternal inheritance, making this technology a well disposed one for gene containment [40]. In addition, the expression of protein in leaves allows the harvest before appearance of reproductive structures. The chloroplast technology overcomes the transgene silencing effect and position effect through site specific recombination which are notable hurdles in nuclear transgene technology [30, 38]. As well as chloroplast technology offers expression of multiple genes through a single transformation event due to efficient transformation of the polycistronic mRNA of the plastids [41]. Multiple genes have been expressed in chloroplast with high transformation

efficiency of up to 46% of the total soluble protein [42]. The proteins expressed in the plant systems need to fold properly and form disulfide bonds. The chloroplast mediated technology allows proper folding, correct disulfide bond formation and formation of oligomers in addition to proper lipid modifications [36, 43, 44]. There are a number of therapeutic proteins whose functionality has been confirmed when expressed in transplastomic plants including biopharmaceutically valued proteins like insulin like growth factor [45], interferon α2b [46], coagulation factor IX [47], bacterial antigens like anthrax protective antigen [48], viral antigens like human papilloma virus L1 [49], dual vaccine to against cholera and malaria [50]. There have been high levels of expression for proteins like cholera toxin B subunit fused to human proinsulin expressing 72% of total leaf protein [51], cholera toxin B subunit fused to human proinsulin containing A,B,C peptides expressing up to 53% of total leaf protein [44], antimicrobial proteins expressing up to 30 % of total leaf protein [52]. Tobacco remains the primary choice of expression system, the reason being ability to obtain large biomass, high efficiency of transformation and rapid scale up. Also other advantages include easy transformation of tobacco vector in to plants and obtain the futile leaf material within a few months from start [38]. Efforts have been made to create and deliver bioactive form of GLP-1 through adenoviral mediated gene transfer [47]. Also, attempts have been made to orally deliver EX4 using nanoparticle [53]. But producing EX4 in plants and delivering them orally may eliminate the need for expensive chemical synthesis, sterile delivery using injections and demand for cold storage. Therefore there is a requirement to further proceed with this concept to make cost convenient and large scale production of EX4 feasible.

Concept of Bioencapsulation and Oral Delivery

On oral delivery of plant derived proteins, they need to be ensured free of degradation from the enzymes in the stomach. It has been thought that the plant cell wall provides protection for the proteins inside the plant cells [36]. A number of proteins have been successfully delivered through bioencapsulation like the coagulation factor IX, human proinsulin [44, 47]. In addition the poor intestinal absorption peeps as another obstacle in the oral delivery method. For overcoming this obstacle and efficient transmucosal delivery, proteins have been fused to CTB which adopts the concept of receptor mediated delivery [54]. The CTB has a strong binding affinity for the gut GM1 receptor, which then facilitates the delivery of fused proteins to the gut associated lymphoid tissue. A number of studies have shown proper functional delivery of the proteins fused to CTB [43, 44, 47, 54]. This affirms the reason to select the chloroplast mediated transformation and fusion to CTB subunit provides a cost effective production of therapeutic proteins.

CHAPTER TWO: MATERIALS AND METHODS

Expression in E.coli

To estimate the fusion protein expression in E.coli, the CTB-EX4 gene was cloned into the plasmid pLDPutr and transformed into XL-10 gold competent cells (Stratgene). Upon successful transformation the positive clones were selected on antibiotic plates containing ampicillin and were inoculated in LB media containing ampicillin, allowed to grow overnight at 37 °C. The next day the cells were centrifuged at max speed and the pellet was resuspended in 1X sample buffer containing 50 mM tris Cl, 100 mM DTT, 2% SDS, 20% glycerol and final volume made up to 1 ml with autoclaved distilled water. The sample was boiled for 5 min and protein concentration was estimated by Bradford assay. Various concentrations of the proteins were loaded on 12% SDS PAGE gel along with untransformed E.coli cells. The membrane after transfer was probed with anti-CTB antibody to verify the expression of fusion protein.

Bombardment and Selection

Bombardment of Wild type Tobacco Leaves

Wild type LAMD tobacco leaves were obtained from seeds germinated on Murashige and Skoog (MS) medium without antibiotic. The gold particle pellet stock stored at -20°C was vortexed. Ten microliter of pLD-CTB-EX4 construct was added for every 100 µl of gold particle in a 1.5 ml micro centrifuge tube. In addition 100 µl of 2.5M $CaCl_2$ and 40 µl of 0.1M spermidine were mixed along with 100 µl of gold particle while vortexing. The mixture was incubated in the shaker at 4°C for 5 to 15 min. The preparation was spun for 1 min at 5000 rpm.

The supernatant was removed and 600 µl of 70% ethanol was added slowly to pellet. The solution was removed immediately and 600 µl of 100% ethanol was added and vortexed to resuspend. The suspension was spun at 5000 rpm for 1 min and the supernatant from the process was discarded and was repeated for 2 times. Then 110 µl of 100% ethanol was added, vortexed and stored on ice until use. The sterilized macrocarrier was placed inside the microcarrier using the insertion cap. Ten microliter of DNA coated gold particle was spread on each macrocarrier and allowed to dry in the laminar air flow hood. Bombardment was carried out using Bio-Rad PDS-1000/He gene gun as illustrated previously [38]. The Abaxial side of the wild type LAMD tobacco leaf was placed up on RMOP media without antibiotics. The leaf material in the uncovered petridish was placed in the target holder placed 9 cm from top of PDS chamber. Rupture disk of 1100 psi and vacuum pressure of 28 inches Hg was used.

Selection and Regeneration of Transplastomic shoots

The leaf pieces after bombardment were incubated in dark for two days at room temperature. The RMOP media was prepared by dissolving 1 pack of MS salt, 1 mg of thiamine, 100 mg of myoinositol, 1 mg of BAP, 0.1 mg of NAA, 30 g of sucrose and the final volume was made to 1 liter with sterile distilled water and pH checked to be 5.8 and finally 5 g of phytoblend was added and was autoclaved. Spectinomycin (200 mg/l) was added for effective selection of LAMD cultivars [55]. Each of the bombarded leaf was cut into small pieces and the bombarded side was placed touching the RMOP media. Then each petridish was wrapped with parafilm and incubated in the culture room. The putative transformants appeared after 5 to 7 weeks. The PCR positive putative transformants were again transferred to fresh RMOP media containing 200 mg/l of spectinomycin for second round of selection and to attain homoplasmy. The regenerated

shoots formed after second round of selection were transferred to ½ MSO rooting media containing 200 mg/l of spectinomycin for the development of roots.

