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LOW-COST PRODUCTION OF PROINSULIN IN TOBACCO AND LETTUCE CHLOROPLASTS FOR INJECTABLE OR ORAL DELIVERY OF FUNCTIONAL INSULIN AND C-PEPTIDE

by

DIANE DARA BURBERRY
B.S. Biology, University of Central Florida, 2006.
B.S. Molecular and Microbiology, University of Central Florida, 2006.

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ABSTRACT

Current treatment for type I diabetes includes delivery of insulin via injection or pump, which is highly invasive and expensive. The production of chloroplast-derived proinsulin should reduce cost and facilitate oral delivery. Therefore, tobacco and lettuce chloroplasts were transformed with the cholera toxin B subunit fused with human proinsulin (A, B, and C peptides) containing three furin cleavage sites (CTB-PFx3). Transplastomic lines were confirmed for site-specific integration of transgene and homoplasmy. Old tobacco leaves accumulated proinsulin up to 47% of total leaf protein (TLP). Old lettuce leaves accumulated proinsulin up to 53% TLP. Accumulation was so stable that up to ~40% proinsulin in TLP was observed even in senescent and dried lettuce leaves, facilitating their processing and storage in the field. Based on the yield of only monomers and dimers of proinsulin (3 mg/g leaf, a significant underestimation), with a 50% loss of protein during the purification process, one acre of tobacco could yield up to 20 million daily doses of insulin per year. Proinsulin from tobacco leaves was purified up to 98% using metal affinity chromatography without any His-tag. Furin protease cleaved insulin peptides in vitro. Oral delivery of unprocessed proinsulin bioencapsulated in plant cells or injectable delivery into mice showed reduction in blood glucose levels similar to processed commercial insulin. C-peptide should aid in long-term treatment of diabetic complications including stimulation of nerve and renal functions. Hyper-expression of functional proinsulin and exceptional stability in

dehydrated leaves offer a low cost platform for oral and injectable delivery of cleavable proinsulin.

ACKNOWLEDGMENTS

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LIST OF ACRONYMS/ABBREVIATIONS

aa – amino acid

aadA – Aminoglycoside 3' adenosyltransferase

Amp – Ampicillin

BAP – Benzylaminopurine

BSA – Bovine Serum Albumin

CP – Control Pellet

CS – Control Supernatant

CTAB – Cetyl trimethylammonium bromide

CTB – Cholera Toxin Beta Subunit

CTB-PFx3 – Cholera Toxin Beta Subunit fused with Proinsulin containing 3 Furin sites

DEB – DNA Extraction Buffer

DMSO – Dimethyl sufloxide

DNA – Deoxyribonucleic Acid

DS – Dialyzed Supernatant

DTT – Dithiothreitol

EDTA – Ethylenediaminetetraacetic Acid

ELISA – Enzyme Linked Immunosorbent Assay

eNOS – endothelial Nitric Oxide Synthase

FT – Flow Through

GM1 – monosialotetrahexosylganglioside

GPGP – Glycine Proline Glycine Proline

Gu-HCl – Guanidine Hydrochloride

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HSA – Human Serum Albumin

IDV – Integrated Density Value

IFNα2β – Interferon α2β

IP – Intraperitoneal

Kb - Kilobase

KDa - Kilodalton

LB – Luria Broth

MAP – Mitogen-Activated Protein

MS – Murashige and Skoog

NAA – Naphthalene Acetic Acid

$Na^+K^+ATPase$ – Sodium Potassium Adenosine Triphosphate pump

NDFS – National Diabetes Fact Sheet

Ni-NTA – Nickel-nitrilotriacetic Acid

OD – Optical Density

PBS – Phosphate Buffered Saline

PC2/PC3 – Prohormone Convertase 2/3

PCR – Polymerase Chain Reaction

Pris – Proinsulin

psbA – Photosystem b/A

RMOP – Regeneration Media of Plants

RMOL – Regeneration Media of Lettuce

RNA – Ribonucleic Acid

RRKR – Arginine Argine Lysine Arginine

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SOC – Super Optimal Broth with Catabolite repression

SSC – Sodium Chloride and Sodium Citrate solution

TEMED – Tetramethylethylenediamine

WT – Untransformed Plant

UTR – Untranslated Region

W1/W2 – Wash 1/2

INTRODUCTION

<u>Type 1 Diabetes</u>

Type I Diabetes is an autoimmune disorder that results from the T-cells of the body's immune system attacking the β-cells of the islets of Langerhans in the pancreas. It represents 5%-10% of all diabetes cases. However, type 1 diabetes accounts for over 90% of diabetes cases in children (Giannini et al, 2009). As of 2007, it was reported that diabetes was the seventh leading cause of death in the US. The main etiology of Type I diabetes is a drop in insulin levels due to the destruction of the insulin-producing pancreatic β-cells, and therefore this leads to abnormally high blood glucose levels. This can lead to symptoms of the disease such as frequent urination, extreme hunger and thirst, weight loss, fatigue, and irritability. However, over time, sustained hyperglycemia leads to a number of other more serious complications such as heart disease, high blood pressure, kidney disease, and nervous system disease (National Diabetes Fact Sheet, 2008). It is these complications from diabetes that ultimately lead to death if left untreated.

Estimated annual costs in 2007 for diabetes care and other indirect costs for the United States alone totaled $174 billion with roughly $116 billion in excess medical expenditures comprised of $27 billion going to direct care, $58 billion to treat diabetic complications, and $31 billion in excess general medical costs. It is estimated that approximately $1 out of every $10 health care dollars is attributed to diabetes (American Diabetes Association, 2008). As the prevalence of diabetes worldwide continues to

increase, these costs are projected to be between $213 and $396 billion by the year 2025 (Giannini et al, 2009). In many developing countries, the annual cost for insulin exceeds 50% of the average annual income. In developed countries, the cost of insulin alone is around $100 per month (Raab et al, 2004). Therefore, there is a great need to reduce the economic burden on people dealing with this disease.

<u>Insulin Production</u>

Insulin is a 51 amino acid peptide hormone with a molecular weight of 5.8 kDa which is derived from its precursor, proinsulin. Proinsulin is produced in the pancreas as a 110 amino acid peptide containing a 24 amino acid signal sequence and the 86 amino acid proinsulin molecule consisting of the B chain (30 aa), C peptide (34 aa), and A chain (21 aa). During insulin biosynthesis in the pancreas, the C-peptide is cleaved off via the action of prohormone convertases 2 and 3 at the B chain/C-peptide and C-peptide/A-chain junctions, respectively, and insulin (51 aa) is formed via disulfide bonds between the A and B chains (Shaw et al, 2002; Steiner, 1998).

Commercial insulin production first began in 1922 and consisted of insulin harvested from bovine or porcine pancreas. Thirty years later, the gene for human insulin was sequenced, and by the 1970's scientists were able to recreate the sequence in order to produce recombinant human insulin. This eliminated previous problems with adverse immune reactions from animal-derived insulin. Recently, insulin analogs such as insulin lispro and insulin aspart have been manufactured to contain amino acid alterations which increase absorption time (Levinson, 2003). Commercial preparations

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of insulin are produced mainly in E. coli (Swartz, 2001) or yeast (Kjeldsen, 2000) as fusion proteins. In E. coli, The A chain and the B chain genes are introduced into two separate colonies, and the fusion peptides are produced individually. Then, the two chains are mixed together and joined by disulfide bonding. The resulting insulin is subsequently purified. Alternatively, insulin is produced from the proinsulin gene. However, C-peptide is removed from the final product.

Although insulin is predominantly made in bacteria or yeast for commercial production, there has been further research focusing on other methods for producing insulin including expression in plants. Earlier attempts at insulin expression in plant tissues have focused mainly on treating the autoimmune aspect of the disease by induction of oral tolerance. Both CTB-insulin produced in potato tubers (Arakawa et al, 1998) and CTB fused with three copies of the insulin B chain expressed in the tobacco nuclear genome (Li et al, 2006) showed expression levels of only 0.1% of total soluble protein. A recent review article reports expression of various proinsulin constructs with or without C-peptide in Arabidopsis seeds that accumulated up to 1% total seed protein (Boothe et al, 2010). However, there is no reference for oral delivery of insulin expressed in plant cells, and there is a need to increase expression levels in large biomass crops in order to further advance this concept.

<u>Advantages of Chloroplasts</u>

Chloroplast genetic engineering in plants offers several advantages over nuclear transformation. Some of these advantages include higher copy number and expression

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levels of the transgene. Each chloroplast may contain up to 100 genomes, while each plant cell may contain up to 100 chloroplasts. Therefore, each plant cell may contain as many as 10,000 chloroplast genomes which results in high expression levels of proteins expressed via the chloroplast genome. Expression levels of up to 70% total soluble protein (Oey et al, 2009), and 72% total leaf protein (Ruhlman et al, 2010) of biopharmaceutical proteins have been reported in transgenic chloroplasts, which are the highest levels reported in published literature in this field. Chloroplasts offer gene containment through maternal inheritance, as the chloroplast genome is not transferred through pollen unlike nuclear genomic DNA (Daniell, 2007; Ruf et al, 2007). The chloroplast vectors are designed with flanking regions which are homologous to the chloroplast genome, and this prevents unwanted positional effects or gene silencing. Furthermore, chloroplasts have the ability to transcribe polycistronic RNA (Quesada-Vargas et al, 2005) and can perform the correct processing of eukaryotic proteins including the ability to carry out post-translational modifications such as disulfide bonding, assembly of multimers, and lipid modifications (Arlen et al, 2007; Arlen et al, 2008; Bally et al, 2008; Daniell et al, 2009a; Daniell et al, 2009b; Davoodi-Semiromi et al, 2009; Lee et al, in press). Finally, plants offer the benefit of low cost production of proteins and the protection of antigen via bioencapsulation (Arlen et al, 2008; Daniell et al, 2009b; Davoodi-Semiromi et al, 2009; Limaye et al, 2006; Verma and Daniell, 2007; Verma et al, 2008; Verma et al, 2010). These advantages make the chloroplast ideal for production of biopharmaceutical proteins and vaccines as well as an ideal vehicle for oral delivery. Several labs have successfully characterized the expression of

biopharmaceuticals such as alpha 1-antitrypsin (Nadai et al, 2009), insulin-like growth factor (Daniell et al, 2009a), Human serum albumin (Fernandez-San Milan et al, 2003), Human interferon alpha (IFNα2b) (Arlen et al, 2007), Cardiotrophin-1 (Farran et al., 2008), Proinsulin (Ruhlman et al, 2007), and coagulation Factor IX (Verma et al, 2010). Ideally, expression of CTB-PFx3 in chloroplasts should provide a low-cost means to produce biologically functional insulin, which could be administered through both the injectable and oral delivery methods. Correct processing of proinsulin involves production of three intramolecular disulfide bonds, whereas CTB requires assembly into pentamers in order to be successfully absorbed into the circulation via the GM1 receptors on the gut mucosa (Limaye et al, 2006). Chloroplasts are capable of performing necessary post-translational modifications which are essential for the functionality of CTB-PFx3 (Arlen et al, 2008; Bally et al, 2008; Daniell et al, 2009b; Davoodi-Semiromi et al, 2009; Lee et al, in press; Limaye et al, 2006; Verma et al, 2010).

Oral Delivery

Oral delivery of biopharmaceutical proteins expressed in plant cells should reduce their cost of production, purification, processing, cold storage, transportation and delivery (Daniell, 2006). Efficient transformation of plastids of edible crops was recently achieved (Kanamoto et al, 2006 Kumar et al, 2004; Lelivelt et al, 2005; Ruf et al, 2001; Ruhlman et al, 2010), further facilitating the oral delivery of therapeutic proteins. The main challenges that arise from the concept of oral delivery of insulin are enzymatic

degradation in the stomach and absorption into the gastrointestinal tract (Agarwal and Khan, 2001). To overcome the first obstacle, human proinsulin with furin cleavage sites was engineered and introduced into the plant chloroplast. The plant cell wall naturally bioencapsulates proteins to prevent enzymatic degradation in the stomach (Daniell et al, 2009b; Davoodi-Semiromi et al, 2009). The antigen is then able to pass into the intestinal tract where it is subjected to mucosal immune responses. To overcome the second obstacle, the proinsulin construct was engineered to have the cholera toxin B (CTB) subunit fused to its N-terminus. CTB binds to GM1 receptors in the lumen of the gut (de Haan et al, 1998; Tsuji et al, 1995), and it has been shown that fusion to the C-terminus of CTB is ideal for pentamerization and GM1 binding (Liljeqvist et al, 1997). It has been previously demonstrated that CTB facilitates uptake into the circulation via binding to the GM1 receptor located on the gut mucosa (Limaye et al, 2006). CTB-conjugated proteins are then delivered to the GALT. Recently, CTB-FIX was successfully expressed and delivered orally to aid in treatment of hemophilia B. After delivery, the antigen was found in multiple tissues of the GALT, specifically the ileum, Peyer's patches, liver, and plasma within two hours (Verma et al, 2010).

