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EXPRESSION OF HETEROLOGOUS PROTEINS IN TRANSGENIC TOBACCO CHLOROPLASTS TO PRODUCE A BIOPHARMACEUTICAL AND BIOPOLYMER

by

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ABSTRACT

The chloroplast has been demonstrated to be an ideal compartment to accumulate certain proteins or their biosynthetic products that would be harmful if they were accumulated in the cytoplasm. Hyper-expression of foreign proteins in chloroplast transgenics has accumulated up to 46% total soluble protein, this is possible due to the ~100 chloroplast genomes per chloroplast and ~100 chloroplasts per cell which can therefore, contain up to 10,000 copies of the transgene. Maternal gene inheritance of plastids in most crop plants results in natural gene containment. Chloroplast transformation also eliminates positional effects that are frequently observed with nuclear transformation and no gene silencing has been observed so far at the level of transcription or translation. Consequently, independent chloroplast transgenic lines have very similar levels of foreign gene expression and there is no need to screen hundreds of transgenic events. The chloroplast genome has also been used in molecular farming to express human therapeutic proteins, vaccines for human or animal use and biomaterials.

In this study we have produced a Nicotiana tabacum cv. petit Havana chloroplast transgenic line that expresses a cholera toxin B subunit (from *Vibrio Cholerae*)-human proinsulin (a,b and c chain) fusion protein, designated CTB-Pris. The pLD-PW vector contains the CTB-Pris gene cloned into the universal chloroplast transformation vector pLD-ctv in which the 16S rRNA promoter drives the aadA gene selectable marker, which confers resistance to spectinomycin; the \( psbA \) 5′ untranslated region (UTR) which enhances translation of CTB-Pris in the presence of light and the \( psbA \) 3′UTR confers transcript stability. The \( trnI \) and \( trnA \) homologous flanking sequences facilitated site-specific integration of transgenes into the tobacco chloroplast genome. Site-specific integration was demonstrated by PCR and Southern blot analysis with probes for
CTB-Pris. Western Blot analysis has demonstrated the presence of abundant CTB-Pris in transgenic plants with both CTB polyclonal and proinsulin monoclonal antibodies. Southern blot analysis has also confirmed that homoplasmy had been achieved in the T_0 generation. The expression levels for CTB-Proinsulin varied between 270µg/100mg to 364.8µg/100mg of plant tissue which equates to ~30% total soluble protein.

In the second study the E. coli *ubiC* gene that codes for chorismate pyruvate-lyase (CPL) was integrated in the tobacco chloroplast genome under the control of the light-regulated psbA 5’ untranslated region. CPL catalyzes the direct conversion of chorismate – an important branch point intermediate in the shikimate pathway that is exclusively synthesized in plastids – to pHBA and pyruvate. pHBA is the major monomer in liquid crystal polymers (LCPs). These thermotropic polyesters have excellent properties, including high strength/stiffness, low melt viscosity, property retention at elevated temperatures, environmental resistance and low gas permeability. The leaf content of pHBA glucose conjugates in fully mature T_1 plants exposed to continuous light (total pooled material) varied between 13-18% DW, while the oldest leaves had levels as high as 26.5% DW. The highest CPL enzyme activity observed in total leaf material was 50,783 pkat/mg of protein, which is equivalent to ~35% of the total soluble protein.

Animal studies in the Daniell lab, suggest that the CTB-Proinsulin producing plants suppress insulitis and clinical symptoms of diabetes in NOD mice. These observations demonstrate the versatility of chloroplast gene expression for production of biopharmaceuticals and biopolymers.
Dedicated to my wife Wendi and son Niko.
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# TABLE OF CONTENTS

LIST OF FIGURES ......................................................................................................................... xi

CHAPTER ONE: INTRODUCTION CTB-PROINSULIN ..................................................................... 1

Diabetes Facts ................................................................................................................................. 1

Insulin and Tolerance ...................................................................................................................... 2

CTB as Mucosal adjuvant .............................................................................................................. 4

CTB-Proinsulin ............................................................................................................................... 5

Historical Aspects of Chloroplast Genetic Engineering .............................................................. 6

Advantages of Plants over E. coli Expression ............................................................................... 8

Advantages of Chloroplast Genetic Engineering over Nuclear transformation ....................... 9

Use of Tobacco for Production of Recombinant Proteins .......................................................... 11

Bioencapsulation of Recombinant Proteins Delays Their Breakdown .................................... 12

Crop Species That Have Been Stably Transformed via Plastid Genome ................................... 12

Transgenic Plants as Bioreactors for the Production of Valuable Pharmaceuticals .................. 16

CPL Introduction .......................................................................................................................... 20

CHAPTER TWO: MATERIAL AND METHODS ........................................................................... 27

Vector Construction of 5’UTR-CTB-Proinsulin ........................................................................ 27

PCR Primer design ......................................................................................................................... 27

PCR Mutagenesis of 5’UTR-CTB by Splicing ............................................................................. 28

PCR Product ligation into PCR2.1 Topo cloning vector ............................................................. 29

Transformation into One Shot competent cells ......................................................................... 29

Mini-Prep plasmid isolation of PCR 2.1 by Alkaline Lysis Method ........................................... 30

Sequencing .................................................................................................................................. 31
Prehybridization and Hybridization .................................................................................................................. 46
CPL Materials and Methods .............................................................................................................................. 47
Construction of the CPL Chloroplast Transformation Vector ............................................................................. 47
Particle Bombardment and Selection of Chloroplast Transformants ................................................................. 48
Confirmation of Site-Specific Integration by PCR ............................................................................................... 49
Southern Blot Analysis to Demonstrate Site-Specific Integration and Homoplasmy. These steps were performed essentially as previously described (Daniell et al., 2004d). .................................. 49
Analysis of pHBA Glucose Conjugates (Performed by DuPont) ................................................................. 50
Determination of CPL Enzyme Activity in Leaf and Stalk Cell-Free Extracts (Performed by DuPont) .............. 52
CHAPTER THREE: RESULTS ............................................................................................................................... 54
CTB-Pris Results ............................................................................................................................................. 54
Vector Construction of pLD-5’UTR-CTB-Human Proinsulin (5CP) and pLD-5’UTR-CTB-Plastid Codon Optomized Proinsulin (5CPTP) ........................................................................................................ 54
E. coli Immunoblot Analysis ............................................................................................................................... 61
PCR Analysis to Screen for Chloroplast Transgenics ......................................................................................... 63
Immunoblot Detection of Fusion Protein in Chloroplast Transgenic Tobacco Plants ................................ 65
Southern Blot Analysis ....................................................................................................................................... 69
Plant Phenotype and Maternal Inheritance ........................................................................................................... 74
CPL Results ...................................................................................................................................................... 77
Vector Construction (Partially Performed at DuPont) ......................................................................................... 77
PCR Confirmation of Chloroplast Transgenics ..................................................................................................... 77
Southern Blotting Analysis to Determine Site Specific Integration and Homoplasmy ................................. 80
LIST OF FIGURES