PCR

The plant genomic DNA was extracted from the wild type leaf and putative transformant shoots using the Qiagen DNeasy Plant mini kit following the provided protocol. The reaction was performed using two different set of primers. The site specific integration of the transgene cassette into the chloroplast genome was checked using the 3P (5'-AAAACCCGTCCTCAGTTCGGATTGC-3') and 3M (5'-CCGCGTTGTTTCATCAAGCC TTACG-3') primers. The 3P primer anneals to the native chloroplast genome and 3M primer anneals to the *aad*A gene. The transgene integration was checked using the 5P (5'-CTGTAGAAGTCACCATTGTTGTGC-3') and 2M (5'-TGACTGCCCACCTGAGAGCGGAC A-3'). The 5P primer anneals to the *aad*A gene and the 2M primer anneals to the trnA gene. The PCR reaction mixture contained 5 µl of 5X PCR reaction buffer, 0.5 µl of each of the 3P and 3M primers or 5P and 2M primers, 0.5 µl of 10 mM dNTP's, 0.75 µl of 50 mM $MgCl_{2,}$ 0.2 µl of Taq DNA polymerase, 0.5 µg of extracted genomic DNA and the final volume was adjusted to be 25 µl with sterile distilled water. The reaction parameters were as follows: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 50 sec, annealing at 58°C for 50 sec and extension at 72°C for 1 min 20 sec; final elongation at 72°C for 10 min. The PCR amplified products were analyzed on 0.8% agarose gel and visualized using ethidium bromide staining.

Southern Blot analysis

Extraction and Restriction Digestion of the Plant genomic DNA

The genomic DNA was extracted from wild type and PCR positive putative transformants using DNeasy plant mini kit (Qiagen) following the provided protocol. Two to three micrograms of extracted genomic DNA was digested using Hind III enzyme. The reaction mixture contained 4 µl of 10X buffer (New England Biolabs), 2 µg of plant genomic DNA and 1 µl of enzyme and final volume adjusted to 40 µl with sterile distilled water. The reactions were incubated overnight at 37°C.

Agarose gel Electrophoresis and Transfer of DNA to membrane

The overnight digested DNA was run on a 0.8% agarose gel at 60 V until completely separated. The DNA was depurinated by soaking in 0.25 N HCl for 10 min and rinsed twice with sterile distilled water for 5 min each to facilitate efficient transfer. The denaturation of DNA was done by soaking the gel in transfer buffer (0.4N NaOH, 1M HCl) for 20 min with gentle rocking. For setting overnight transfer the gel was placed upside down facing the presoaked nylon membrane followed by filter paper cut to the same size of the gel and gentle pressure was applied by placing a stack of paper towels folded to fit gel size. Weights were placed on top to balance and to ensure even contact between gel and membrane. The DNA was transferred overnight. The following day the membrane was rinsed with 2X SSC (3M NaCl, 0.3M sodium citrate) 2 times for 5 min each. After drying the membrane on a filter paper, the membrane was cross linked using GS gene linker UV chamber at C3 setting.

Preparation of flanking probe

The flanking probe was generated by digesting pUC-CT DNA with BamH I and Bgl II enzyme and generating 0.81 kb fragment containing the chloroplast flanking regions trnI-`trnA

by gel elution. Forty five micro liter of the flanking DNA fragment was denatured at 95°C from 5 min and immediately placed on ice for 2 to 3 min. The denatured probe was added to the ready to go DNA labeling beads (General Electric) and the tube was mixed gently. Five microliter of radioactive ^{32}P was also added to the mixture and mixed well. The mixture was incubated at 37°C for an hr. The radiolabelled probe was purified by using G50 (Probe Quant) microcolumns. The column was vortexed for the resuspension of the resin. The column was centrifuged at 3000 rpm for one min after breaking the bottom plug. The probe DNA was added to the column and transferred to a new tube and again centrifuged at 3000 rpm for 2 min.

Hybridization and Autoradiography

The overnight transferred membrane was prehybridized using 10 ml of QuickHyb prehybridization solution (Stratgene) at 68°C for 1 hr using a rotary hybridizing chamber (Fischer Biotech). In the meantime 5-10 µl of the probe solution was added to 100 µl of salmon sperm DNA in an eppendorf tube, vortexed and heated at 94°C for 5 min. The probe preparation was then added to the bottle with the prehybridized membrane and incubated for 1 hr at 68°C. The membrane was washed twice with 50 ml of wash buffer 1 (2 X SSC & 0.1% SDS) at room temperature for 15 min per wash and washed twice with 50 ml of wash buffer 2 (0.1X SSC and 0.1% SDS) at 60°C for 15 min per wash. The radioactivity was checked using a Geiger counter and membrane was wrapped in a plastic wrap. X-ray film was exposed to the hybridized membrane in dark and kept at -80°C for 12-72 hr depending on the radioactive count. The exposed X-ray film was developed in an automated processor.

Multiplication and confirmation of inheritance

Southern positive plants were multiplied and around 30 plants were transferred to Jiffy peat pots (Harris Seeds) and then acclimatized in the incubation chamber for 2 weeks before transfer to the green house for collection of biomass. Seeds were collected from the self pollinated transplastomic plants and were germinated in spectinomycin containing ½ MS media along with the wild type seeds for 3 weeks in incubator room to check inheritance.

Protein Extraction and Bradford assay

The leaf material was harvested according to different time points and maturity level from plants in green house and was grounded using liquid nitrogen to make a fine powder. Three hundred microliter of freshly prepared plant extraction buffer (100 mM Nacl, 10 mM EDTA, 200 mM tris HCl pH 8.0, 0.05% Tween-20, 0.1% SDS, 14 mM BME, 200 mM sucrose, 2 mM PMSF, protease inhibitor cocktail tablet) was added to 100 mg of finely grounded plant leaf material. The leaf tissue was homogenized with hand held homogenizer for 5 min on ice, avoiding overheating problems. The homogenate was further centrifuged at 14,000 rpm for 5 min at 4°C to obtain the supernatant and pellet fractions and the pellet was resuspended in plant extraction buffer. The concentration of the total leaf protein was determined using the Bio-Rad protein assay dye reagent. The standard curve was plotted by making serial dilution from 1mg/ml BSA up to 0.03125 mg/ml. The plant homogenate, supernatant and pellet samples were diluted with water in the ratio of 1:10, 1:20 and 1:40. To the 96 well assay plate, 10 µl of sample/standard was added to each well in duplicates. Two hundred microliter of diluted

Bradford dye reagent was added to each well and the absorbance was measured at 595 nm within 30 min using a plate reader.