C-Peptide

The production of chloroplast-derived, orally-deliverable functional insulin should provide many benefits including a lower cost of production and the possibility of delivering the C-peptide which is currently lacking in commercially available insulin. This is of importance because type 1 diabetics fail to produce C-peptide, an essential

molecule which once was thought to be biologically inactive. C-peptide is located in between the B chain and A chain of the proinsulin molecule. Only recently, C-peptide has been shown to function independently of insulin. C-peptide acts via G-protein activation which signals the cell to open Ca^{2+} channels allowing for an influx of cellular Ca^{2+}. This leads to stimulation of eNOS, Na^+,K^+-ATPase, and the MAP kinase pathway (Wahren, 2004). This action of C-peptide is thought to be the mechanism behind the beneficial effects seen upon restoration of C-peptide levels in type 1 diabetic animals and patients (Hills and Brunskill, 2009; Johansson et al, 2000; Sima and Li 2005). Stimulation of the activities of Na^+,K^+-ATPase and endothelial nitric oxide synthase (eNOS) is essential for nerve function (Ekberg et al, 2003; Wahren et al, 2007), and the stimulation of Na^+,K^+-ATPase also leads to correction of renal structure and function (Rebsomen et al, 2008).

<u>Furin Protease</u>

Furin is an endoprotease present in the constitutive secretory pathway and cell surface of virtually all cells (Taylor et al, 2003). The consensus sequence for furin cleavage is the C-terminal arginine in the amino acid sequence Arg-X-Lys/Arg-Arg (Thomas, 2002). The introduction of furin consensus sequences at the B-chain/C-peptide and the C-peptide/A-chain has been demonstrated to increase the processing of proinsulin to mature insulin in a wide variety of non-neuroendocrine cells, including fibroblasts, myoblasts, epithelial cells and lymphocytes (Croze and Prud'homme, 2003; Fujimoto et al., 2005; Groskreutz et al., 1994; Hay and Docherty, 2003; Ito et al., 2005;

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Nishigori et al., 1996; Oh et al., 2006; Scougall et al., 2003; Scougall and Shaw, 2003; Shaw et al., 2002; Short et al., 1998; Tatake et al, 2007; Yamasaki et al., 1999; Yanagita et al., 1992; Yasutomi et al., 2003). In contrast, native proinsulin is processed in the pancreatic β-cells via prohormone convertases PC2/PC3. Non-neuroendocrine cells therefore do not possess the ability to process proinsulin into mature insulin. This processing is necessary for function of insulin, so addition of furin cleavage sites is necessary to facilitate processing in the gut. Although several different furin cleavage sites have been engineered, there seems to be the best processing (87%) of human proinsulin to unmodified mature human insulin using the RRKR furin cleavage sequence in adult fibroblasts (Shaw et al, 2002). Therefore, this is the sequence that has been engineered into our proinsulin constructs. Tobacco was used as a model system to quantify the production and facilitate the purification of CTB-PFx3 as well as monitor its functionality. Subsequentially, CTB-PFx3 was produced in lettuce for oral delivery studies.

MATERIALS AND METHODS

<u>Preparation and Transformation of Competent E. Coli Cells</u>

Competent cells were created according to the method of Inoue et al (1990). Inoue transformation buffer (55mM $MnCl_2 \cdot 4H_2O$, 15mM $CaCl_2 \cdot 2H_2O$, 250mM KCl, 10mM PIPES pH 6.7) was filter sterilized through a 0.45 µm Nalgene filter and stored at -20°C until ready for use. Meanwhile, XL-1 blue E. coli was inoculated in 3ml LB broth containing 7mg/L chloramphenicol and left to incubate overnight at 37°C. The next day, 50 ml LB broth containing 1 ml of 1M $MgCl_2$ was inoculated from the overnight culture and incubated under vigorous shaking at 37°C. Once the OD reached 0.4-0.6, the contents of the flask were cooled on ice for 10 minutes and then the cells were transferred to oakridge tubes and centrifuged at 3000rpm in a Sorvall rotor for 10 minutes at 4°C. The medium was removed, and the cells were gently resuspended by swirling in 80 ml ice-cold Inoue buffer. Cells were spun under the same conditions, and the buffer was discarded. The cells were resuspended in 20 ml Inoue buffer, and 1.5 ml of DMSO was added slowly. The cells were mixed by swirling and incubated on ice for 10 minutes. 200µl aliquots of the suspension were then quickly dispensed into chilled, sterile eppendorf tubes, snap-frozen in liquid nitrogen, and stored at -80°C.

For transformation, competent cells were thawed on ice and split into 100µl aliquots in pre-chilled 14 ml polypropylene round bottom tubes (Falcon). 3µl of the plasmid DNA was added to the cells and incubated on ice for 30 minutes. Cells were then heat shocked in a 42°C water bath for 45 seconds and rapidly returned to ice for 3

minutes. Cells were spun for 1 minute at 5000 rpm and the pellet was resuspended in 800μl SOC media, warmed for 3 minutes in a 37°C water bath, and then incubated for 2 hours at 37°C in a shaker. 100μl of cells was spread onto LB agar plates containing 50mg/L ampicillin. The remaining cells were centrifuged at 5000 rpm for 2 minutes and all but 100μl of the supernatant was removed. The cells were resuspended in the remaining media and spread on the LB/Amp plates. All plates were incubated at 37°C overnight.

Vector Construction

The CTB-PFx3 construct was created from pLD-5'UTR-CTB-Pins as previously described (Ruhlman et al, 2007). The Proinsulin gene was modified in order to include furin cleavage sites in between the B-chain/C-peptide and the C-peptide/A-chain junctions as well as between the CTB/B-chain fusion site. The pLD-5'UTR-CTB-Pins vector was digested with NcoI and SmaI and the resulting portion containing the 5'UTR was ligated into the pGEM-Pris(fx3) vector containing the mutated proinsulin. This was subsequently digested with EcoRI and NotI and subcloned into the pLD-g10-RecA vector, replacing the g10-RecA gene with the 5'Utr CTB-Pris(fx3) gene. The final vector was designated pLDutr-CTB-Fx3Pris. To create the lettuce vector, pLDutr-CTB-Fx3Pris was digested with SmaI and XbaI and the resulting Fx3Pris fragment was ligated into the 12.13.9.11 CTB-GPGP-FIX vector containing the lettuce-specific psbA 3' and 5' UTRs, replacing the FIX gene. To create the final vector, the pLspsbACTB-Fx3 Pris was digested with NdeI and NotI which was cloned into pLsLFHPAG vector containing the

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lettuce flanking regions, and this vector was designated as pLsLF-CTB-Fx3Pris. After vector construction, the CTB-PFx3 genes were sequenced (GENEWIZ). Sequences were confirmed to be correct using BLAST search.

Bombardment and Selection

Seed Sterilization

Wild type Nicotiana tabacum cv. Petit Havana or Lactuca sativa cv. Simpson Elite seeds were sterilized in a disinfecting solution of 1.5% bleach and 0.1% Tween-20 and grown on sterile Murashige and Skoog (MS) medium. Seeds were poured into an eppendorf tube and 1ml of 70% ethanol was added for 30 seconds. 1ml of disinfecting solution was added for 20 minutes with gentle tapping every 5 minutes. Seeds were then washed with 1ml of dH_2O three times and then sprayed onto MS medium. After germination, plants were allowed to grow for ~3 weeks until large enough for bombardment.

Preparation of Media

RMOP was made by dissolving 1 package of MS salts, 30g sucrose, 100mg myo-inositol, 1mg benzylaminopurine (BAP), 0.1mg α-naphthalene acetic acid (NAA), and 1mg thiamine hydrochloride made up to 1 liter in dH_2O and brought to pH 5.8. 6g Phytoblend (Cassion Laboratories) was then added. For RMOL, this recipe was changed to 1 package of MS salts, 30g sucrose, 100mg myo-inositol 0.2mg BAP, 0.1mg NAA, 10mg thiamine hydrochloride, 1 mg pyridoxine hydrochloride, 1mg nicotinic acid,

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and 1mg glycine made up to 1 liter in dH_2O before bringing the pH to 5.8 and addition of phytoblend.

Preparation of Gold Particles

Gold particles were vortexed at 4°C for 30 minutes. 6µg pLDutr-CTB-Fx3Pris or pLsLF-CTB-Fx3Pris was added for every 50µl of gold particles while vortexing. Additionally 50µl of 2.5M $CaCl_2$ and 20µl of 0.1M spermidine were added per 50µl gold while vortexing. The mixture was vortexed for 5 minutes and then kept on ice for 2 minutes. The gold preparation was then spun at 14,000rpm for 10 seconds. The clear supernatant was discarded, and the pellet was washed with 140µl 70% ethanol. The gold prep was again spun for 10 seconds at 14,000rpm and the supernatant discarded. Next, the prep was washed twice with 60µl cold 100% ethanol and kept on ice until use.

Bombardment of Leaves

Bombardment was done using the Bio-Rad PDS-1000/He gene gun as described previously (Verma et al, 2008). Sterile tobacco leaves were placed abaxial side up whereas sterile lettuce leaves were placed abaxial side down on MS medium and were bombarded under sterile conditions using pLDutr-CTB-Fx3Pris or pLsLF-CTB-Fx3Pris coated with gold particles prepared according to Kumar and Daniell 2004. Sterile films were placed in the groove of the macro carrier and stop screen placed in the macro carrier holder. 7µl of the gold preparation was swirled onto the center of the film and let dry. Rupture discs of 1100 and 900 psi were used for bombardment of tobacco and lettuce, respectively.

Tissue Regeneration and Selection

Bombarded leaf pieces were incubated in the dark for 2 days at room temperature and then leaf pieces were transferred to regeneration media of plants (RMOP) containing 500mg/L spectinomycin for tobacco or regeneration media of lettuce (RMOL) containing 100mg/L spectinomycin (Ruhlman et al, 2010). The appearance of green shoots was seen after 4-6 weeks. These shoots were allowed to grow and the leaves were cut and subsequently transferred to RMOP or RMOL containing 500mg/L or 100mg/L spectinomycin, respectively, for the second round of selection. After new shoots formed, these were transferred for the third round of selection to MS containing 500mg/L spectinomycin for tobacco or 100mg/L spectinomycin for lettuce and allowed to establish roots. Plants were then transferred to the greenhouse.

PCR

Total plant genomic DNA was extracted using the Qiagen DNeasy kit according to the manufacturer's protocol. PCR was performed using primers corresponding to the 3P (5'-AAAACCCGTCCTCAGTTCGGATTGC–3') and 3M (5'CCGCGTTGTTTCATCAA GCCTTACG-3') regions of the gene cassette in order to determine site-specific integration. The 3P primer anneals to the native chloroplast genome, and the 3M primer anneals to the aadA gene. PCR was also performed using the 5P (5'-CTGTAGAAGTCA CCATTGTTGTGC-3') and 2M (5'-TGACTGCCCACCTGAGAGCGGACA-3') regions of the gene cassette in order to determine transgene integration. The 5P primer anneals to the aadA gene, and the 2M primer anneals to the native trnA gene (Verma et al 2008).

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For lettuce, the primer 16 SF (5'-CAGCAGCCGCGGTAATACAGAGGA-3'), which also lands on the native chloroplast genome, was used in place of 3P. Cycling parameters were as follows: 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C (3P/16SF/3M) or 56.4°C (5P/2M) for 1 minute, and 72°C for 2 minutes; final extension at 72°C for 10 minutes. The reactions were held at 15°C and then run on a 1% agarose gel for visualization of bands.