CTB-Proinsulin Figures

Figure 1 A: Codon Optimized and Human Proinsulin Gene Synthesis........................................... 57
Figure 1 B: PCR Gene Synthesis of Human and Codon Optimized Proinsulin .................................. 58
Figure 1 C: PCR’ing of the psbA 5’ UTR ..................................................................................... 58
Figure 1 D: Slicing by Overlap Extension ......................................................................................... 59
Figure 1 E: Final Construct and Site of Integration into the Chloroplast Genome......................... 60
Figure 1 F: Following PCR Mutagenesis ......................................................................................... 61
Figure 2: E. coli SDS-PAGE ........................................................................................................... 62
Figure 3 A: 3P3M and 5P2M PCR Primers ..................................................................................... 64
Figure 3 B/C: PCRing of Human (5CP) and Codon Optimized (5CPTP) Proinsulin T₀ transgenic
tobacco plants: Fig 3B .................................................................................................................. 65
Figure 4 A: Western blot analysis of 5CP and 5CPTP chloroplast transgenic, probed with
proinsulin antibody ........................................................................................................................ 67
Figure 4 B: Western blot analysis of 5CP chloroplast transgenic, probed with CTB polyclonal
antibody and Quantified Using a Spot Densitometer ..................................................................... 68
Figure 5 A: Probes used for Southern blotting ................................................................................ 70
Figure 5 B: Photo Illustration Depicting Wild Type and Chloroplast Transgenics....................... 70
Figure 5 C: Southern Blot Hybridization with Gene Specific Probe .................................................. 71
Figure 5 D: Southern Blot Hybridization with Flanking Sequence Probe ........................................ 72
Figure 5 E: Southern Blot Hybridization with Flanking Sequence Probe ........................................ 73
Figure 6 A: Photograph depicting 5CP plants .................................................................................... 75
CPL Figures

Figure 1: Schematic representation of gene cassette integrated into chloroplast genome:........... 79

Figure 2: Figure 2a/b. Southern blot analysis to determine gene specific integration and homoplasmy......................................................................................................................... 81

Figure 3: Photo depicting transgenic plants...................................................................................... 86
CHAPTER ONE: INTRODUCTION CTB-PROINSULIN

Diabetes Facts

Diabetes is a disease in which the body does not produce or properly utilize insulin. Several forms of diabetes exist the predominant forms are: Type I diabetes which results from the body’s failure to produce insulin and Type II diabetes is the result of insulin resistance in which the body no longer properly utilizes insulin. All people with type 1 diabetes need to take insulin because their bodies do not make enough of it. Insulin helps turn food into energy for the body to work. Healthy eating, exercise, and losing weight may help you lower your blood glucose (also called blood sugar) when you are diagnosed with type 2 diabetes. If these treatments do not work, you may need one or more types of diabetes pills to lower your blood glucose. After a few years, you may need to take insulin shots because your body is not making enough insulin. The insulin molecule is a hormone which is required by the body to allow glucose entry into cells that is required for energy. Diabetes can be associated with serious complications and premature death, but people with diabetes can take steps to control the disease and lower the risk of complications.

As of 2002, the American Diabetes Association estimated that there were 18.2 million people in the United States or 6.3% of the population, who have diabetes. An estimated 13 million have been diagnosed and unfortunately 5.2 million people are unaware that they have the disease. Complications that can arise from diabetes include the following: heart disease, kidney disease, eye complications, nerve damage, foot complications, skin problems, gastroparesis, and depression. Diabetes was the sixth leading cause of death listed on U.S. death certificates in 2002, this ranking was based on the 69,301 death certificates in which diabetes was listed as the
underlying cause of death. Altogether, diabetes contributed to 213,062 deaths. Therefore, it is essential to find a prevention for this dreadful disease.

**Insulin and Tolerance**

Insulin is secreted by pancreatic β-cells which is regulated by the glucose sensing system. The exact metabolic functions include the stimulation of the storage of glucose in the liver, glucose uptake, and utilization in fat and muscle cells. The insulin polypeptide is made up of an A chain (21 amino acids) and a B chain (30 amino acids) which are disulfide bonded together between cysteine residues. Before processing it is preproinsulin which is located within the secretory granules of the β-cell, then processed to release proinsulin, insulin and C peptide. The C peptide which is part of proinsulin prior to processing has remained a mystery until recently where human trials have demonstrated that the proinsulin C-peptide stimulates the activities of Na+,K+-ATPase and endothelial nitric oxide synthase, both of which are enzyme systems of importance for nerve function and known to be deficient in type 1 diabetes (Ekberg et al., 2003).

The major destruction of β-cells occurs predominantly from autoreactive T-cytotoxic cells and T-helper 1 cells reactive to β-cell autoantigens such as insulin. An important discovery was that not only do β-cells produce insulin but isolated cells within the thymus (where T-cells mature) and the spleen have mRNA’s coding for insulin, organs essential for the immune system. Hanahan (1998) has shown that small amounts of molecules expressed within the thymus lead to tolerance. Studies in man have indicated that different that the difference sizes of a variable nucleotide tandem repeat (VNTR) 5’ of the insulin gene are associated with type 1 diabetes, and the form of the VNTR that contain the greater number of repeats is associated with greater insulin message in the human thymus as well as protection from diabetes (Pugliese et al., 1997, Vafiadis et al., 1997). This has lead to the hypothesis that the influence contributed by the insulin
gene locus may relate to the variation in expression of the insulin message within the thymus, leading to tolerance. Thus if insulin is the critical antigen for the development of diabetes the increased presence within the thymus may lead to the deletion or inactivation of insulin specific T cells which go through positive and negative selection in the thymus.