Western Blot analysis

To check for the expression of the CTB fused transplastomic protein, western blot analysis was performed. The samples were diluted using 2X sample loading buffer(3.55 ml dH_20, 1.25 ml 0.5M Tris-HCl pH 6.8, 2.5 ml glycerol, 2 ml 10% SDS, 0.2 ml 0.5% bromophenol blue), boiled and were loaded in the wells of 12% SDS-PAGE gel and ran at 110 V for the separation of samples according to the molecular weight. The transfer of proteins to the nitrocellulose membrane was carried out using a Bio-Rad electro blotting apparatus at 85 V for 1 hr. The transferred membrane was soaked in PBS-T (PBS with 0.1% tween-20) and incubated at room temperature for 5 min. Membranes were then soaked in PTM (PBST with 4% dry milk) and incubated at room temperature for 1 hr with gentle rocking. CTB-EX4 protein was detected using anti-CTB rabbit primary antibody (Sigma) diluted in PTM (1:3000) and anti-EX4 rabbit primary antibody (Abcam) diluted in PTM (1:2000). The membrane was incubated overnight at 4°C with gentle rocking. The next day after discarding the primary antibody solution, the membrane was once washed with PBS-T for 5 min at room temperature. Then goat anti-rabbit IgG conjugated to horse radish peroxidase (HRP) secondary antibody diluted in PTM (1:4000) was added and incubated at room temperature for 1.5 hr with gentle rocking and membrane was washed thrice with PBS-T, 10 min each and once with PBS, 10 min. Supersignal West Pico chemiluminescent substrate (Thermo Scientific) was added and the chemiluminescent signal was

detected on autoradiography film which was developed using an automated X-ray film processor.

Densitometric Studies for quantification of protein

The quantification of the transplastomic protein was carried out by immunoblot analysis along with various known concentrations of the purified CTB standards (Sigma). To plot the standard curve CTB standards of 50, 100, 150 ng were loaded. Also varying extracts of CTB-EX4 plant homogenate were added and the values obtained were directly compared to the standard curve using the Alphaimager and Alpha ease FC software. The amount of CTB-EX4 proportional to the concentration of total leaf protein was determined as per calculations provided in Verma et al, 2008.

Lyophilization

The CTB-EX4 leaf material was frozen in liquid nitrogen and then lyophilization was done to remove the water from leaf material to facilitate the increase of protein concentration. The process was carried out with the aid of the in Freezone Benchtop Freeze Dry Systems (Labconco) in vacuum for 48 hr at -50° C at 0.036 mBar. The leaf material after lyophilization was stored at room temperature to carry out further tests.

CTB-GM1 ELISA

The CTB contains the binding site for the intestinal epithelial cells, GM1. The receptors require correct pentameric confirmation of the CTB subunit for the efficient binding to the receptor [56, 57]. For the ELISA assay fresh weight leaf material and lyophilized leaf material

along with the CTB standard were used. The 96 well microtiter plate was coated with GM1 ganglioside receptor (Sigma G-7461) by incubating 100 µl GM1 (3 µg/ml) in bicarbonate buffer, (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at 4°C overnight. One hundred micro liter of PTM (3% dry milk) was coated as a control. The following day the plate was washed three times with PBS-T and sterile water. Two hundred micro liter of PTM (3% dry milk) was added and incubated for 2 hr at 37°C for blocking. The plate was again washed three times with PBS-T and sterile water. Various concentrations of protein from leaves of transformed and untransformed plants diluted in plant extraction buffer were added to the wells and incubated overnight at 4°C, including CTB protein as control. The plate was washed thrice with PBS-T and sterile water and incubated with 1:3000 dilution of rabbit anti-CTB primary antibody (Sigma) for 1 hr at 37°C. Following washing three times with PBS-T and sterile water, the plate was incubated with 1:4000 dilution of anti-rabbit IgG, conjugated to horse radish peroxidase (Southern biotech, USA) for 1 hr at 37°C. The plate was washed three times with PBS-T and water. Then, 100 µl of substrate was added and kept in dark for 20-30 min depending on the color development. The reaction was stopped by adding 50 µl of 2 M H_2SO_4. Absorbance was measured at 450 nm by using a microplate reader.

***Invitro* cell culture assay**

Culture and maintenance of β-TC6 Cells

The mouse pancreatic tumor cell line, β-TC6 was a gift from Dr.Dinender Singla (University of Central Florida). The cells were regularly cultured in formulated Dulbecco's Modified Eagle's Medium (ATCC-catalog #30-2002) supplemented with 15% heat inactivated

fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified 5% CO_2 - 95% air incubator at 37°C.

Insulin secretion assay

The β-TC6 cells grown on 12 well culture plates that had reached 70-80% confluency were incubated in glucose free Krebs-ringer bicarbonate buffer for 1 hr at 37°C. The buffer was then removed and the cells were incubated in Krebs-ringer bicarbonate buffer with the various concentrations of transplastomic peptides, commercial peptide and glucose (10 mM) for 45 min at 37°C. PBS was used as the negative control. The molar concentration of the partially purified protein was calculated based on the molecular weight of the CTB protein since quantification was based on densitometry values using purified CTB standards. The culture media was harvested and stored for insulin secretion assay. The content of insulin secreted into the supernatant was determined with the ultrasensitive rat insulin ELISA kit (Crystal Chem. Inc.,) pursuing the manufacturer's protocol. The insulin standards and the media along with the sample diluents were added to the insulin antibody coated 96 well microplate and were incubated for 2 hr at 4°C. The plate was then washed five times with 1X wash buffer and anti-insulin enzyme conjugate was added to each well and incubated for 30 min at room temperature. The plate was washed seven times with 1X wash buffer and then enzyme substrate solution was added and incubated for 10 min at room temperature in the dark. Finally 1M sulfuric acid was added to stop the reaction. Absorbance was measured at 450 nm using the plate reader within 30 min and concentrations were calculated using the standard curve.