Southern Analysis

DNA Extraction and Purification

Total plant DNA was extracted using the cetyl trimethylammonium bromide (CTAB) method (Allen et al 2006) followed by phenol chloroform extraction to remove contaminating salts and proteins. DNA extraction buffer (DEB) was made with 2.5ml 1M Tris pH 8.0, 1ml 0.5M EDTA, 7ml 5M NaCl, 5ml 10% CTAB, 50µl β-mercaptoethanol, and brought up to 25ml with dH_2O. 1ml of DEB was pre heated at 65°C for 15 min and then added to 100mg leaf tissue ground to a fine powder in liquid nitrogen. Samples were incubated at 65°C for 1 hour with intermittent mixing. Next, 2/3 volume chloroform:isoamyl alcohol (48:2) was added and tubes were mixed by gentle inversion for 20 minutes. Samples were centrifuged at 10,000 rpm for 10 minutes. The top layer was removed into a new tube and 2/3 volume cold isopropyl alcohol was added which precipitated the DNA. Samples were centrifuged for 10 minutes at 10,000 rpm and washed twice with 500µl 70% ethanol. The pellet was dried in a DNA Speed Vac (Savant) and subsequently dissolved in Tris-EDTA buffer (TE) containing 0.1 mg/ml

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RNase. Samples were incubated at 37°C for 30 minutes and stored at 4°C until ready for phenol chloroform purification.

Phenol chloroform purification was performed by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the DNA sample followed by centrifugation at 12,000 rpm for 15 minutes. The upper layer was removed into a new tube and an equal volume of chloroform was added in order to remove phenol and inhibit DNA digestion. Samples were centrifuged for 15 minutes and the upper layer was again removed to a new tube. 1 ml chilled 100% ethanol and 1/10 volume 3M sodium acetate pH 5.2 was added and incubated at -20°C for 1 hour to precipitate the DNA. Samples were centrifuged for 10 minutes and the pellet was washed twice with 500µl 70% ethanol. The DNA pellet was vacuum dried and resuspended in dH2O for Southern analysis.

Restriction Digestion of Plant Genomic DNA

Transgenic and untransformed tobacco DNA was digested using AflIII, and lettuce DNA was digested using BgIII in a reaction containing 2µl 10X buffer (New England Biolabs), 3µg plant genomic DNA, 2µl BSA, and 1µl enzyme made up to 20µl with dH$_2$O. The reactions were incubated overnight at 37°C.

Agarose Gel Electrophoresis and DNA Transfer

Southern analysis was performed according to lab protocol (Kumar and Daniell, 2004). The entire 20µl restriction digestion samples were separated on a 0.8% agarose gel at 50V for 4h in Tris-acetic acid-EDTA (TAE) buffer. The gel was depurinated for 15

minutes in 0.25N HCl, rinsed 2 times for 5 minutes each in dH$_2$O, and soaked in transfer buffer (0.4N NaOH, 1M HCl) for 20 minutes. All steps were performed with gentle rocking. DNA was transferred to a nylon membrane (Whatman) overnight on the benchtop. The gel was placed face down directly on a layer of plastic wrap followed by the membrane which was soaked for 5 minutes in transfer buffer, a piece of filter paper cut to the same size as the gel, then a stack of absorbent paper towels with a heavy weight on top. Parafilm was placed around the edges of the gel in order to prevent lateral transfer, and the transfer was allowed to take place over night. The next day, the membrane was rinsed with 2X SSC (0.3M NaCl, 0.03M Sodium Citrate) 2 times for 5 minutes each and DNA was crosslinked to the membrane at 150 mJoule in GS Gene Linker (Bio-Rad).

Generation and Labeling of Probes

Flanking sequence probe was generated by digesting the pUC-CT vector (Verma et al, 2008) with BglII and BamHI in order to generate a 0.81kb fragment corresponding to the trnI/trnA region of the native chloroplast genome. Probe was labeled with ^{32}P using Ready-to-Go DNA labeling beads (General Electric). 45μl of probe DNA was denatured by boiling for 5 minutes and then placed on ice for 2-3 minutes. DNA was briefly centrifuged and added to the Ready Mix tube along with 5μl ^{32}P which was then incubated at 37°C in the radioactivity hood for one hour. A G50 column was vortexed to suspend the resin. The bottom plug was broken off and the column was spun at 3000 rpm for one minute. The column was transferred to a new eppendorf tube and the probe

DNA was added and spun at 3000rpm for 2 minutes. The radioactivity of the probe was measured in a scintillation counter (X) in order to determine the proper amount of probe needed to hybridize to the membrane.

Probe Hybridization and Audioradiography of Membrane

The membrane containing the crosslinked DNA was placed in a cylindrical bottle with the top facing outward and the underside touching the wall of the bottle ensuring that no air bubbles were present between the bottle and the membrane. 10 ml of Stratagene Quick-Hyb solution was added to the bottle and incubated for 1 hour at 68°C in the rotating hybridization incubator (FisherBiotech). Meanwhile, 100µl of salmon sperm DNA was added to a fresh eppendorf tube. A calculated amount of labeled DNA probe was added and the mixture was boiled for 5 minutes. This solution was then added to the pre-hybridized membrane and incubated for 1 hour at 68°C. Next, the incubation temperature was brought down to 27°C to cool. The probe was saved or discarded and the membrane was washed twice with Wash I solution (2X SSC and 0.1% SDS) for 15 minutes each at room temperature. Next, the membrane was washed twice with Wash II solution (0.1X SSC and 0.1% SDS) for 15 minutes each at 60°C. The radioactivity was checked with a Geiger Counter, and the membrane was wrapped in plastic wrap for audioradiography. The membrane was placed in a cassette and exposed to x-ray film from 4 hours up to 2 days at -80°C. When ready, the cassette was removed from the freezer and allowed to thaw before the film was removed in the dark room and placed in the developer.

Bradford Assay

Young, mature, and old leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. 500µl of plant extraction buffer (100mM NaCl, 200mM Tris-HCl pH 8.0, 0.1% Triton-X, 400mM sucrose, and Roche complete mini protease inhibitor cocktail tablet) was added to 100mg of frozen leaf tissue and vortexed for 10min at 4°C. Plant tissue homogenates were further separated into supernatant and pellet fractions via centrifugation (14000 rpm for 5 min at 4°C), and the pellet was resuspended in 500µl plant extraction buffer for analysis. Total leaf protein concentration was determined using the Bio-Rad protein assay dye reagent concentrate. Dye was diluted 1:5 in water and filtered through Whatman filter paper. The standard curve was made using 0.4mg/ml BSA serially diluted down to 0.006mg/ml. Plant homogenate, supernatant, and pellet samples were diluted in water 1:5, 1:10, 1:20 and 10ul of sample and BSA standard were loaded in duplicate to a 96 well plate. 200µl of diluted Bio-Rad dye was added to each sample and standard and the plate was read on a plate reader at 595nm.

SDS-PAGE and Immunoblot Analysis

Homogenate, supernatant, and pellet samples were diluted in 2X sample loading buffer (3.55 ml dH2O, 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) and loaded onto a 12% SDS-PAGE (Bio-Rad) gel. Samples were separated at 100-150V until the dye front reached the bottom of the gel. After separation, the gel was either stained with Coomassie Brilliant Blue R-250 (Bio-Rad) or

transferred to a nitrocellulose membrane (Bio-Rad) for immunoblot analysis. Transfer was carried out using a Bio-Rad transfer cassette at 85V for 1 hour. Membranes were briefly rinsed in dH2O and blocked in PBS-Tween-20 + 3% dry milk (PTM) for 30 minutes to 1 hour. CTB-PFx3 was detected using anti-CTB primary antibody (Sigma) diluted 1:4000 for 1.5 hours followed by goat anti-rabbit IgG-HRP (Southern Biotech) at a dilution of 1:5000 for 1 hour. Proteins were subsequently detected on autoradiography film using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Densitometric Quantification of CTBPFx3

Immunoblots for quantification were performed using known concentrations of CTB protein standard (Sigma) of 25, 50, 100, 200, and 400ng in order to create a standard curve. Total protein concentration was determined via the Bradford assay (Bio-Rad). Varying concentrations of CTB-PFx3 plant homogenate were directly compared to the standard curve using Alphaimager and Alphaease FC software. The percentage of CTB-PFx3 relative to the concentration of total leaf protein and mg CTB-PFx3 per gram leaf tissue were calculated according to the formula published in Verma et al, 2008.

Protein Solubilization and Furin Cleavage

CTB-PFx3 homogenate was separated into supernatant and pellet fractions. The pellet fraction containing the CTB-PFx3 in an insoluble form was resuspended in 1.5ml of plant extraction buffer per 100mg ground plant tissue along with 100-300mM dithiothreitol (DTT, Promega) and 6M Guanidine Hydrochloride (Gu-HCl, Sigma). Samples were rocked for 16-18h at 4°C. The samples were then centrifuged at 14,000

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rpm for 5 minutes at 4°C, and the supernatant containing soluble CTB-PFx3 was separated from the pellet fraction. The sample was subsequently dialyzed using Spectra/Por molecularporous membrane tubing (Fisher Scientific) in 20mM sodium phosphate pH 7.8, 500mM NaCl overnight at 4°C. For cleavage with furin protease, 1µg of dialyzed sample was incubated with 100mM HEPES pH 7.5, 0.5% Triton X-100, 1mM $CaCl_2$, 1mM 2-mercaptoethanol, and 2 units furin (New England Biolabs) at 25°C for 16h.

Affinity Purification

After solubilization and dialysis of the CTBPFx3, the resulting supernatant was passed over a column (Clontech) consisting of 2ml Ni-NTA Agarose (Qiagen) which was equilibrated with 20mM sodium phosphate pH 7.8 and 500mM NaCl (Binding buffer). The column was subsequently washed with 30ml binding buffer and then with 30ml wash buffer (20mM sodium phosphate pH 6.0 and 500mM NaCl). Samples were eluted from the column in 1ml fractions using 10ml elution buffer (20mM sodium phosphate pH 6.0, 500mM NaCl, and 250mM imidazole). All steps were carried out at a flow rate of 0.5ml/min. Fractions were assayed for protein concentration via absorbance units at 280 nm using the SmartSpec 3000 (Biorad) and Bradford assay to determine the concentration in mg/ml. Purified CTB-PFx3 (1µg) was incubated with furin and cut and uncut samples were run on a 16% Tricine-SDS gel. Samples were also subsequently run on 12% SDS-PAGE gel and subjected to coomassie stain and immunoblot in order to determine the percent purity.

Sodium Phytate Precipitation

Sodium phytate precipitation was done to remove Rubisco from purified samples. Purified samples were treated with 10mM $CaCl_2$ and 10mM sodium phytate (phytic acid sodium salt hydrate from Sigma) for 10 minutes at 37°C. Afterwards, samples were spun in a tabletop centrifuge at room temperature for 10 minutes at 14,000rpm. Supernatant was removed, placed in a fresh tube, and stored at -20°C until use.

Silver Stain

Tricine-SDS gel was incubated in fixative (45% dH_2O, 45% methanol, 10% acetic acid) for 40 minutes. The gel was then washed with 50% methanol 2X for 5 minutes each and then with dH_2O 2X for 5 minutes each. Next, the gel was rocked in Hypo solution (20mg $Na_2S_2O_3$ in 100 ml dH_2O) for exactly 1 minute and then washed in dH_2O 3X for 5 minutes each. Silver solution (200mg $AgNO_3$ and 100µl formaldehyde in 100 ml dH_2O) was added and the gel was rocked for 30 minutes. The gel was washed in dH_2O 4X for 5 minutes each and then developer solution (6g Na_2CO_3, 100µl of hypo, 100µl formaldehyde in 300 ml dH_2O) was added. The gel was vigorously shaken in developer solution until protein bands appeared, and then the reaction was stopped by addition of 5% acetic acid.

Animal Studies to Determine Functionality

All procedures performed in this study are based on an approved protocol in accordance with UCF-IACUC. Female C57BL/6 mice weighing 17-20 grams each were

purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were divided into four groups with 6 mice per group. Samples were prepared for delivery via IP injection by dilution in sterile PBS. Commercially purified insulin and purified CTB-PFx3 derived from transgenic tobacco were normalized via immunoblot and subsequently diluted to give each mouse a dosage of 0.04U/kg of commercially prepared insulin (Roche) and 8µg/mouse of plant-derived purified CTB-PFx3. IP injections were delivered in a volume of 100µl. Blood glucose levels were measured by bleeding of the tail vein before sample administration and again at 15, 30, 60, 120, and 180 minutes after sample administration (Accu-Chek, Aviva). A second injection was given after 180 minutes and blood glucose measurements were taken at 15, 30, 60, 120, and 180 minutes after the second IP injection. Food pellets were removed for the duration of the study. For the oral study, female C57BL/6 mice were divided into four groups with 5 mice per group. Commercially purified insulin was diluted in sterile PBS and given at a dose of 0.04U/kg. IP injections were delivered in a volume of 100µl. Oral samples were prepared from material ground in liquid nitrogen and stored at -80°C until the day of the experiment. Prior to gavage, 3 grams of wild type and transgenic leaf material were mixed with 500µl 20X PBS and homogenized using a General Laboratory Homogenizer (GLH-2596, Omni International) for five minutes. 200µl (~200mg) of leaf material containing approximately 0.5mg CTB-PFx3 was administered orally to mice via a 20 gauge gavage needle. Blood serum was taken by bleeding of the tail vein before sample administration and again at 15, 30, 60, 120, and 180 minutes after sample administration. A second

dose was given after 180 minutes and blood glucose measurements were taken at 15, 30, 60, and 120 minutes after the second dose.