Oral administration of antigens represents a potential way to induce oral tolerance. Tolerance refers to a state of lowered systemic responsiveness toward priming with an antigen following oral delivery. Both an active and a passive form of tolerance can be induced dependant upon the amount of dose and route of administration. The passive form is the functional inactivation of antigen specific lymphocytes and is selective for only pre-existing effectors. The active form of tolerance operates through the action of regulatory lymphocytes that are able to downmodulate inflammation by bystander suppression of effector cells. Bystander suppression is viewed as a form of immunoregulation rather than tolerance. Mucosal immunity generated by oral delivery is a protective immune response manifested by Th2 type cytokines such as IL-4, IL-10, and TGF-β. Antigen taken orally leads to presentation in the intestinal mucosa, which is able to generate protective Th2 T cells in the gut-associated lymphoid tissue (GALT) such as the peyer’s patches. The antigen utilized is important because its nature will determine the type of cytokines produced by the antigen specific T-cells and must direct the mucosal derived T-cells to migrate to the organ of interest, and must then be able to downregulate the localized immune response. Previous studies have demonstrated that insulin given orally to non-obese diabetic (NOD) mice was demonstrated to reduce the level of diabetes by 50% and the protection was associated with the development of Th-3 type response, specifically TGF-β producing T-cells (Zhang et al., 1991).
CTB as Mucosal adjuvant

Oral tolerance induced by autoantigens has been applied successfully as a therapeutic tool in experimental models of autoimmune diseases (Strobel et al., 1998). The basic mechanism of oral tolerance in humans is currently a work in progress and the oral antigen administration regimens have resulted in limited success when applied to patients (Garside et al., 1999, Pozzilli et al., 2000, Krause et al., 2000). A possible explanation for the limited success could be due to the fact that the doses of the orally administered antigens to humans was too low compared to those of mice, especially when you take into consideration the surface area of the intestinal absorptive epithelium (Pozzilli et al., 2000). Therefore, CTB may provide the necessary costimulation required to overcome an inefficient presentation of insulin to the mucosal T-cells, resulting from the limited transport of native insulin over the epithelial layer. In order to effectively treat human autoimmune diseases with orally administered antigens, large doses are required. In order for oral tolerance to become a realistic therapy for human autoimmune diseases, adjuvants that possess the ability to enhance the tolerogenic potential of orally delivered antigens need to be identified. The coupling of autoantigens in this case insulin to the non toxic cholera toxin B subunit (CTB) dramatically increases their tolerogenic potential (Bergerot et al., 1997). This effect is mediated by the ability of CTB to act as a transmucosal carrier, although CTB may have a direct affect on the immune system (Burkart et al., 1999, Li et al., 1996). The current limitation for using CTB conjugated to insulin is the production process and this is the current limitation for its use on an industrial scale as an oral vaccine (Bergerot et al., 1997). Therefore, this limitation can be overcome by the hyperexpression of CTB-proinsulin fusion protein within transgenic tobacco chloroplasts.
CTB-Proinsulin

Arakawa and colleagues (1998) were able to generate nuclear transgenic potato plants expressing CTB-proinsulin and followed this work with animal studies in NOD mice. They expressed CTB-proinsulin that was able to form pentamers by disulphide bonds, which must occur in order to bind to the intestinal epithelium receptor GM$_1$. Their proinsulin was conjugated to CTB by a flexible glycine, proline, glycine, proline, hinge region, which allows proper folding of each individual fusion protein by lowering the steric hindrance. Upon feeding to NOD mice the CTB-proinsulin potato plants were able to induce both a systemic and intestinal anti-CTB antibody response, demonstrating an effective delivery to the intestinal mucosal immune system. The researchers were also able to detect anti-proinsulin IgG antibodies. The presence of anti-proinsulin IgG1 rather than IgG2a isotype indicates that a Th2 response was predominant which indicates a class switch from the Th-1 response, which is predominant in diabetes. They also observed a lack of insulitis in the NOD mice which are predisposed to develop diabetic symptoms. With their data pooled together the conclusion was that feeding microgram quantities of potato expressing CTB-proinsulin to mice effectively suppressed the development of autoimmune diabetes in NOD mice and that plant synthesized CTB-proinsulin was effective at 100-fold lower concentrations than those reported for unconjugated autoantigens (Arakawa et al., 1998). These results reinforce the importance and effectiveness of conjugating CTB to autoantigens, in this case proinsulin, in order to induce tolerance and thus protection from diabetes symptoms. Therefore, it is quite feasible to hyper-express this fusion protein, CTB-proinsulin in transgenic tobacco chloroplasts in order to continue further testing on NOD mice at much higher quantities.
Historical Aspects of Chloroplast Genetic Engineering

The age of chloroplast genetic engineering began in the 1980’s, it was then that it became possible to introduce isolated intact chloroplasts into protoplasts and regenerate plants (Daniell et al. 2002). The early research on chloroplast genetic engineering in vascular plants was dedicated to the development of chloroplast systems capable of efficient, prolonged protein synthesis and expression of foreign genes (Daniell and McFadden 1987). With the invention of the Gene Gun by John Sanford (reviewed in 1993), it became possible to genetically engineer plastids without the tedious need to isolate the plastids. Chloroplast transformation in higher plants was brought to life through the use of autonomously replicating chloroplast vectors in dicot plastids (Daniell et al. 1990) and transient expression in monocot plastids (Daniell et al. 1991).