CHAPTER THREE: RESULTS

Regeneration and confirmation of transgene integration by PCR

The pLDputr-CTB-EX4 construct was created with a GPGP hinge region and furin cleavage site by Dr.Kwang-Chul Kwon (Figure 2B). The expression of the fusion protein was confirmed by western blot analysis of the transformed E.coli cells (Figure 2G). The presence of fusion protein was visualized by presence of a band at ~ 17 kDa. Chloroplast transformation vector pLDputr-CTB-EX4 was bombarded into LAMD tobacco leaves which had low nicotine content and regenerated on media containing 200 mg/l spectinomycin for selection. Four green shoots appeared by the end of 5-7 weeks. The putative transformants were confirmed for transgene integration by 3P-3M and 5P-2M polymerase chain reaction (PCR) analysis. The 3P primer anneals to the native chloroplast genome and 3M primer anneals to the *aad*A gene (Figure 2B) and produced a 1.65 kb PCR amplified fragment of the integrated transgene cassette into chloroplast genome (Figure 2C), eliminating the nuclear transformants and spontaneous mutations. The 5P primer anneals to the *aad*A gene and the 2M primer anneals to the trnA gene (Figure 2B) and produced a distinct band of 2.119 kb in size, confirming integration of transgene (Figure 2D). Thus the PCR analysis indicated the site specific integration of the transgene in the tobacco chloroplast genome. The PCR positive shoots were allowed for two more rounds of selection before further confirmation by Southern blot analysis.

Southern blot analysis to evaluate homoplasmy

In order to confirm the site specific transgene integration and evaluate homoplasmy of transplastomic lines, Southern blot analysis was performed. The genomic DNAs of the wild type and three independent transplastomic plant lines were digested with Hind III restriction enzyme. The anticipated products of Southern blot analysis are represented in Figure 2A and Figure 2B. Wild type yielded a 7.67 kb fragment and the transplastomic chloroplasts yielded a 9.77 kb fragment when digested with Hind III (Figure 2E) confirming stable and site specific integration of the transgene cassette. Two transplastomic lines showed homoplasmy while one showed heteroplasmy. The homoplasmic lines were multiplied and used for further study. All of the transplastomic seeds germinated were healthy but wild type seeds showed bleached phenotype in the germination medium containing spectinomycin showing lack of Mendelian segregation (Figure 2F).