RESULTS

Vector Construction

The CTB-PFx3 gene construct was inserted into the pLD vector as previously established by the Daniell lab (Verma and Daniell, 2007; Verma et al, 2008) or the pLsLF vector (Ruhlman et al, 2007; Ruhlman et al, 2010) under the control of the light-regulated psbA region located within the 5' UTR. Both vectors contain the *aadA* gene which confers resistance to spectinomycin and are driven by the upstream prrn promoter. Chloroplast vectors include native tobacco or lettuce DNA flanking regions (*trnI/trnA*) in order to facilitate homologous recombination. The CTB gene was separated from the proinsulin gene via a Glycine-Proline-Glycine-Proline (GPGP) hinge to avoid steric hindrance, followed by a furin cleavage site (RRKR) to enable removal of the CTB gene following delivery. Two additional furin cleavage sites were engineered at the B chain/C-peptide and the C-peptide/A chain junctions, replacing the native PC2 and PC3 cleavage sites as previously described (Shaw et al, 2002). The location of engineered cleavage sites and disulfide bonds are compared with native proinsulin in Figure 1. The location of CTB fusion is also shown.
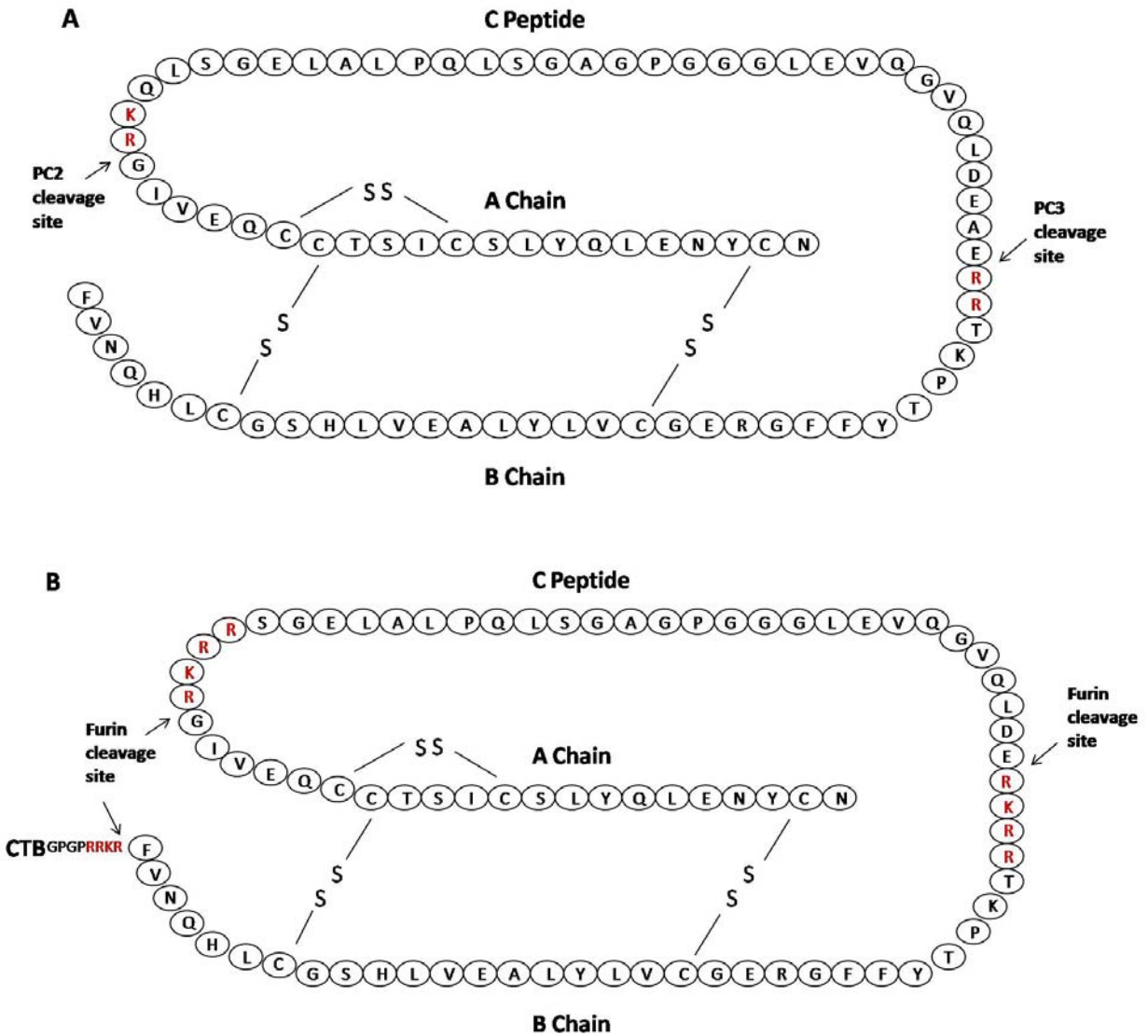
Figure 1: Location of Furin cleavage sites.

(A) Native proinsulin. (B) Proinsulin molecule modified to contain furin cleavage sites and fused to CTB.

Chloroplast Transformation and Evaluation of Homoplasmy

Chloroplast vectors pLDutr-CTB-Fx3Pris and pLsLF-CTB-Fx3Pris were bombarded into tobacco and lettuce leaves, respectively. After 4-6 weeks, green shoots appeared and were subjected to 3 rounds of selection. Presence of the transgene was confirmed via PCR (data not shown), and homoplasmy was confirmed by Southern blots. Transformed and untransformed tobacco DNA was digested with AflIII and probed with a 0.81kb sequence complimentary to the *trnI/trnA* region of the native chloroplast genome. This produced a 4.2kb fragment in the untransformed tobacco (Figure 2A) and a 6.4kb fragment in the transplastomic tobacco (Figure 2B). Both transplastomic lines A and B showed only one hybridizing fragment of 6.4kb size, and did not show a fragment of the same size as the untransformed plant, indicating that all the chloroplast genomes had integrated the foreign gene and confirmed homoplasmy (Figure 2C).

Figure 2: Southern analysis of transgenic tobacco containing CTB-Proinsulin with three furin cleavage sites.

(A) Map of untransformed tobacco plastome. Digestion with AflIII yields a 4.2kb fragment. Broken line represents probe annealing site. **(B)** Map of transformed tobacco plastome. Digestion with AflIII yields a 6.4kb fragment. **(C)** Southern analysis from T_1 generation. A and B represent independent transplastomic lines. WT, untransformed plant.

Similarly, transformed and untransformed lettuce DNA was digested with BglII which produced a 3.75kb fragment in the untransformed lettuce (Figure 3A) and a 6.3kb fragment in the transplastomic lettuce (Figure 3B). Only the 6.3kb band was present in all transplastomic lines tested, confirming homoplasmy (Figure 3C).
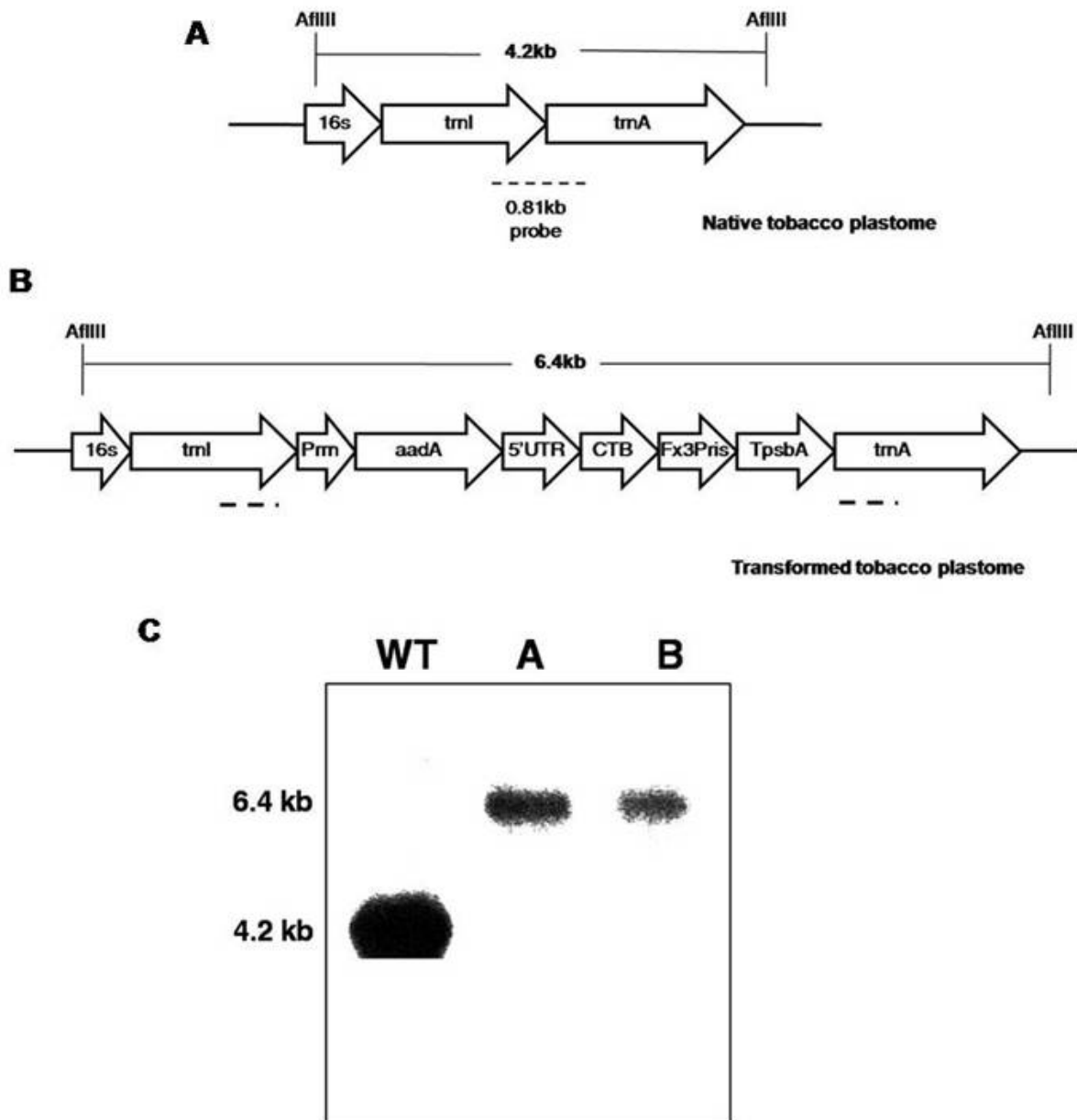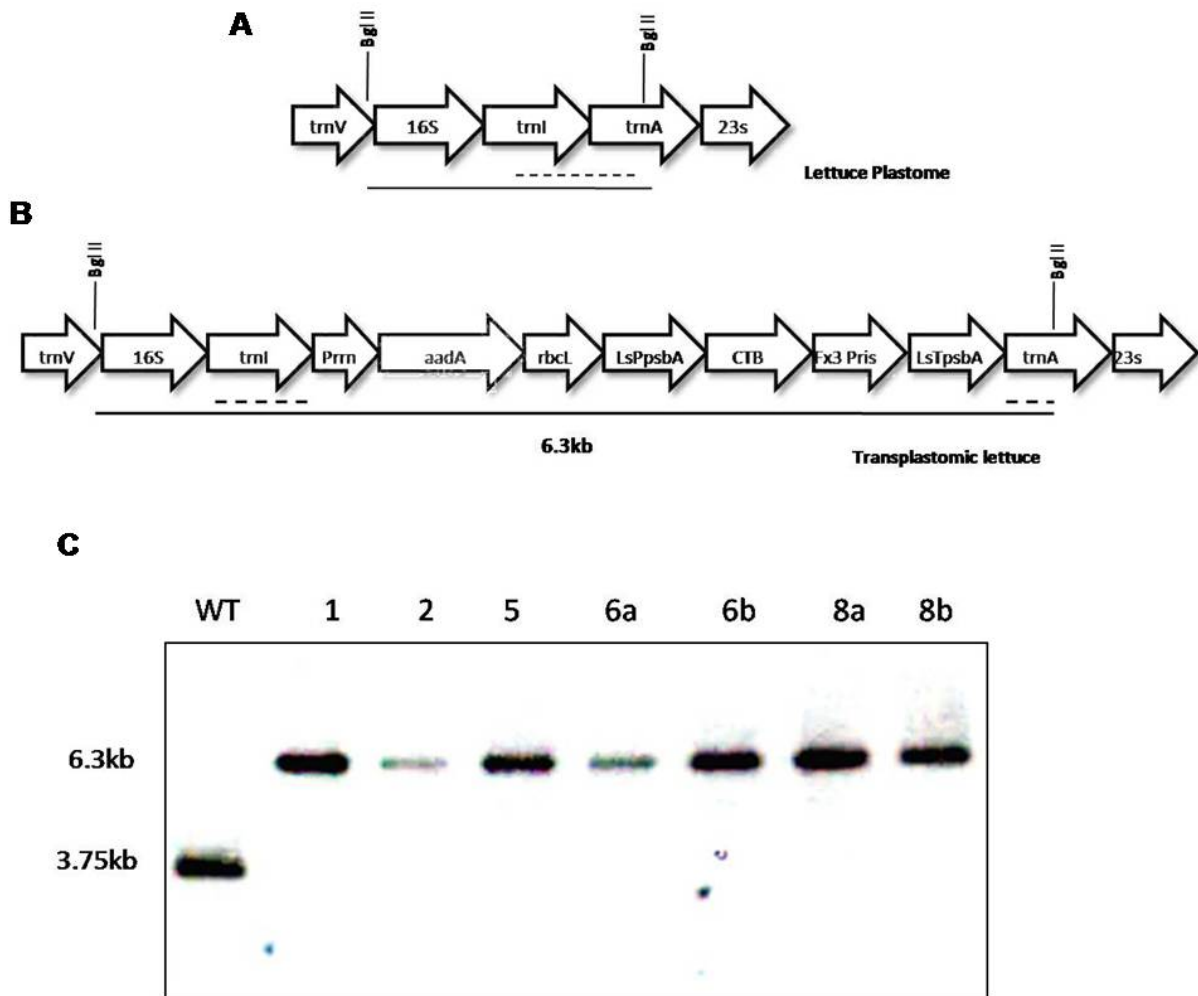
Figure 3: Southern analysis of transgenic lettuce containing CTB-Proinsulin with three furin cleavage sites.