In 1998, Boyton and colleagues performed the first successful chloroplast genome complementation experiment. This was reported using *Chlamydomonas reinhardtii*, which is a unicellular green alga which contains a single chloroplast that occupies approximately sixty percent of the cell volume (Boyton et al., 1998). The procedure consisted of photosynthetically incompetent mutants that contained a deletion in the *atpB* gene, the mutants did not possess chloroplast ATP synthase activity. The natural *atpB* gene was bound to tungsten microprojectile particles and shot into cells spread on agar plates utilizing a gunpowder charge (Klein et al., 1987). The Chlamydomonas single large chloroplast provided an ideal target for the delivery of foreign DNA. The native *atpB* gene that was introduced into the alga cells was able to reverse the deletion mutant phenotype. Utilizing light for selection it was demonstrated that photoautotrophic growth could be restored and the native gene carried by a vector could
successfully integrate into the Chlamydomonas chloroplast genome by homologous recombination, replacing the deleted \textit{atpB} gene. Plastids are endosymbionts of cyanobacteria and have inherited an efficient \textit{recA} type system that facilitates homologous recombination (Cerruti et al., 1992). It was then shown that the foreign DNA flanked by chloroplast DNA sequences is incorporated and stable within the \textit{Chlamydomonas} chloroplast genome; the \textit{uidA} gene that was introduced was transcribed but the translated product was not observed (Blowers et al., 1989). Daniell et al., (1990) was the first to demonstrate foreign gene expression in cultured tobacco cell plastids by biolistic delivery of autonomously replicating chloroplast vectors, this was followed by leaves, calli, and somatic embryos of wheat plants (Daniell et al., 1991). In past years, foreign genes were introduced and expressed only in isolated intact plastids (Daniell and McFadden, 1987). In 1991 Goldschmidt-Clermont transformed the \textit{Chlamydomonas} single chloroplast genome with the selectable marker gene \textit{aadA} that confers resistance to the antibiotics spectinomycin and streptomycin (Goldschmidt-Clermont, 1991), this was a major contribution to the field since the majority of higher plants that are currently transformed via their chloroplast genome utilize this selectable marker. This work was followed by the demonstration of stable integration of the \textit{aadA} gene introduced into the chloroplast genome (Svab and Maliga 1993). The field is currently growing at a rapid pace and there are now biotechnology companies that solely rely on this technology. Plastid genetic engineering technology is currently being applied to edible crops such as potato, tomato, carrot, and soybean (Sidorov et al., 1999; Ruf et al., 2001; Kumar et al., 2004a; Duformantel et al., 2004) and the agriculturally important crop cotton (Kumar et al., 2004b).
Advantages of Plants over E. coli Expression

The chloroplast transformation system contains many advantages over expression in E. coli. The current production of recombinant protein within microorganisms is quite expensive, they require costly purification methods, and in order to scale up for mass production, fermenters which are quite expensive must be used. Producing pharmaceuticals within plants can circumvent this cost. The current production costs can be drastically reduced because in order to farm plants, all that is required is land and transgenic seeds, which when compared to fermenters is inexpensive and can be scaled up in one growing season at a much lower cost. It has been estimated that the cost of recombinant protein production in tobacco leaves will be on average 10-50 times cheaper than the current methods of E. coli fermentation (Kusnadi et.al., 1997).

Another advantage of plants is that they provide a heat stable environment for recombinant proteins and the technology also exists to harvest, store, and purify recombinant proteins from plant material. In addition, each transgenic tobacco plant can produce up to one million seeds (Daniell et al., 2001a).

Recombinant proteins expressed in plants are capable of attaining their natural confirmation such as disulphide bonding which E. coli cannot do. This has been demonstrated with the expression of CTB within transgenic tobacco chloroplasts (Daniell et al., 2001b). GM1 binding assays have demonstrated this since in order for CTB to bind GM1 receptor it must form a pentamer which are held together by disulphide bridges.

Finally, as stated earlier it is quite costly and time consuming to purify recombinant proteins from E. coli. As an example, insulin purification from E. coli by chromatography accounts for
30% of the production cost and 70% of the set up cost (Petrides et. al., 1995). If expressed in crop plants purification would not be necessary, but purification from a plant such as tobacco would be required but it would be less expensive and free of any human pathogens. It must be pointed out that the use of tobacco to express recombinant protein is used as a model system, because of the already established protocols and the less time required to create transgenics. This technology will eventually be utilized on crop plants for pharmaceutical production.

**Advantages of Chloroplast Genetic Engineering over Nuclear transformation**

Chloroplast genetic engineering reached a crucial point when it was demonstrated that transformation with the Bacillus thurigiensis (Bt) cry2Aa2 operon accumulated 46.1% total plant protein, this was the highest protein total ever accumulated in plants by genetically engineering plants and also the first time an operon/polycistrionic message was expressed (DeCosa et al., 2001). The resulting protein had accumulated in intact cuboidal crystals. This was achieved because of the ability of the plastid to express prokaryotic genes due to the plastids prokaryotic nature as opposed to the eukaryotic nature of nuclear transformed plant expressed proteins. Therefore, plastid transformation not only possesses the ability to accumulate proteins in large amounts but several other advantages also exist. Although transgenic chloroplasts are present in the pollen, the foreign gene cannot escape to other crops due to the inability of plastid DNA to be passed onto the egg cell. The majority of crop plants plastid genes are strictly maternally inherited thus gene containment. Even though the pollen in plants have been demonstrated to contain metabolically active plastids, their DNA is lost during pollen maturation and therefore is not present in the next generation (Nagata et al., 1999).
The chloroplast is an excellent place to accumulate foreign proteins or their biosynthetic products that otherwise would be harmful if present in the plant cytoplasm and therefore no physiologically damaging effect is observed in a plastid transgenic plant (Bogorad, 2000). This has been observed in the accumulation of the cholera toxin B subunit (CTB), when it accumulated in large quantities in chloroplast transgenic tobacco plants no physiologic side effect was observed (Daniell et al., 2001b). Although very small quantities of LTB an E. coli toxin with similar structure, function and immunochemistry was shown to be toxic when expressed via the nuclear genome because of its presence within the cytoplasm (Mason et al., 1998). Also, trehalose which is utilized by the pharmaceutical industry as a preservative was shown to be toxic when present in the cytoplasm but was non toxic when accumulated within plastids (Lee et al., 2003).

Foreign genes introduced into the plastid genome are integrated into spacer regions by homologous recombination of chloroplast flanking sequences present in the bombarded vector. Therefore, site-specific integration is directed to a specific region this eliminates the position effect observed in nuclear transgenic plants. Thus, as opposed to nuclear transgenics which vary in expression therefore must be screened to determine which line is highly expressed, plastid transgenic lines express the same level of foreign protein within the range of physiologic variations (Daniell et al., 2001b). Yet another advantage of plastid transformation is no gene silencing is observed, which is a concern for nuclear transformants. It has been demonstrated that no gene silencing in plastid transformants at the level of transcription has been observed despite the accumulation of 169-fold higher than observed in nuclear transformants (Lee et al., 2003).
Also, no gene silencing at the translational level have been observed despite the massive accumulation of foreign protein of levels up to 46% (DeCosa et al., 2001).

**Use of Tobacco for Production of Recombinant Proteins**

Tobacco although a non food or non feed crop has been demonstrated to be an ideal crop for chloroplast genetic engineering, due to its ease of genetic manipulation. Tobacco has been shown to be an excellent biomass producer with in excess of forty tons of fresh leaf weight per acre based on multiple mowing seasons and a prolific seed producer with up to a million seeds per plant, which demonstrates why this crop is an ideal candidate for large scale commercial production. This model has been extensively utilized for the large scale production of therapeutic proteins. Kusnadi (1997) has estimated that the cost of production of recombinant proteins in tobacco leaves will be approximately fifty fold lower than in E. coli fermentation systems currently in use.