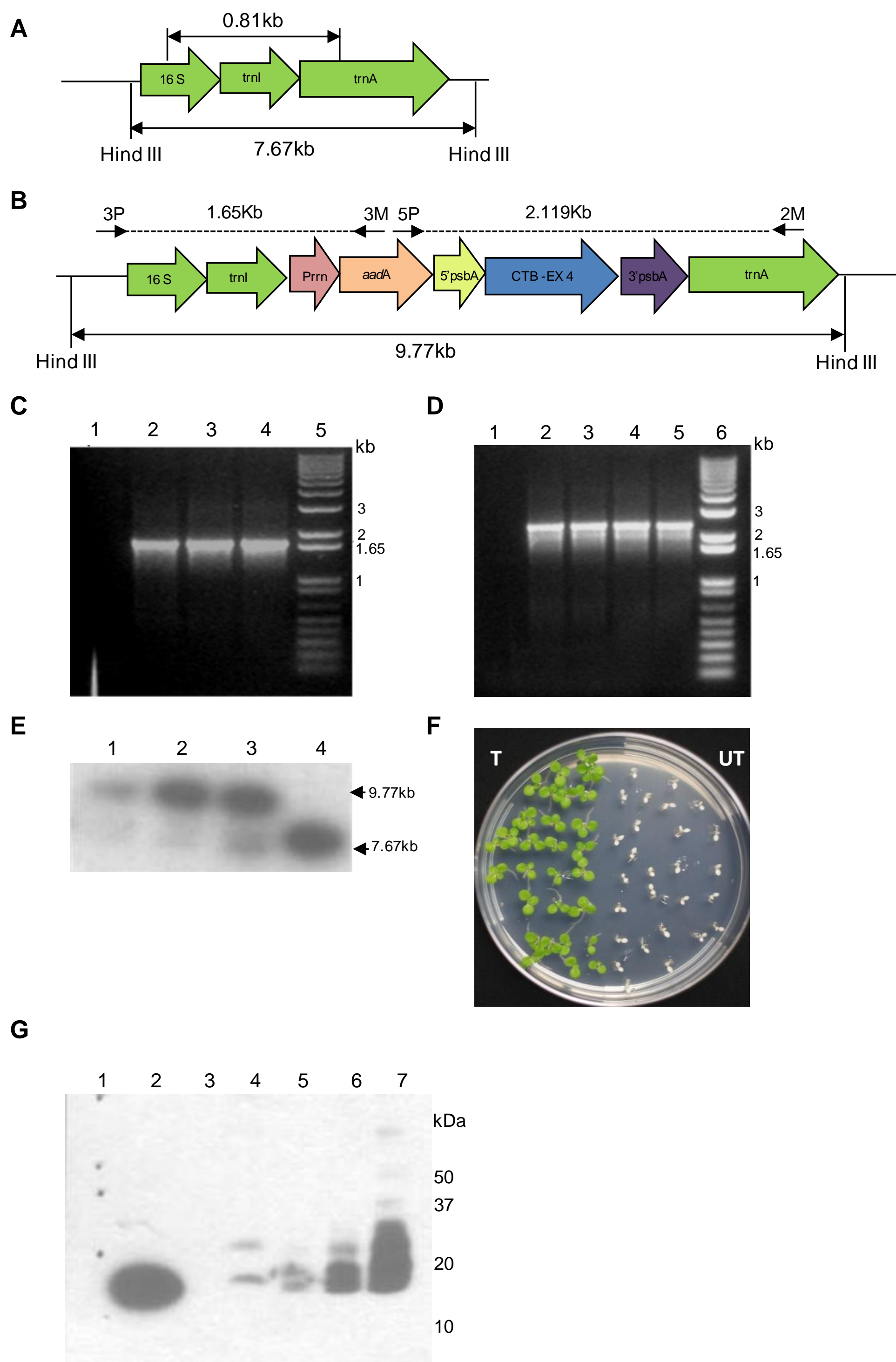

Figure 2: Evaluation of transgene integration and inheritance

(A) Schematic representation of the wild type tobacco chloroplast genome. The 0.81 kb fragment represents the region complementary to the probe used for Southern blots. (B) Schematic representation of the transplastomic chloroplast genome. (C) The amplification of genomic DNA fragment with 3P and 3M primer set, which produced a 1.65 kb distinct band. Lane 1, wild type. Lane 2, line E5. Lane 3, line E6. Lane 4, line E9. Lane 5, 1 kb DNA size marker. (D) The amplification of genomic DNA with 5P and 2M primer set, which produced a 2.119 kb distinct band. Lane 1, wild type. Lane 2, line E5. Lane 3, line E6. Lane 4, line E9. Lane 5, positive control (CTB-EX4 containing pLD-putr plasmid). Lane 6, 1 kb DNA size marker. (E) Southern blot analysis of CTB-EX4 transplastomic plants. Lane 1-3, transplastomic lines of CTB-EX4 arrowed at 9.77 kb. Lane 4, wild type arrowed at 7.67 kb. (F) Evaluation of inheritance of CTB-EX4 transplastomic seeds on spectinomycin-containing (200 mg/l) MS medium. The transplastomic plants did not follow Mendelian segregation which was evident by absence of wild type plants in the next generation plants. (G) Western blot analysis of protein expressed in E.coli probed with anti-CTB antibody showing expression of CTB fusion protein. Lane 1, Protein marker. Lane 2, CTB standard (25 ng). Lane 3, Blank. Lane 4, Untransformed E.coli protein extract (20 µg). Lane 5, Transformed E.coli extract (5 µg). Lane 6, Transformed E.coli extract (10 µg). Lane 6, Transformed E.coli extract (20 µg).

Selection and regeneration of transplastomic shoots

The shoots were selected and regenerated in RMOP media with spectinomycin (Figure 3A). The PCR positive shoots were allowed for rooting the ½ MS media containing spectinomycin (Figure 3B). The plants were confirmed to be homoplasmic by Southern blot analysis and around 30 plants were transferred to Jiffy peat pots, acclimatized in the incubation chamber for 2 weeks before transfer to the green house for collection of biomass. After moving to green house the plants were allowed for optimum growth and expression of protein. Absence of pleitropic effects in the transplastomic plants can be visualized in comparison with the wild type plant (Figure 3C).

Figure 3: Selection and regeneration of transplastomic shoots

(A) Four to six weeks after gene gun bombardment, transplastomic shoots appeared. (B) PCR positive shoots transferred to ½ MS media for rooting and molecular characterization. (C) Transplastomic homoplasmic plants transferred to green house compared to wild type plants for absence of pleitropic effects. T: Transformed plant, UT: Untransformed plant.

Quantification of EX4 expression

The leaf material from the transgenic plants was tested for the presence of the fusion protein using anti CTB and anti EX4 primary antibody. The leaf material was harvested according to the leaf age and duration of illumination from plants in the green house. Leaf material from transplastomic plant and wild type plant were ground to fine powder in liquid nitrogen. The young leaves represent the top, smaller leaves. The mature leaves represent the middle, fully grown leaves. The old leaves represent the bottom, senescent leaves. The protein was homogenized using homogenizer and was divided into homogenate, supernatant and pellet fractions. Western blots probed with anti CTB rabbit antibody and anti EX4 rabbit antibody showed the presence of a ~ 17 kDa CTB-EX4 fusion protein in the transplastomic lines (Figure 4E). The expression of CTB-EX4 was more in homogenate and pellet than the supernatant indicating the presence of multimers (Figure 4A). A predominant band at ~ 17 kDa represents the monomeric form of the fusion protein and ~ 34 kDa represents the dimer. The exposure for longer time resulted in visualization of tetramers and pentamers. Quantification of the transplastomic plant was carried out by the densitometric analysis of the transplastomic protein. Varying concentrations of the CTB standard protein were loaded on the gel along with the 5 μg of transplastomic protein extracts. The expression levels varied according to the developmental stage of the leaves and duration of exposure to illumination (Figure 4B and 4C). Figure 4B and 4C shows that the mature leaf harvested at 6 pm showed the highest level of expression which is due to the light and developmental regulation of the psbA regulatory elements. The transplastomic CTB-EX4 protein expression of mature leaf varied between ~ 9.6-12.8% of the total leaf protein. The lyophilization of transplastomic leaf material showed that ~ 90% of weight

was reduced when compared to the fresh weight leaves. Also the western blot probed with anti CTB antibody showed similar pattern of bands comparable to the fresh weight and there was up to ~14% increase in the transplastomic protein content (mg /gram of leaf) (Figure 5A).

ELISA to evaluate pentameric confirmation

The ability of CTB fusion protein to bind to the GM 1 receptor was evaluated by GM1 ELISA. The assay confirmed the binding the fusion proteins the GM1 receptors invivo and formation of the pentameric confirmation. The absorbance values were similar to the purified CTB standards. The ELISA also showed that there was proper folding and disulfide bond formation since the pentameric confirmation was required (Figure 4D and 5C).