(A) Map of untransformed lettuce plastome. Digestion with BglII yields a 3.75kb fragment. Broken line represents probe annealing site. **(B)** Map of transformed lettuce plastome. Digestion with BglII yields a 6.3kb fragment. **(C)** Southern analysis from T_0

generation. 1, 2, 5, 6 and 8 represent independent transgenic lines. Lowercase letters a and b represent different samples from the same line. WT, untransformed plant.

Detection and Quantification of Cleavable Proinsulin

Coomassie staining and immunoblotting were used to determine whether the transgenic plants were expressing CTB-PFx3. Transplastomic and untransformed young, mature, and old leaves were ground to a fine powder in liquid nitrogen and protein was extracted with the addition of 300-500µl plant extraction buffer. Young leaves are the new, light green, smaller leaves growing at the top of the plant. Mature leaves are large, dark green, and grow towards the middle of the plant. Old leaves are the bottom-most leaves that are beginning to turn brown (Figure 4).
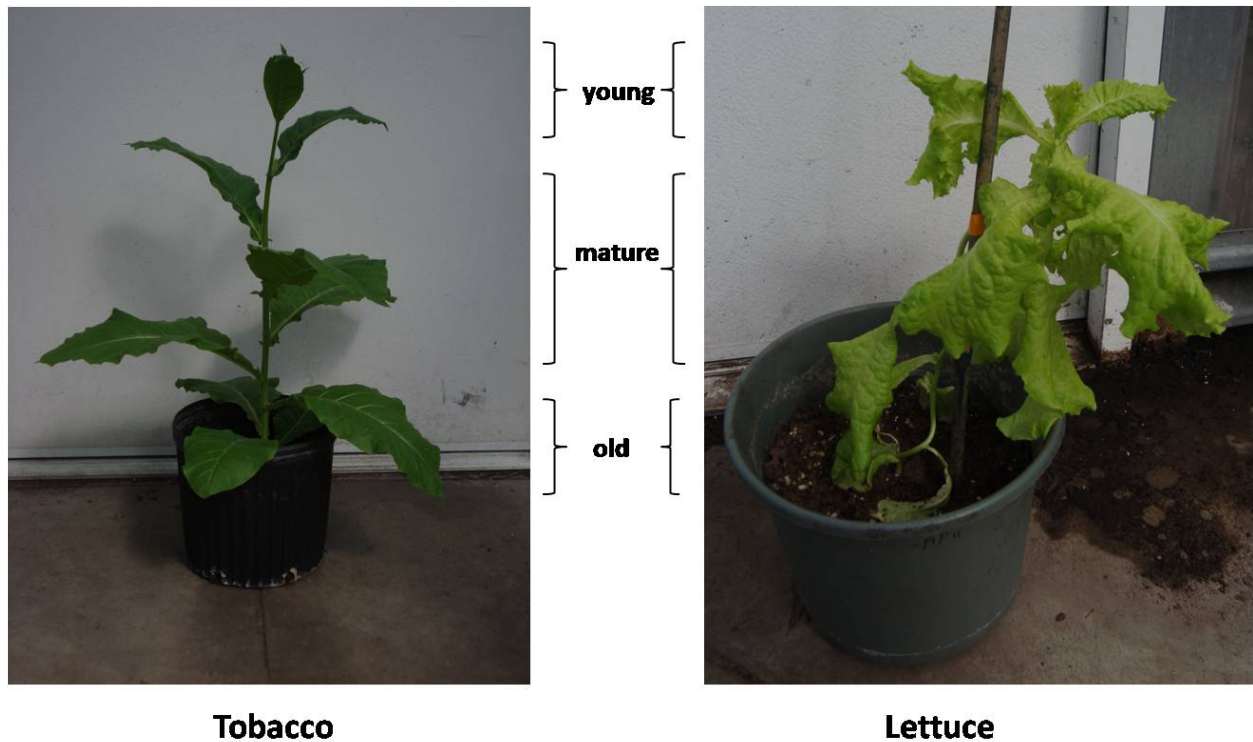
Figure 4: Diagram of relative leaf ages in tobacco and lettuce plants.

Samples were spun down and the resulting supernatant, pellet, and homogenate fractions were collected and run on a 12% SDS-PAGE gel (Figure 5A). A polypeptide was seen at 22kDa in the pellet and homogenate fractions, but not in the supernatant, indicating that the CTB-PFx3 was expressed in the form of insoluble inclusion bodies. Immunoblots were performed using anti-CTB antibody to further confirm the presence of transgene expression. Young, mature, and old homogenate samples were loaded in equal volumes along with wild type homogenate and recombinant CTB protein (Figure 5B). A prominent band at 22kDa represents the monomer size of the CTB-PFx3, but it is clear that the protein forms dimers, trimers, tetramers, pentamers, and multimers of

44kDa, 66kDa, 88kDa, and 110kDa sizes. Varying dilutions of the CTB-PFx3 homogenate were loaded onto a 12% SDS-PAGE gel along with known concentrations of recombinant CTB protein, and spot densitometry was performed in order to quantify expression levels (Figure 5C). Different concentrations of CTB were loaded onto the gel in order to generate a standard curve (Figure 5D). CTB-PFx3 was expressed in levels up to 47% in old leaves (Figure 5E, Table 1) and as much as 2.92 mg/g leaf tissue (Figure 5F).

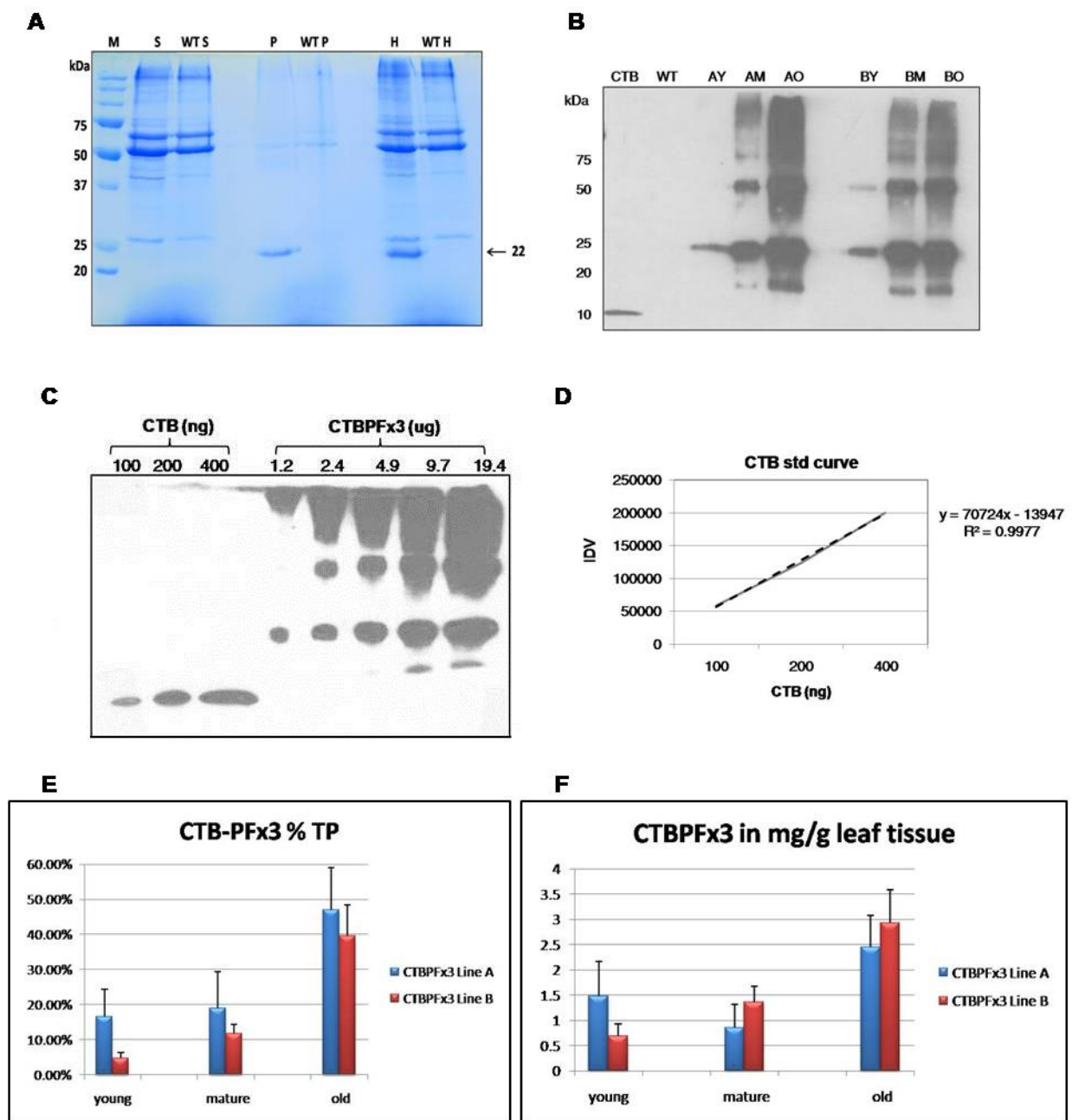**Figure 5:** Characterization of CTB-Proinsulin expressed in tobacco chloroplasts.

(A) Coomassie stain. M, marker; S, supernatant; P, pellet; H, homogenate; WT, untransformed plant. S&H: 30ug load. P: 7.5ug load. **(B)** Western blot probed with anti-

CTB antibody showing CTB-PFx3 expression of young, mature, and old leaf total protein. 1µg per lane. CTB: 100ng CTB standard. **(C)** CTBPFx3 total protein was loaded in varying concentrations and compared to known quantities of CTB standard protein using densitometry. Blot was probed using anti-CTB antibody. **(D)** Plot of integrated density values (IDV) for quantification of CTBPFx3 based on standard curve. Broken line shows data points. Solid line: trend line. **(E)** Percent total protein for young, mature, and old leaves based on densitometry values. **(F)** Quantification of young, mature, and old leaves in mg CTBPFx3 per gram total leaf tissue.