Utilizing chloroplast transformation technologies researchers have been able to hyper express valuable pharmaceutical products such as elastin derived polymers a wound healing agent (Guda et al., 2000), Human Serum Albumin (Fernandez San-Millan 2003), Maganin, a broad spectrum topical agent, systemic antibiotic, wound healing stimulant, and potencial anticancer agent (DeGray et al., 2001), interferons, cytokines that interfere with viral replication, insulin-like growth factor, Guy’s 13 monoclonal antibody that inhibit *Streptococcus mutans* to protect against dental caries (Daniell 2004) and subunit vaccine antigens such as CTB (Daniell et al., 2001b).
Agronomic traits conferred via chloroplast genetic engineering technology include the following: Insect resistance by expressing the Cry2Aa2 gene (DeCosa et al., 2001), herbicide resistance by expressing glyphosate a broad spectrum herbicide that non selectively eliminates weeds by inhibiting 5-enolpyruvylshikimate-3-phoshate synthase (EPSPS) (Daniell et al., 1998), the antimicrobial peptide MSI-99, offers protection against prokaryotic organisms such as *Pseudomonas aeruginosa* a major plant pathogen, the BADH gene which confers salt tolerance and the yeast trehalose phosphate synthase gene which confers drought tolerance (Lee et al., 2001) to name a few.

**Bioencapsulation of Recombinant Proteins Delays Their Breakdown**

Bioencapsulation of recombinant proteins within plant cells offers protection from immediate digestion by the acidic environment of the stomach (Walmsley and Arntzen, 2000, Yu and Langridge, 2001). The current evidence for bioencapsulation is demonstrated for vaccines. In human clinical trials, plants cells have been shown to be sufficient for subunit vaccine protection from digestion which lead to an effective systemic and mucosal response without the need of an adjuvant (Tacket et al., 2000, Kapusta et al., 1999, Tacket et al., 2000, Walmsley and Arntzen, 2000). Thus, demonstrating that plant cells are capable of protecting recombinant proteins from digestion and the recombinant proteins are able to maintain their structure.

**Crop Species That Have Been Stably Transformed via Plastid Genome**

Daniell et al., (1998) proposed the use of a “universal vector” that contained the DNA flanking sequence from one plant species, be used to transform another unknown genome sequence of another species. Following this concept both the potato and tomato plastid genomes have been
successfully transformed utilizing flanking sequences from tobacco. The one drawback was that the transformation efficiency was low if the flanking sequence DNA homology was not high. The currently established tobacco plastid transformation vector that contains the tobacco endogenous flanking sequence (100% homology) have been demonstrated to be extremely efficient. But, when Petunia flanking sequences were used to transform the tobacco plastid genome the transformation efficiency was much lower than if the tobacco flanking sequence was used (DeGray et al., 2001).

To Date, tobacco has been by far the most extensively utilized plastid transformation system. This is due to its ease of genetic manipulation due to an already established system. More recombinant proteins have thus far been produced in tobacco by nuclear and chloroplast transformation than all the other plant species combined. Tobacco has been shown to be the ideal plant species for scale up. A single tobacco possess the ability to produce upwards of one-million seeds and one-acre of tobacco plants can produce more than forty metric tons of leaf material in multiple harvests per year (Cramer et al., 1999). Also, the machinery that is required for the harvesting and processing of tobacco leaves is already in place for commercialization. Plus, an alternate use for this hazardous crop could be demonstrated to be quite effective. Kusnadi, (1997) has estimated that the production of recombinant proteins such as biomaterials, within tobacco plants would be 50-fold more cost effective than the current method of E. coli fermentation systems. Tobacco is also a not used as human food or fed to animals. Thus, it would make senses to utilize this crop for pharmaceutical protein production. Containment has been an issue, but this can be overcome by harvesting the plants before reproductive structures appear and ensure complete containment of the transgene.
Potato has recently been transformed via the plastid genome by optimizing the bombardment procedure and selection process (Sidorov et al., 1999). The vector that was used was originally designed for tobacco plastid transformation. The flanking sequence identities are approximately 98% identical. The vector utilized the aadA gene for selection and GFP for visual screening. Following bombardment and the regeneration of shoots, the potato plants were quantified for expression of GFP and shown to be 5% total soluble protein (tsp) within the green tissues and 0.5% tsp within the microtubers. The researchers explained that the difference in expression within the microtubers was due to the lower plastid DNA copy number coupled with the lower transcriptional/translational activities seen in plastids of this tissue type (Sidorov et al., 1999). The plastid transformation in potato plants is a positive step for this technology since this was the first time it was successfully applied to a crop plant with encouraging results, which can be applied to other crop plants. But since 1999 no useful traits have thus far been engineered in the potato plant, this may be due to the very low transformation efficiency. Therefore, the transformation efficiency in potato must be greatly improved.

Tomato as of 2001 has been genetically engineered via the plastid genome. The research was painstakingly slow, taking 2 years from the date of bombardment of leaf material until the harvest of the first plastid transformants. The transformed tomato plants were able to produce fruits that contained viable seeds which transmitted the transgene in a maternally inherited fashion as was expected (Ruf et. al., 2001). The success of tomato plastid transformation produces many possibilities, the foremost being the ability to engineer a crop that is suitable for
human consumption without cooking. But, since the initial publication, no useful traits have thus far been engineered, again this may be due to the low efficiency of transformation.

In the two examples given above the plastid genomes were transformed using fully mature plastids as the recipients of transgene and regenerated via direct organogenesis. However, in order to effectively transform more economically useful crops it is essential to be able to transform non-green cells that contain proplastids and be able to regenerate these plants by somatic embryogenesis and achieve homoplasmy without subsequent rounds of regeneration. This requires the knowledge of the regulatory sequences in non-green plastids and the ability to reach homoplasmy without repetitive regeneration. Thus, using carrot and cotton as two model systems, the transformation of non-green plastids and the regeneration of these transgenic plants by somatic embryogenesis has recently been demonstrated (Kumar et al., 2004a,b). These results have demonstrated a procedure for plastid transformation of several useful crop species monocots such as corn, wheat, barley, and sugarcane. But the current limitation in achieving plastid transformants in these crop species is the need for their plastid genomes to be fully sequenced. This is currently underway, and once this has been accomplished many more crop plants will be successfully transformed via their plastid genomes.