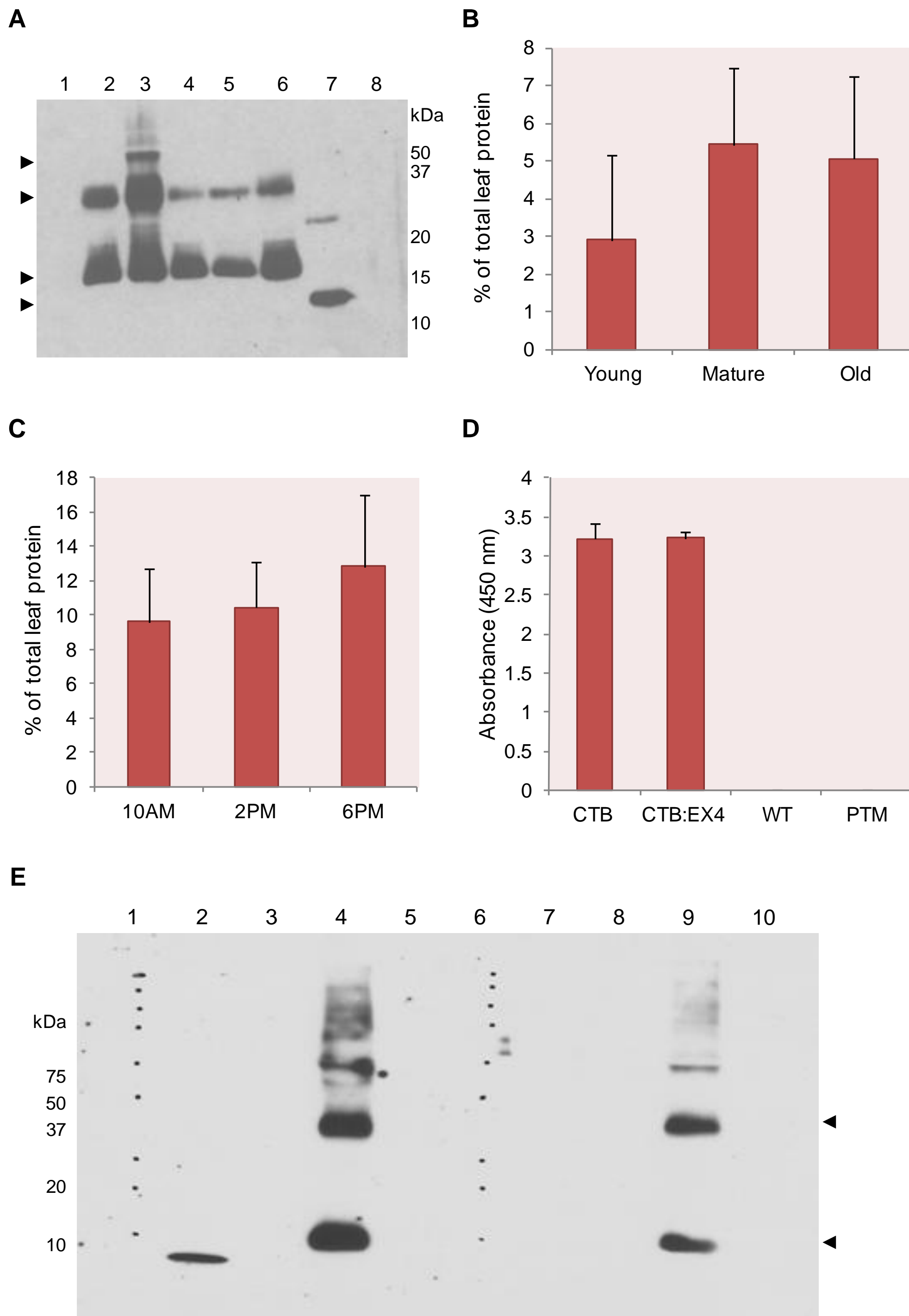

Figure 4: **Quantification and analysis of EX4 expression.**

The extracted plant proteins, of different leaf age and duration of illumination, were run on 12% SDS-PAGE gel.

(A) Western blot analysis of total leaf protein probed with anti-CTB antibody showing expression of CTB fusion protein. Lane 1, Wild type. Lane 2, Transplastomic line 5 (10 µg). Lane3, Transplastomic line 6 (5 µg). Lane 4, Transplastomic line 9 (15 µg). Lane 5 and lane 6, Supernatant and pellet of transplastomic line 9 (20 µg) respectively. Lane 7, CTB standard (50 ng). Lane 8, protein marker. Marked aero indicates the presence of monomeric and multimeric forms of the transplastomic protein. (B) Percentage of fusion protein in total leaf protein according to leaf age. (C) Percentage of fusion protein in total leaf protein according to duration of illumination. (D) GM1 ELISA assay for the presence of CTB-EX4 functional pentamers. CTB: purified CTB standard (10 ng), CTB-EX4: Fresh weight transplastomic plant extract (5 µg of total protein), WT: wild type, PTM: PBST with dry milk. (E) Western blot analysis of total leaf protein probed with anti-CTB antibody and anti EX4 antibody showing expression of CTB fusion protein. Lane 1&6, protein marker. Lane 2&7, CTB standard (25 ng). Lane 3&10, Wild type plant protein extract (15 µg). Lane 4, Transplastomic plant protein extract (15 µg) probed with anti CTB antibody. Lane 9, Transplastomic plant protein extract (15 µg) probed with anti EX4 antibody. Lanes 1-5 were probed with anti-CTB primary antibody. Lanes 6-10 were probed with anti-EX4 primary antibody. Marked aero indicates the presence of monomeric and dimeric forms of the transplastomic protein.