Transgenic lettuce plants were prepared in the same way as tobacco, and varying dilutions of the samples were loaded onto a 12% SDS-PAGE gel for spot densitometric analysis of protein concentration (Figure 6A). Different concentrations of recombinant CTB protein was loaded onto the gel in order to create a standard curve (Figure 6B). Of the four lines tested, CTB-PFx3 was found to account for up to 53% of the total leaf protein (Figure 6C, Table 2) and as much as 3.28 mg/g leaf tissue (Figure 6D, Table 2). The majority of expressed protein was found in the old leaves, and expression levels were comparable to levels observed in tobacco.
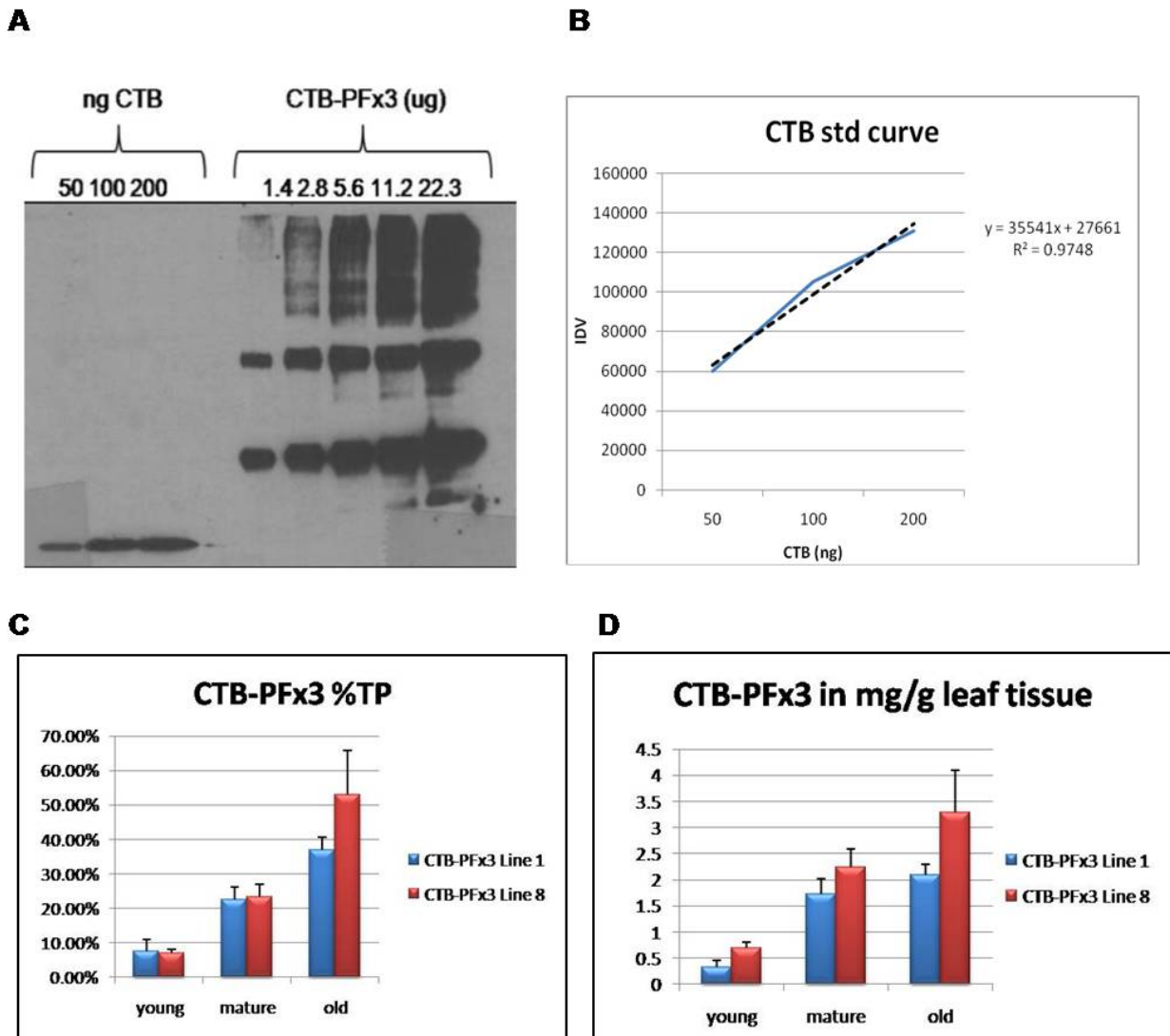
Figure 6: Characterization of CTB-Proinsulin expressed in lettuce chloroplasts.

(A) CTBPFx3 total protein was loaded in varying concentrations and compared to known quantities of CTB standard protein using densitometry. Blot was probed using anti-CTB antibody. **(B)** Plot of integrated density values (IDV) for quantification of CTBPFx3 based on standard curve. Broken line shows data points. Solid line: trend

line. **(C)** Percent total protein for young, mature, and old leaves based on densitometry values. **(D)** Quantification of young, mature, and old leaves in mg CTB-PFx3 per gram total leaf tissue.

Additionally, due to the high levels of expression observed in old leaves, senescent and dried lettuce leaves were tested to investigate stability of CTB-PFx3. Senescent leaves that were completely brown and dry were harvested from transplastomic lettuce plants and protein was extracted as previously described. Leaf homogenate ranging from 0.125µg to 2µg total leaf protein was loaded on a 12% SDS-PAGE gel and probed with CTB and insulin antibodies (Figure 7, A and B). High levels of expression up to 39% total leaf protein were detected even in senescent lettuce leaf material, further demonstrating that due to the insolubility of CTB-PFx3, it is highly stable and protected from proteolytic degradation.
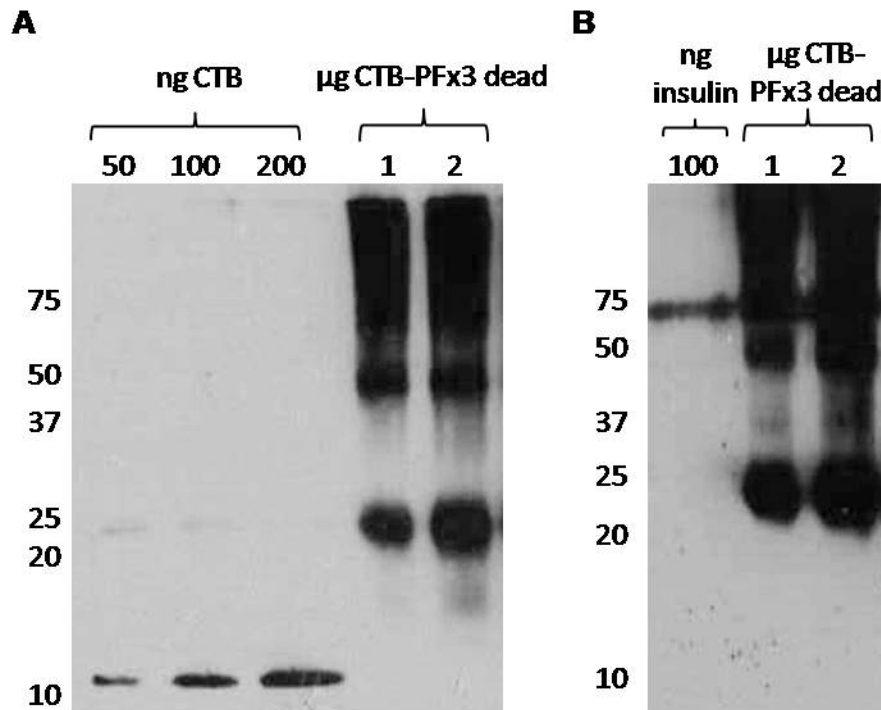
Figure 7: Expression of CTB-Proinsulin in dried senescent lettuce leaves.

(A) CTB-PFx3 total protein from dead leaf homogenate was loaded in 1 or 2 µg concentrations along with varying dilutions of CTB standard protein. Immunoblot was probed with anti-CTB antibody. **(B)** CTB-PFx3 total protein from dried leaf homogenate was loaded in 1 or 2 µg concentrations along with 100ng of insulin standard protein (shown in multimer form). Immunoblot was probed with anti-insulin antibody.

<u>Solubilization and Furin Cleavage of Proinsulin</u>

To prepare the CTB-PFx3 for purification and cleavage assays, it was feasible to remove the protein as inclusion bodies and convert into a soluble form. Previously, in our lab, insoluble inclusion bodies of Human serum albumin (HSA) expressed in

tobacco chloroplasts were solubilized and successfully purified (Fernandez-San Millan et al, 2003). Solubilization was achieved using high quantities of Guanidine Hydrochloride (Gu-HCl) and DTT. DTT was added to plant pellet resuspended in plant extraction buffer containing 6M Gu-HCl. No CTB-PFx3 was found in the supernatant fraction after protein extraction, and this protein was entirely seen in the pellet fraction (Figure 8A). Addition of 100mM DTT achieved some solubility, but addition of 200 or 300mM DTT achieved almost complete solubility of CTB-PFx3. Therefore, 300mM DTT was used for all subsequent solubilizations. After solubilization, the denatured protein was refolded in 20mM sodium phosphate pH 7.8 and 500mM NaCl. To determine if the engineered cleavage sites were functional, a furin cleavage assay was performed (Figure 8B). Upon addition of furin and immunoblot visualization with CTB antibody, it is seen that the 22kDa polypeptide representing the size of the CTB-PFx3 monomer disappears, and a polypeptide is seen at ~11kDa representing CTB alone as well as a polypeptide at ~14kDa which most likely represents an incomplete cleavage between CTB and proinsulin consisting of CTB fused to the B chain of proinsulin, with cleavage occurring at the B chain/ C peptide junction. The proinsulin 2-3kDa cleavage products were not detected by immunoblot with insulin antibody most likely due to the small size of the protein. Silver stain was performed to visualize the 2-3kDa products (Figure 8C). In this gel, the monomer of CTB-PFx3 is seen to fade after addition of furin, and a large band appears at the 2-3kDa size. One large band is visualized because the A chain, C peptide, and B chain cleavage products of proinsulin are 2kDa, 2.5kDa, and 3kDa respectively, which are too close in size to be resolved independently.
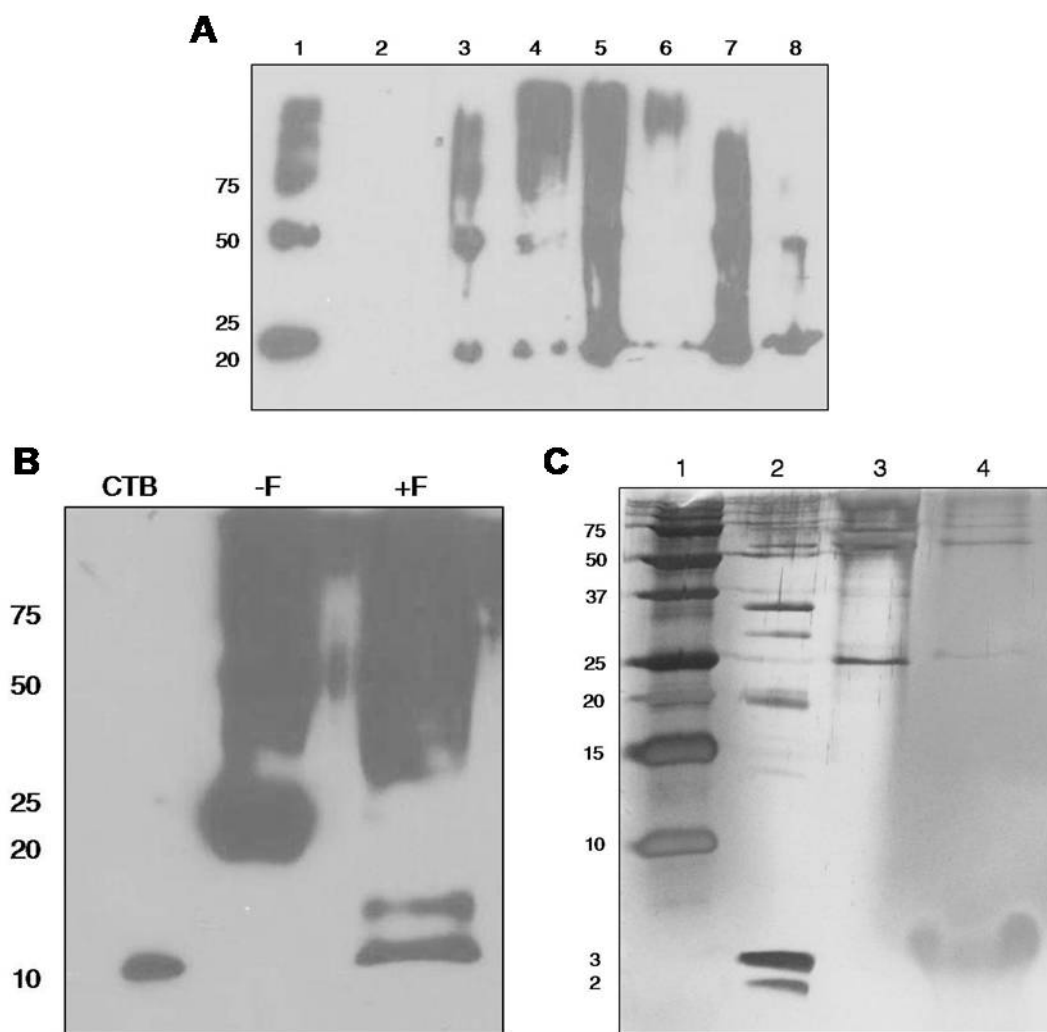
Figure 8: Solubilization and furin digestion of CTB-proinsulin.