Carrot is one of the world’s most important vegetables for animal and human health. The crop offers a great source of vitamin A, vitamin C, fiber, and carbohydrates. Carrot is a biennial plant whose life cycle is completed in 2 years. The initial year the edible taproot appears, the following year the plant flowers in response to cold weather. Therefore, carrot is an environmentally safe crop and is protected against gene flow in the pollen and thus seeds. Therefore, there is no
concern about contamination of food crops by transgene flow that is of concern to the public. Kumar and colleagues (2004a,b) was the first to report stable and highly efficient plastid transformation in both carrot and cotton utilizing non-green tissues as explants and regenerated by somatic embryogenesis. The flanking sequences used for particle bombardment were doubled in length to 2kb on both sides in order to maximize transformation efficiency by homologous recombination. The transformation of the carrot plastid genome was demonstrated to be just as efficient as the transformation of tobacco. This was the first plant species in which stable plastid transformation was achieved with the use of explants and stable transgene expression in proplastids. The above examples were included in order to demonstrate that crop plants that are regularly consumed by humans could be transformed via their plastid genomes. This project could some day be extended to these crop plants in order to facilitate oral delivery of pharmaceutically significant therapeutic proteins such as insulin.

**Transgenic Plants as Bioreactors for the Production of Valuable Pharmaceuticals**

There are currently several arguments for the utilization of transgenic plants for the purpose of producing pharmaceuticals in mass quantity. Plant systems have been shown to be more economical than the currently utilized industrial facilities using fermentation. The technology currently exists for the harvesting and processing of plant and plant products on a mass scale, and the expensive purification requirement can be eliminated if the plant tissue containing the recombinant protein is consumed as food such as, orally delivered pharmaceutical proteins. When plants are directed to target proteins within stable intracellular compartments such as the chloroplast, or expressed directly in the chloroplast, the amount of pharmaceutical protein can
approach industrial scale levels. Furthermore, the health risks due to contamination with human pathogens and toxins can effectively be minimized.

An astonishing feature of chloroplast expression is the ability to accumulate large quantities of recombinant proteins as much as 46% total soluble protein observed for the CRY protein (DeCosa et al., 2001). The first stably expressed pharmaceutical protein in transgenic chloroplasts was GVGVP, a protein based polymer that is used for several medical applications such as: prevention of post surgical adhesions and scars, wound coverings, artificial pericardia, tissue reconstruction and programmed drug delivery (Guda et al., 2000).

Another pharmaceutically important protein expressed via the chloroplast genome was human somatotrophin (Staub et al., 2000), this protein is utilized to treat hypopituitary dwarfism in children, turner syndrome, chronic renal failure, and HIV wasting disease. Human somatotrophin was able to reach levels up to 7% tsp, and upon purification it was demonstrated that the protein was properly disulfide bonded and structurally identical to native human somatotrophin. The results demonstrated that the chloroplast possess the proper machinery to fold eukaryotic proteins and add disulfide bonds in the correct positions. This research demonstrates the ability of the chloroplast to hyper-express human blood proteins.

Another human blood protein, human serum albumin (HSA) which accounts for approximately 60% of the protein in serum is the most widely distributed intravenous protein and is currently prescribed in multigram quantities for the replacement of blood volume in cases of trauma and various other clinical situations. HSA is a 66.5kDa monomeric protein that contains 17 disulfide
bonds. The current worldwide need for HSA is in excess of 500 tons, valued at $1.5 billion. There is currently no system for mass producing HSA that is commercially feasible. The current estimates by industry officials, suggest that the cost effective yield for pharmaceutical production is 0.1mg of HSA per gram of fresh weight (Farran et al., 2002). Fernandez San-Millan and colleagues (2003) was able to express HSA, utilizing chloroplast transformation of tobacco plants achieving levels up to 11.2% tsp. The high levels of expression did not affect the phenotype of the plant and the HSA protein could be visualized in inclusion bodies that formed, also, the chloroplast had increased in size in order to accommodate the foreign protein. These inclusion bodies not only protected HSA from proteolytic degradation but also facilitated single step purification by centrifugation. Since the HSA protein has a chemical and structural function complex studies are required to fully demonstrate the functionality of chloroplast derived HSA (Watanabe et al., 2001).

Interferon alphas (IFNα) a human cytokine of the immune system, which acts by interfering with viral replication and proliferation has been expressed within the chloroplast of transgenic tobacco plants. They are recognized as potent enhancers of the immune response and have been used for many clinical treatments. The specific subtype, IFNα2B was approved in 1986 by the Food and Drug Administration to treat patients with hairy cell leukemia. IFNα 2B has also been proven beneficial for the treatment of various viral and malignant diseases. The currently marketed IFNα2B is produced in E.coli expression systems which require in vitro processing and purification and the current cost of treatment is $26,000 per year. Just recently, IFNα2B has been used to treat patients suffering from West Nile virus and the cost of the 2-week treatment is $2500 per patient. IFNα2B is currently administered by injection and severe side effects are
common. Up to 20% of these patients develop anti-IFNα2B antibodies which are undesirable because this lessens the effectiveness of the treatment. The side effects have been linked to the route of administration and dosage parameters. It has been shown that oral administration of human IFNα is therapeutically effective for the treatment of various infectious diseases. Thus, the production of IFNα2B in transgenic tobacco chloroplasts for oral delivery may eliminate some of the negative side effects. This can be quite effective since bioencapsulation by plant cells can protect recombinant proteins from the low pH encountered within the gut and plant expressed pharmaceuticals are pathogen free. Upon expression of IFNα2B in transgenic tobacco chloroplasts the recombinant protein achieved levels up to 18.8% tsp. the functionality of the chloroplast derived IFNα2B was demonstrated by its ability to protect HeLa cells against the cytopathic effect of encephalomyocarditis virus (Falconer, 2002). The chloroplast derived IFNα2B was demonstrated to be just as effective as the commercially produced Intron A. Therefore, the expression levels that were achieved and the demonstration of proper functionality make this ideal for purification and further use in orally deliverable IFNα2B for preclinical trials (Daniell 2004).