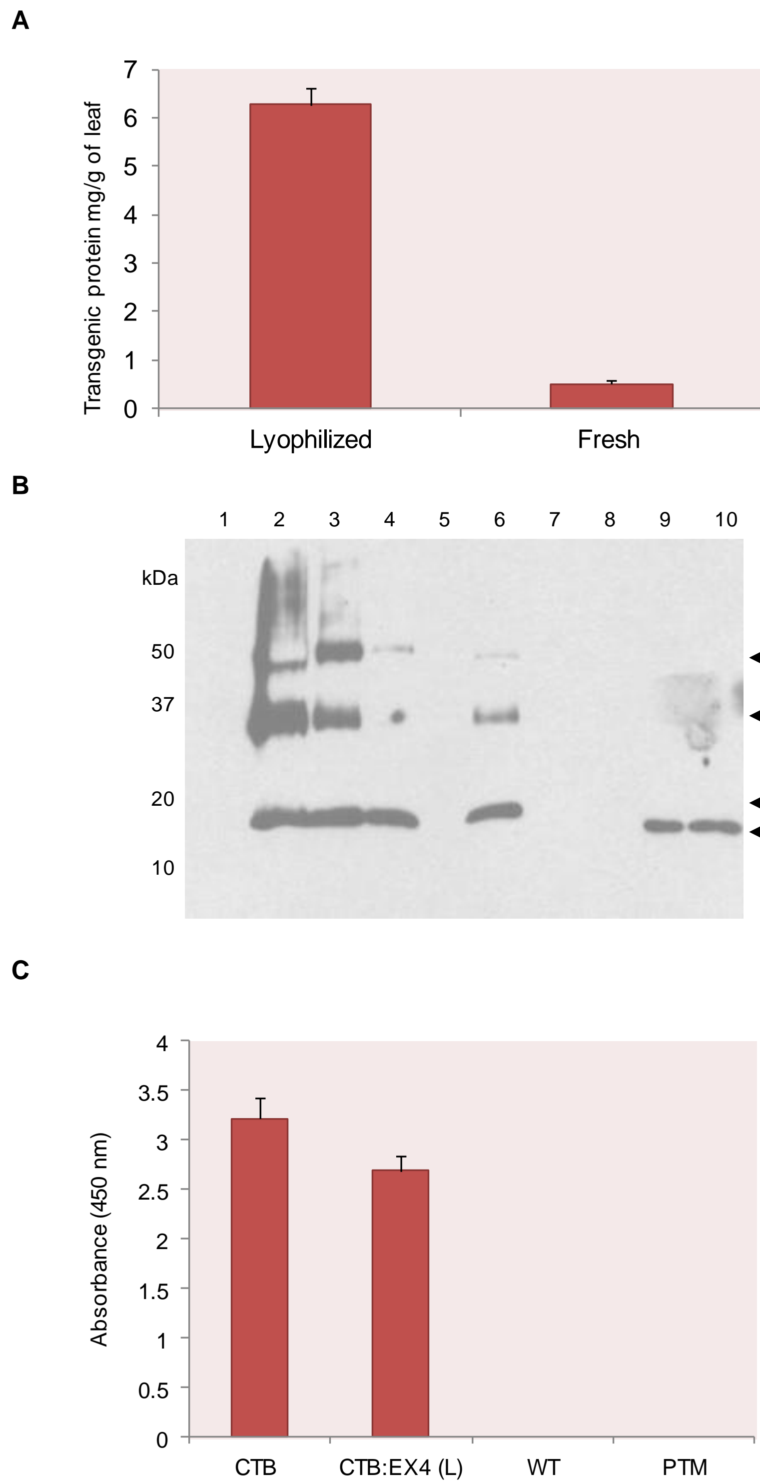

Figure 5: Analysis of lyophilized material

(A) Amount of transplastomic protein in lyophilized sample and fresh weight sample. (B)Western blot analysis of lyophilized and fresh weight material. Equal quantity of lyophilized and fresh weight material extracted. Lane 1, Protein marker. Lanes 2-4, Lyophilized protein extracts (1X, 1:10X, 1:20X respectively). Lane 5, blank. Lanes 6-8, Fresh weight protein extracts (1X, 1:10X, 1:20X respectively). Lanes 9-10, CTB standards (25 ng and 37.5 ng respectively). Marked aero indicates the presence of monomeric and multimeric form of the transplastomic protein (C) GM1 ELISA assay for the presence of CTB-EX4 functional pentamers. CTB: purified CTB standard (10 ng), CTB-EX4 (L): Lyophilized transplastomic plant extract (5 μg of total protein), WT: wild type, PTM: PBST with dry milk.

***Invitro* cell culture to evaluate functionality of transplastomic protein**

The functionality of CTB-EX4 was tested with the β-TC6 cells by incubating with the commercially available EX4 peptides and partially purified CTB-EX4 in 10 mM of glucose. The insulin secretion was observed in concentration based increment of partially purified CTB-EX4 protein (up to ~16 ng/ml) along with commercially available EX4 (~17 ng/ml) (Figure 6) when measured by the ELISA kit (crystal chem.). The molar concentrations were calculated based on CTB protein and it is a partially purified protein. This might account for the difference in insulin secretion levels of the commercial protein and partially purified transgenic protein. The furin cleaved transplastomic protein showed a slightly higher increase in insulin secretion compared to its non cleaved counterpart. This could be explained by the fact that the furin enzyme liberated the EX4 from the fusion protein. For the furin assay, the samples were incubated in a buffer containing $CaCl_2$ at 30°C, overnight. The requirement of calcium for exocytosis of insulin from

the pancreatic beta cells could be the reason for furin assay samples showing a higher insulin secretion compared to the same concentration samples incubated in normal PBS buffer [25]. Taken together, CTB-EX4 fusion protein was expressed without interference of normal structural pentameric formation of CTB in tobacco chloroplasts and maintained the functionality of EX4 in the *in vitro* assay.

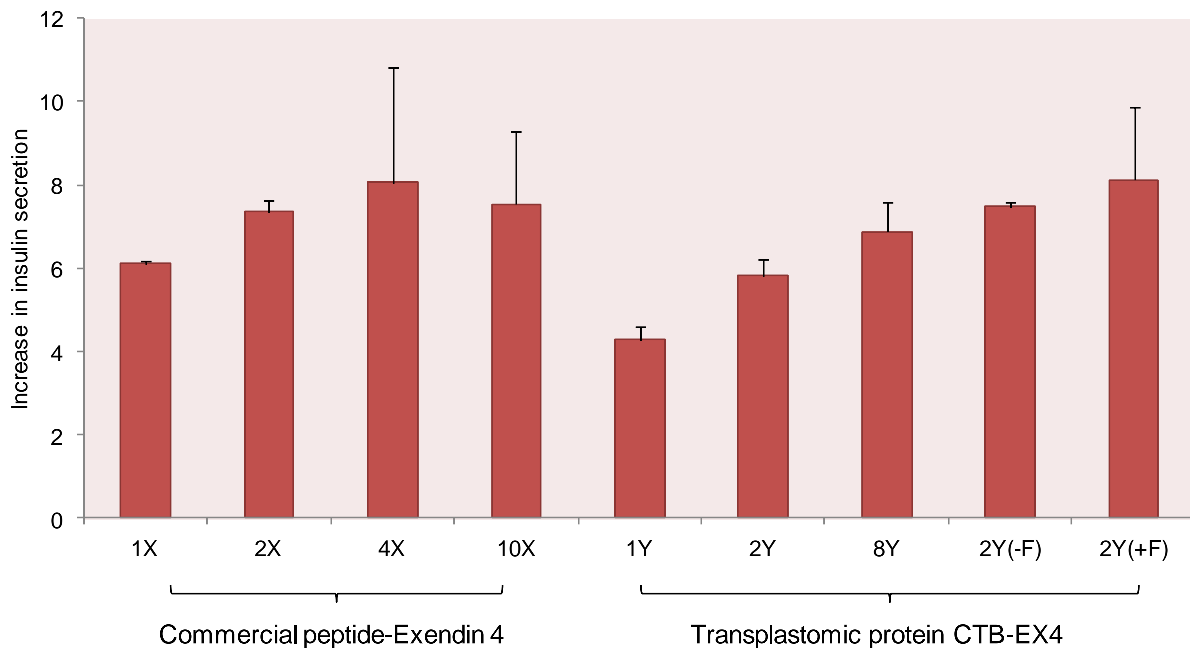

Figure 6: Insulin Secretion assay of partially purified CTB-EX4 with β-TC6 cell line. .