(A) Solubilization of CTB-PFx3 in 6M Gu-HCl. 5µg per lane. Lane 1, control pellet. Lane 2, control supernatant. Lane 3, supernatant + 100mM DTT. Lane 4, pellet +100mM DTT. Lane 5, supernatant +200mM DTT. Lane 6, pellet +200mM DTT. Lane 7, supernatant +300mM DTT. Lane 8, pellet +300mM DTT. **(B)** Furin digestion of CTB-

PFx3. CTB, 100ng CTB standard protein. –F, CTBPFx3 before furin cleavage. +F, CTBPFx3 after addition of furin. 1µg per lane. **(C)** Silver stained gel after affinity purification of CTBPFx3. Lane 1, precision plus ladder. Lane 2, Mark 12 low molecular weight ladder. Lane 3, purified CTBPFx3 1ug load. Lane 4, purified CTB-PFx3 after addition of furin. 1ug per lane.

<u>Purification and Enrichment of Proinsulin using Nickel Affinity Chromatography</u>

Solubilized and dialyzed plant extracts containing CTB-PFx3 were passed over a Ni-NTA column to facilitate purification. Fractions were eluted and run on an SDS-PAGE gel and visualized by coomassie staining (Figure 9A). The 55kDa Rubisco large subunit is seen prominently in the wild type sample. This is typical as Rubisco is the most abundant protein in plants and probably the most abundant protein on earth (Jensen, 2000). The 22kDa band of CTB-PFx3 is seen prominently in the control pellet (CP), but not in the control supernatant (CS), again showing that the protein is in the form of inclusion bodies. After solubilization and dialysis, CTB-PFx3 is detected in the dialyzed supernatant fraction (DS). Approximately 30ml of dialyzed supernatant were passed over the Ni-NTA column. No protein is seen to elute in the wash fractions (W1 and W2). The two eluted fractions with the highest optical density (OD) at 280nm were run on the gel. The 22kDa polypeptide representing CTB-PFx3 is seen prominently along with Rubisco, but it is seen that the contaminating protein bands are much less abundant; therefore, the total CTB-PFx3 protein content was purified and enriched compared to the starting material.

Purification was repeated in order to achieve a greater level of purification by removing Rubisco. In order to enhance purification, old leaves were prepared in the same way as in the previous purification as they contain less Rubisco, and samples were run over a Ni-NTA column. After purification, additional Rubisco was removed using sodium phytate precipitation (Krishnan and Natarajan, 2009), and samples were loaded on an SDS PAGE gel for coomassie stain (Figure 9B).

The resultant purified fractions were loaded in equal quantity onto an SDS-PAGE gel for immunoblot analysis along with the dialyzed supernatant, flow through, and wash fractions. Blots were probed with either anti-CTB antibody (Figure 9C) or anti-insulin antibody (Figure 9D). In both blots, the purified CTB-PFx3 is shown to be highly enriched compared to the dialyzed supernatant. Additionally, the CTB-PFx3 monomer is enhanced, which would provide the functional, cleavable proinsulin. Densitometric analysis showed that the original dialyzed supernatant contained 27% CTB-PFx3, whereas after the first purification, this increased to an average of 57% purity (range determined by densitometry to be between 42% - 72%), a nearly 2-fold increase. After the second attempt at purification followed by sodium phytate precipitation, the sample was shown to be about 87% pure (range determined by densitometry to be between 75% - 98%), over a 3-fold increase from the original dialyzed supernatant and a 1.5-fold increase from the first purification (Figure 9E). The starting concentration of the CTB-PFx3 in the dialyzed supernatant was ~30,000ng/ml (0.03 mg/ml) which increased to ~320,000ng/ml (0.32 mg/ml) after the first purification and to ~140,000ng/ml (0.14mg/ml) after the second purification representing a total enrichment greater than

10 fold and 4.5 fold, respectively (Figure 9F). The lower concentration in the second purification is most likely due to the fact that less total sample volume was loaded onto the column resulting in a lower concentration but achieving higher purity.
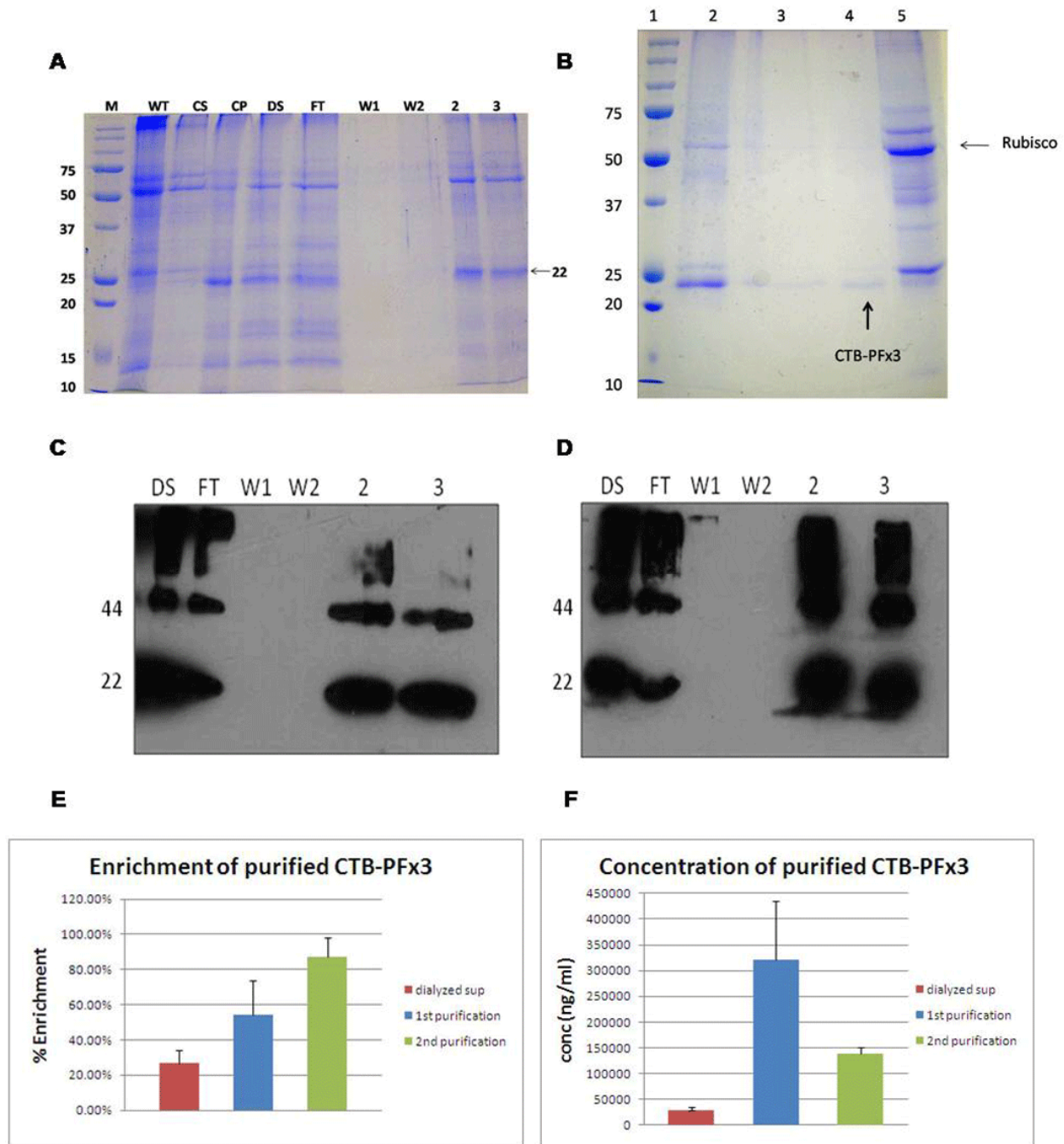
Figure 9: Purification of CTB-Proinsulin by affinity chromatography.

(A) Coomassie stained gel. DS = dialyzed supernatant; FT = flow through; W1 = wash 1; W2 = wash 2; 2,3 = purified fractions; WT = wild type; CS = control supernatant; CP = control pellet **(B)** Coomassie stained gel showing second purification of CTB-PFx3: Lane 1: Marker. Lane 2: control pellet (before purification). Lane 3: Purified CTB-PFx3, Lane 4: Purified CTB-PFx3 after phytate precipitation to remove Rubisco. Lane 5: Control supernatant. All 10ug load. **(C)** 1µg of purified CTB-PFx3 samples was loaded using CTB primary antibody. DS = dialyzed supernatant; FT = flow through; W1 = wash 1; W2 = wash 2; 2, 3 = purified fractions **(D)** 1µg of purified CTB-PFx3 samples was loaded using insulin primary antibody. DS = dialyzed supernatant; FT = flow through; W1 = wash 1; W2 = wash 2; 2, 3 = purified fractions **(E)** Densitometry shows that after purification, CTB-PFx3 makes up ~57% of the eluted fractions after the first purification and ~87% after the second purification as compared to ~27% of the dialyzed solubilized supernatant before passing over the nickel column. **(F)** The concentration of CTB-PFx3 increases roughly 10 fold (~320000ng/ml) after the first purification and 4.6 fold (~140,000ng/ml) after the second purification over nickel column than the initial concentration of dialyzed solubilized supernatant (~30000ng/ml).

Functional Evaluation of Cleavable Proinsulin

CTB-PFx3 purified from transplastomic tobacco as well as commercially produced purified insulin (Roche) was injected into female C57BL/6 mice along with PBS as a negative control. Blood glucose measurements were taken by tail vein bleed before intraperitoneal (IP) injection and at 15, 30, 60, 120, and 180 minute time

intervals. Another injection was given after the 180 minute time point, and blood glucose measurements were again taken at 15, 30, 60, 120, and 180 minute time points after the second injection. Blood glucose levels were found to be significantly lower than PBS control in all groups at the 120 minute time point after the second injection (Figure 10a), similar to commercial insulin.