Human insulin like growth factor 1 (IGF-1) is produced in the liver and is a potent multifunctional anabolic hormone. The IGF-1 protein possesses three disulfide bonds and functions in the regulation of cell proliferation and differentiation in a variety of human cell and tissue types. It plays a role in the renewal of tissue and its repair. Persons with cirrhosis take approximately 600mg of IGF-1 a year at a cost of $30,000 per mg (Nilsson et al., 1991). The current production platform is E. coli although it is not produced in its mature form since E.coli lacks the ability to add disulfide bonds. IGF-1 was recently produced in transgenic tobacco
chloroplasts and it accumulated up to 32% tsp of the recombinant protein (Ruiz, 2002). The aforementioned examples of production of pharmaceuticals of clinical significance, demonstrate that transgenic plants offers an attractive alternative to the current methods of production. Besides the ability to deliver these pharmaceuticals orally, the low cost of purification without the need for post-translational modifications make chloroplast genetic engineering technology the wave of the future. For example, developing countries that cannot afford medicines due to the high cost associated with the production and storage could simply be given seeds to grow if the transgenic crop can be consumed orally and quantities of pharmaceutical remain constant within the plant. One major concern about plant derived pharmaceuticals is the degradation within the stomach when take orally, but this is overcome because plant cells contain cellulose and sugars, which provide protection to the recombinant protein and allows a slow gradual release of the pharmaceutical also known as bioencapsulation (Mor et al., 1998). These examples demonstrate the feasibility of utilizing chloroplast transgenic technology for the production of clinically important recombinant proteins.

**CPL Introduction**

All plants normally produce pHBA, albeit usually in small quantities. Radioisotope studies with *Lithospermum erythrorhizon* suggest that this compound is derived from the CoA ester of \( p \)-hydroxycinnamic acid (pHCA-CoA) through a \( \beta \)-oxidation-like mechanism (Löscher and Heide, 1994). However, earlier studies with the same plant species (Yazaki et al., 1991) and carrot cell cultures (Schnitzler et al., 1992), supported a cleavage mechanism that occurs via intermediacy of \( p \)-hydroxybenzaldehyde. Notwithstanding our current ignorance of the detailed plant biosynthetic pathway, a number of studies have shown that it is possible to dramatically elevate
pHBA levels in plants through metabolic engineering. Indeed, two different “single-enzyme”
pathways have been described, both involving microbial proteins that have no known plant
counterparts. One of these enzymes is chorismate pyruvate-lyase (CPL). Its substrate is
chorismate, an important branch point intermediate in the shikimate pathway, which is largely, if
not entirely, synthesized in chloroplasts and other types of plastids (Hrazdina and Jensen, 1992;
Herrmann and Weaver, 1999). Using nuclear transformation and a chloroplast-targeted version
of \textit{E. coli} CPL (“TP-UbiC”), Heide and co-workers were able to generate transgenic tobacco
plants that had leaf levels of pHBA that were three to four orders of magnitude greater than wild
type plants (Siebert et al., 1996). Virtually all of the compound (>98%) accumulated in the
vacuole as two glucose conjugates - a phenolic glucoside and a glucose ester - that both
contained a single glucose molecule attached to the aromatic hydroxyl or carboxyl group of
pHBA by a 1-O-\textendash-D linkage. Glucose conjugation, which takes place in the cytosol via distinct
UDP-glucosyltransferases (Lim et al., 2002), is required for vacuolar uptake and recent studies
have shown that the pHBA phenolic glucoside and glucose ester are transported by separate
carriers that have different mechanisms (Bartholomew et al., 2002). The highest leaf content of
pHBA glucose conjugates in nuclear-transformed tobacco plants was 0.52 % dry weight (DW),
which is equivalent to 0.24 % “free” pHBA after correcting for the mass of the attached glucose
residue. Nevertheless, the CPL-expressing tobacco plants were perfectly healthy and
phenotypically indistinguishable from non-transformed control plants (Siebert et al., 1996). TP-
UbiC was subsequently expressed in tobacco cell cultures (Sommer and Heide, 1998; Sommer et
al., 1998) and hairy root cultures of \textit{Lithospermum erythrorhizon} (Sommer et al., 1999), and the
results were remarkably similar. In none of these studies, however, did the leaf content of pHBA
glucose conjugates exceed 1.0% DW. Although these experiments are very encouraging and
constitute a major breakthrough for plant metabolic engineering, a commercially viable pathway for pHB̈A production in plants will require 10- to 20-fold higher levels of product accumulation.

The other microbial protein that has been used to elevate pHB̈A levels in tobacco is the 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) of *Pseudomonas fluorescens* (Mayer et al., 2001). This enzyme catalyzes a sequential hydration and retro-aldol cleavage of p-hydroxycinnamoyl-CoA (pHCA-CoA) - a key intermediate in the phenylpropanoid pathway - to yield p-hydroxybenzaldehyde (Gasson et al., 1998; Mitra et al., 1999). In tobacco, the vast majority of the aldehyde was oxidized by endogenous plant enzymes that remain to be elucidated, and the metabolic fate of the resulting pHB̈A was glucosylation and vacuolar uptake similar to the situation with CPL. The HCHL route to pHB̈A production in plants is technically less complicated than the CPL pathway from a metabolic engineering perspective since pHCA-CoA is synthesized in the cytosol. Hence, there is no need to target the foreign protein across a lipid bilayer into an intracellular organelle to gain access to its substrate. In HCHL-expressing tobacco plants, pHB̈A glucose conjugates in leaf tissue accumulated to ~2.9% DW, which translates to ~1.3% non-glucosylated pHB̈A (Mayer et al., 2001). Although this represents a 5- to 6-fold improvement over CPL-mediated pHB̈A production, the HCHL-expressing tobacco plants suffered a severe depletion of phenylpropanoids that resulted in numerous phenotypic abnormalities, including leaf chlorosis, stunted growth, male sterility, and altered lignin content. Clearly, the ability of these plants to replenish pHCA-CoA could not keep pace with the massive diversion of this compound to pHB̈A glucose conjugates. These results strongly suggest that substrate availability, *not* enzyme activity, sets an upper threshold on pHB̈A accumulation in tobacco plants that hyperexpress HCHL, at least in leaf tissue.
Although it is conceivable that the current limitation for CPL-mediated pHBA production in plants is carbon flux through the plastid shikimate pathway, other explanations seem more likely. One potential area for improvement may lie in the design of a better chloroplast targeting sequence to achieve higher levels of enzyme activity in the intracellular compartment of interest. Indeed, there was a positive correlation between CPL specific activity and accumulation of pHBA glucose conjugates in several of the studies cited above. Furthermore, in none of these studies was there any indication that saturation had been achieved with respect to enzyme. Most naturally occurring chloroplast proteins are nuclear-encoded and synthesized on cytosolic ribosomes as larger molecular weight precursors with a cleavable N-terminal transit peptide. Following chloroplast protein import, the transit peptide is proteolytically removed to yield the “mature” polypeptide. Although hundreds of transit peptide sequences are now known, our ability to manipulate them to achieve optimal chloroplast targeting of a foreign protein is, at best, still a matter of trial and error. Simply attaching a chloroplast transit peptide to the N-terminus of the passenger protein does not guarantee success. Even very subtle changes in the vicinity of the natural cleavage site of the Rubisco small subunit precursor can lead to diminished chloroplast uptake (Wasmann et al., 1988) and/or aberrant proteolytic processing (Robinson and Ellis, 1984; 1985).