Commercially available EX4 and partially purified CTB-EX4 were treated to β-TC6 cells. 1X for the commercial peptide represents 5 nM concentration calculated based on the molecular weight of EX4. 1Y for the partially purified protein represents 32 nM concentration calculated based on the molecular weight of the CTB protein. The graph was normalized to PBS value which was used as negative control. Secreted insulin was assayed after incubating in Krebs-Ringer buffer containing 10 mM glucose for 45 min at 37°C. (-F): non furin treated sample. (+F): furin treated sample.

CHAPTER FOUR: DISCUSSION

Type 2 diabetes, a non communicable disease is caused by high levels of blood glucose level which is contributed by various factors including environmental factors, insulin resistance and β cell malfunction [2, 3]. T2D affects a vast majority of the population in the mankind and requires cost effective treatment, which otherwise poses the threat of growing into a pandemic [1, 6]. Exenatide, an injectable insulinotropic agent demonstrates appreciable antidiabetic effects in clinical trials of patients with type 2 diabetes [58, 59] and requires cold storage and needle usage, conditioning the requirement for other technologies. Genetically engineered plants expressing auto antigens including human IA-2 [60], human glutamic acid decarboxylase [61] proinsulin [43], insulin and C peptide [44] have been developed to treat type 1 and 2 diabetes. In this study, we report the expression of CTB fused EX4 in tobacco chloroplast and the functionality comparable to commercial EX4 as shown in Figure 6. We have used the chloroplast transformation for the production of orally deliverable form of the drug, which is a very efficacious and inexpensive method to produce biopharmaceutically important proteins [36]. The system has several advantages associated with production, purification, storage and transportation charges when compared to chemical production [36, 38, 43, 54]. It has been reported that gene containment by maternal inheritance, multiple gene expression in single transformation, proper folding and lipid modifications are the well disposed traits of the chloroplast mediated transformation technology [36, 38, 43, 54].

For unimpeded absorption in the intestine the EX4 was fused with CTB, since the CTB has high affinity to the intestinal GM1 receptors and affirms delivery [44, 47, 54]. The construct had

been stably integrated in the chloroplast genome which was confirmed when PCR positive homoplasmic shoots were obtained in the LAMD cultivars (Figure 2). Since tobacco is not a food crop there is minimal risk of escape of the transgene. The nicotine content of tobacco is a concern when a transplastomic protein is expressed in tobacco plants. In this study, so LAMD cultivar which contains low nicotine content was used [46, 55]. The expression of the transplastomic protein in the tobacco system had no pleitropic effects on the plants as the plants transferred to green house were healthy and had normal growth pattern, comparing to wild type plants (Figure 3C). The gene containment and absence of Mendelian segregation had been verified by germination of the seeds collected from the green house plants in spectinomycin containing media (Figure 2) which was similar to the earlier reports [39, 40]. High level of transplastomic protein expression was recorded by western blot in the leaves harvested from the transplastomic plants (Figure 4A). The densitometric analysis established that the mature leaf harvested at 6 pm showed the highest amount of expression (up to 13% of the total leaf protein). This could be explained by the facts that the younger leaves possess fewer chloroplasts and the chloroplast operon promoter *psbA* is developmentally regulated and the older leaves show a decrease in photosynthetic and Rubisco activity [62]. The level of expression is also dependent on the exposure to light [63]. We report here that the incretin fusion protein is stably expressed between up to ~9-13% of transplastomic leaf protein in mature tobacco plants and transplastomic protein content per gram of lyophilized leaf increased ~14 fold compared to the fresh weight. The lyophilization of leaf material resulted in removal of ~90 % of water content from the leaves and so the lyophilized leaf material could be stored in room temperature and more amount of transplastomic protein can be delivered orally. This expression level would be considered

significant in terms of production cost and storage. In addition, the transplastomic plants are easier to grow and harvest the leaf material. The current cost of exenatide with 10 mcg injection twice a day would cost between $1800-$2200 annually which is quite expensive in comparison to the other third line agents available in the market [64]. But producing exenatide in the chloroplast transformation system with appreciable level of expression may provide a solution to the existing problem and would significantly cut the cost expensive incretin treatment which would make the drug be available at a cheaper cost comparative to the available market drugs.

The pentameric confirmation of the transplastomic protein is required for efficient binding to the intestinal GM1 receptors and effective release when orally delivered which has been established in previously published reports [47, 54]. This report is consistent with the previous reports, which was affirmed by the CTB-GM1 ELISA where the absorbance values were similar to that of the positive control for the fresh weight and lyophilized leaf material. This might be considered a pre confirmatory test revealing that orally delivering the transplastomic protein will render the functional transplastomic peptide.

The β-TC6 cell line, a mouse pancreatic beta cell line was selected for the functionality study of plant derived CTB-EX4 since they exhibit the property of glucose mediated insulin secretion which is triggered by binding of GLP-1 to GLP-1 receptor on the surface of them [65, 66]. The *in vitro* study revealed that the transplastomic protein increased the insulin secretion from the pancreatic beta cell line. The insulin secretion was dose-dependent as shown in commercially available EX4 (Figure 6). There is an increase in the insulin secretion with the furin treated sample compared to the non treated sample. The release of EX4 peptide from the CTB subunit

during furin cleavage assay, allows the uninterrupted binding of the EX4 peptide to the GLP-1R on the pancreatic- β cell surface and thus triggering exocytosis of insulin from the vesicles by increasing intracellular Ca^{2+} concentration [25]. The mechanism of action of furin enzyme in the body on oral delivery of furin cleavage site containing transplastomic peptide had been previously illustrated [54]. The data indicates on oral delivery of transplastomic tobacco leaf expressing CTB-EX4 will be able to lower blood glucose level by stimulating insulin secretion. Future studies would inquire the effect of oral delivery of the transplastomic peptide to lower blood glucose levels, effect on the pancreatic β cell mass and effect on body weight. To orally deliver the therapeutic proteins, edible and stable lettuce transformation system has been established previously [44, 67]. Although the studies initially performed were in tobacco chloroplast, production of therapeutic peptide in lettuce transformation system is required. Future study would require expression of EX4 in lettuce chloroplast to develop an edible transformation system. To summarize, the transgene was stably integrated and expressed to high levels using the chloroplast technology. The expressed protein was also found to be functional regulating the glucose metabolism by increasing the insulin secretion in β-TC6 cells.

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