Additionally, transplastomic lettuce and tobacco plants along with untransformed lettuce were delivered to mice by oral gavage. Commercially produced purified insulin was injected as a control. Blood glucose measurements were taken by tail vein bleed before gavage or injection and at 15, 30, 60, 120, and 180 minute time intervals. Another dose was given after the 180 minute time point, and blood glucose measurements were again taken at 15, 30, 60, and 120 minute time points after the second dose. Blood glucose levels were found to be significantly lower than untransformed control in all groups at the 120 minute time point after the second injection (Figure 10b). Food pellets were removed from cages for the duration of both studies.
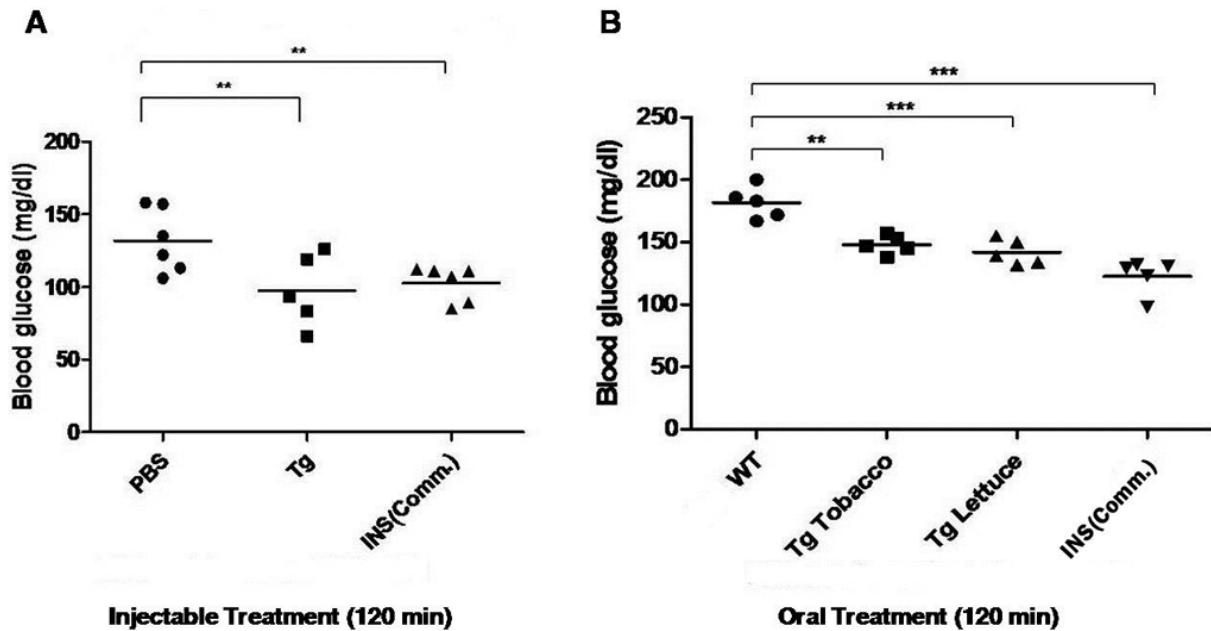
Figure 10: Functional evaluation of CTB-Proinsulin in mice delivered by injection or oral gavage.

ANOVA (Dunnett's multiple comparison test) was performed on all groups of mice. (a) Mice were administered two IP injections of PBS only (PBS), purified CTB-PFx3 (Tg), or commercial Insulin (INS Comm.). Blood glucose levels were measured after the first 15 minutes and each hour (up to three hours) after each injection. Blood glucose levels were statistically lower (**$P<0.01$) 2 hours after the second injection in all groups compared to the PBS only negative control. (b) Mice were administered two oral gavages of untransformed lettuce (WT), tobacco expressing CTB-PFx3 (Tg tobacco), lettuce expressing CTB-PFx3 (Tg lettuce), or two IP injections of commercial Insulin (INS Comm.). Blood glucose levels were measured after the first 15 minutes and each

hour (up to three hours) after each injection. Blood glucose levels were statistically lower (**P<0.01; ***P<0.001) 2 hours after the second injection in all groups compared to the untransformed (WT) control.

DISCUSSION

Existing treatments for type 1 diabetes are painful, costly, and currently available insulin does not include the C-peptide. Here, we have expressed CTB-Proinsulin with three furin cleavage sites in both tobacco and lettuce chloroplasts in order to produce insulin that may be processed in any cell in the body. This will significantly reduce the cost of production, as well as facilitate the possibility of delivering the C-peptide which should aid in the treatment of diabetic complications. One acre of farm land can yield about 40 metric tons of tobacco with three harvests (Arlen et al, 2007) or 11 metric tons of lettuce biomass in a single harvest. Assuming the expression level of CTB-PFx3 is around 3mg/g of leaf tissue, and taking into consideration a 50% loss of protein during the purification process, this would yield about 20 million daily doses of insulin. It is estimated that 220 million people worldwide have diabetes, 10% of these having type 1 diabetes. Therefore, one acre of biomass could produce enough insulin to treat a large type 1 diabetic population, which would be a major economic advantage.

There are many disadvantages to insulin injections which are currently the most widespread form of administration. These include, pain, itching, allergy, and lipodystrophy at the injection site. Additionally, these disadvantages often cause noncompliance of patients with their insulin regimen. Therefore, alternative approaches to insulin delivery are necessary to help diabetic populations meet their medical needs. Some of the recent approaches to address these problems include buccal spray insulin, inhalable insulin, oral anti-diabetic drugs, and oral delivery of insulin. Buccal spray

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insulin is absorbed by the mucosa of the mouth and has a faster onset than injectable insulin. Limitations of this approach include a shorter duration of action and a dosage requirement that is 5-7 times higher than injectable insulin as well as mild side-effects such as dizziness (Pozzilli et al, 2010). Inhalable insulin faces similar challenges. The FDA approved inhalable insulin, Exubera, was pulled off the market due to poor sales and adverse side effects such as reduced lung function (Siekmeier and Scheuch, 2008). Oral anti-diabetic drugs are used mainly to treat type 2 diabetics as they improve insulin production, so there is still a need to deliver functional insulin to people with type 1 diabetes and type 2 diabetics who require insulin. The main challenges that arise in advancing the concept of oral delivery of insulin are protection from enzymatic degradation in the stomach and efficient absorption into the GALT (Agarwal and Khan, 2001). Approaches to overcome these challenges include encapsulation of insulin using nanoparticles and the delivery of insulin along with a protease inhibitor (Iyer et al, 2010). A novel approach to circumvent these obstacles is the expression of insulin in plant cells.

Insulin has never been produced without a fusion protein as it is highly unstable and is prone to N-terminal degradation (Boothe et al, 2010). Therefore, we fused proinsulin with CTB in order to protect the N terminus of insulin from degradation as well as provide a means to facilitate purification and uptake of orally delivered insulin into the GALT. CTB binds to GM1 receptors in the lumen of the gut (de Haan et al, 1998, Tsuji et al, 1995), and it has been shown that fusion to the C-terminus of CTB is ideal for pentamerization and GM1 binding (Liljeqvist et al, 1997; Limaye et al, 2006). Recently,

CTB fused with blood clotting factor IX was successfully expressed and delivered orally to aid in treatment of hemophilia B. After delivery, the antigen was found in different tissues of the GALT, specifically the ileum, Peyer's patches, liver, and plasma within two hours (Verma et al, 2010). Our CTB-proinsulin construct did not contain a His tag or any other tag to facilitate purification. However, CTB contains three histidine residues that are in close enough proximity when CTB is properly folded, which is enough to allow binding to anion exchange resins such as Ni^{2+} or Co^{2+} (Dertzbaugh and Cox, 1998). This way, purification of the CTB-PFx3 is facilitated by the CTB fusion. Additionally, purification is enhanced by the fact that a majority of the plant proteins are discarded with the supernatant, thus the initial sample to be purified contains only those proteins which were insoluble, eliminating a majority of endogenous plant soluble proteins. When expressed in plants, the insulin is also protected from enzymatic degradation in the stomach via bioencapsulation (Davoodi-Semiromi et al, 2010; Limaye et al, 2006; Verma et al, 2010). Therefore, advancing the concept of oral delivery of insulin by expressing it in plant cells should reduce its cost, facilitate purification and processing, eliminate the need for cold storage, and simplify transportation and delivery (Daniell et al, 2009b).

Additionally, none of the alternative or currently used approaches to insulin delivery include the C-peptide. C-peptide is generally used as a marker to determine the extent of insulin release in patients. A large portion of diabetic patients who undergo extensive insulin therapy to maintain normal blood glucose levels still develop long-term complications such as neuropathy and nephropathy. C-peptide has been shown to

alleviate these complications in various studies, and this suggests that diabetic patients should receive supplemental C-peptide in addition to insulin (Hills and Brunskill, 2009). For these reasons, we have created cleavable proinsulin, which is able to be processed into properly folded and functional insulin as shown by functionality studies in mice. Upon cleavage, C-peptide is available for circulation, where it may interact with its G-protein-coupled receptor and in turn activate the Na^+/K^+ ATPase and MAPK pathways, which are partly responsible for the beneficial effects seen in alleviating diabetic complications. This is the first time that the complete proinsulin gene has been expressed in plants with the possibility of in vivo processing outside the pancreas, into functional insulin and C-peptide.

Upon hyper-expression of CTB-PFx3 in tobacco and lettuce chloroplasts, we noticed expression in the form of insoluble inclusion bodies, similar to insulin inclusion bodies observed in E. coli (Williams et al, 1982) or plants (Boothe et al, 2010). The functionality of CTB stems from its ability to pentamerize in order to bind to GM1 receptors (Liljeqvist et al, 1997) and has been demonstrated to form functional pentamers in chloroplasts (Daniell et al, 2001; Limaye et al, 2006; Verma et al., 2010). Additionally, insulin and proinsulin may form self-aggregates of monomers, dimers, tetramers, and hexamers (Pekar and Frank, 1972) which may further contribute to their aggregation in chloroplasts. Furthermore, the majority of proinsulin accumulation was seen in older leaves as opposed to mature leaves, which is often observed among foreign (soluble) proteins expressed in chloroplasts (Daniell et al, 2001; Koya et al, 2005; Arlen et al, 2008; Daniell et al, 2009a). This may account for the high levels of

expression seen in old leaves, as proinsulin may be protected from proteases which break down endogenous soluble leaf proteins with age (Martin and Thimann, 1972; Vierstra, 1993). Stability of CTB-PFx3 was even seen in senescent and dried leaves which should facilitate harvest, field drying, storage, and transportation of this valuable protein. This is the first report of stable accumulation of a therapeutic protein in dried senescent leaves.

Quantification of total CTB-PFx3 expression in chloroplasts showed up to 47% and 53% in tobacco and lettuce old leaves, respectively. Attempts made to determine protein quantification by means of enzyme-linked immunosorbent assay (ELISA) were unsuccessful most likely due to the high quantity of protein aggregates which either inhibited binding to the ELISA plate or reduced the ability of the CTB primary antibody from binding to the proper epitopes on the CTB molecule. Addition of DTT to the sample extraction buffer or boiling of samples prior to coating of the ELISA plate with CTB-PFx3 improved the detection of signal, but not enough to yield an accurate quantification. The denaturation of the CTB-PFx3 monomer and multimers during the immunoblot procedure most likely facilitated better binding of the CTB primary antibody, allowing access to the desired epitopes. For densitometry studies, only the monomer and dimer bands were used for the quantification, as the higher multimers tended to form less defined bands. Therefore, the percentages reported are likely an underestimation of the total expressed CTB-proinsulin.

Mice that were given oral CTB-PFx3 experienced changes in blood glucose levels but levels were significantly lower at 120 minutes after the second dose when

compared to mice given untransformed lettuce. This demonstrates that proinsulin is delivered to the circulation and is properly cleaved and folded. In a recently published study, we observed blood clotting factor IX fused with CTB in the circulatory system 2 hours after oral delivery (the earliest time point in this study) of CTB-FIX expressed in chloroplasts (Verma et al, 2010). Therefore, detection of plant-derived insulin activity in less than two hours is quite reasonable. At this point, the percentage of orally delivered protein absorbed by the GALT and delivered into the circulatory system has not been fully investigated, although several bioencapsulated chloroplast-derived therapeutic proteins have been orally delivered and shown to be fully functional. Additionally, injected CTB-PFx3 must be processed into CTB and insulin when compared with the commercial insulin, which is already in its correct functional conformation. Therefore, determining the actual amount of CTB-PFx3 mice received and time required for processing would require further investigations.

CONCLUSIONS

This is the first time that the complete proinsulin gene has been expressed in plants with the possibility of in vivo processing outside the pancreas into functional insulin and C-peptide. It is also the first report of stable transgene expression present in senescent leaf tissue. There is a need for low-cost insulin as well as a need for developing oral insulin in order to avoid the pain and inconvenience associated with insulin delivery via injection or pump. Additionally, the currently available commercial insulin preparations do not include the C-peptide, which has been shown to alleviate diabetic complications such as neuropathy, nephropathy, and problems of the circulation. Proinsulin containing furin cleavage sites at the B chain/C-peptide and C-peptide/A chain junctions was fused with CTB, and a furin cleavage site was present at the point of fusion. This was expressed in tobacco and lettuce chloroplasts at levels up to 47% and 53%, respectively, in old leaves. These high levels of expression led to the formation of insoluble inclusion bodies, which were solubilized using high concentrations of Gu-HCl and DTT. CTB-PFx3 was either orally delivered to mice or injected after purification, and results showed significant reduction in blood glucose levels as compared to negative controls. Future studies would investigate long-term effects of the C-peptide delivery on diabetic complications in addition to the effects of CTB-PFx3 on blood glucose levels.

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