It is occasionally observed that chloroplast uptake of foreign proteins can be improved by including a small portion of the mature N-terminus of the transit peptide donor in addition to the transit peptide and scissile bond (Van den Broeck et al., 1985; Schreier et al., 1985). However, this approach is still associated with a high degree of unpredictability that is inextricably linked
to the passenger protein. For example, in an attempt to improve plant pHBA production, Heide’s group fused the Rubisco small subunit transit peptide and first 21 amino acid residues of the “mature” polypeptide to the N-terminus of *E. coli* CPL (Sommer and Heide, 1998; Sommer et al., 1998). Surprisingly, however, this manipulation resulted in much lower levels of pHBA than the artificial fusion protein that was used in the earlier studies, TP-UbiC. One of the obvious pitfalls of this strategy is that cleavage of the transit peptide results in a CPL variant with an unnatural N-terminal extension that could have detrimental effects on catalytic activity and/or enzyme stability in the chloroplast compartment.

An alternate approach to express foreign proteins or enzymes that function within chloroplasts would be to directly integrate and express transgenes via the chloroplast genome. Such an approach has additional advantages including high levels of transgene expression (Daniell et al., 2002; Devine and Daniell, 2004), transgene containment (Daniell, 2002) and multi-gene engineering (DeCosa et al., 2001; Ruiz et al., 2003; Daniell & Dhingra, 2002; Lossl et al., 2003). Moreover, the chloroplast is an ideal compartment to accumulate certain proteins or their biosynthetic products that would be harmful if they were accumulated in the cytoplasm (Daniell et al., 2001a; Lee et al., 2003; Leelavathi et al., 2003; Daniell et al., 2004a). Chloroplast transformation also eliminates positional effects that are frequently observed with nuclear transformation and no gene silencing has been observed so far at the level of transcription (Lee et al., 2003) or translation (DeCosa et al., 2001). Consequently, independent chloroplast transgenic lines have very similar levels of foreign gene expression and there is no need to screen hundreds of transgenic events. Because of these advantages the chloroplast genome has been engineered
to confer several useful agronomic traits, including herbicide resistance (Daniell et al., 1998), insect resistance (McBride et al., 1995; Kota et al., 1999), disease resistance (DeGray et al., 2001), drought tolerance (Lee et al., 2003), salt tolerance (Kumar et al., 2004a) and phytoremediation (Ruiz et al., 2003). The chloroplast genome has also been used in molecular farming to express human therapeutic proteins (Guda et al., 2000; Staub et al., 2000; Fernandez San-Milan et al., 2003; Leelavathy and Reddy, 2003; Daniell et al., 2004b,c), vaccines for human (Daniell et al., 2001a; 2004c; Daniell, 2004) or animal use (Molina et al., 2004) and biomaterials (Guda et al., 2000; Lossl et al., 2003). Although most of these studies were done in tobacco, highly efficient stable plastid transformation of major crop species has recently been reported for carrot (Kumar et al., 2004a), cotton (Kumar et al., 2004b) and soybean (Dufourmantel et al., 2004).

The present study is the first attempt to utilize chloroplast transformation for metabolic engineering to generate plants that accumulate large amounts of a small aromatic compound of significant commercial value, pHBA. Towards this goal we have stably integrated the unmodified *E. coli* *ubiC* gene into the tobacco chloroplast genome and studied the consequences of hyperexpression of this enzyme in leaf and stem tissue. Another distinguishing feature of this work is that the pHBA levels reported are based on separately processed “total leaf” and “total stalk” material that was obtained from fully mature first- and second-generation plastid-transformed plants. It should also be emphasized that this is the first time that stalk production of pHBA has been examined in either CPL- or HCHL-expressing tobacco plants. Finally, our experiments provide unequivocal evidence that the current limitation for CPL-mediated pHBA production in nuclear-transformed plants is achieving high enough levels of enzyme activity in

25
the chloroplast compartment – the site of chorismate synthesis – and that this obstacle is easily circumvented using plastid transformation. Until now high-level production of pHBA in plants has been elusive.
CHAPTER TWO: MATERIAL AND METHODS

Vector Construction of 5’UTR-CTB-Proinsulin

The vectors termed pLDW-5CP (5’UTR-Cholera Toxin B subunit-Human Proinsulin) and pLDW-5CPTP (5’UTR-Cholera Toxin B subunit-Plastid Proinsulin) were initially bombarded into petit Havana tobacco plants. It was determined after several western blots that the expected fusion protein was not being expressed. Sequencing of the bombarded constructs showed a point deletion, which lead to a frameshift mutation within the CTB gene. This explained the inability for the western blots to show proper expression of the fusion protein. Therefore it was necessary to correct this point deletion by PCR mutagenesis.

PCR Primer design

Following sequencing, it was determined that at position 185 within the CTB gene an A had been deleted. PCR primer sets termed for 5c repair

CAAAAAAAAGCAATTGAAAGGATGAAGGATACCC and rev 5c repair
GGGTATCCTTCATCCTTCAATTGCTTTTTTTGTGAATCTATATGTTGACTACC

TGGTAC were designed and ordered from Invitrogen. These primer sets were designed to insert an A at position 185 and substitute an A for G without changing amino acid sequence in order to create an mfeI restriction site that was later used for screening clones. The two primer sets were designed with the QUICK PRIME and DNA STAR softwares. The primers were diluted to a 100µM and stored at -20°C.
**PCR Mutagenesis of 5’UTR-CTB by Splicing**

The mutagenesis was performed by PCR followed by sequence verification of the previously spliced together products. The left PCR reaction contained 0.6µl of template DNA (5’UTR-CTB), 5.0µl of 10x Pfu buffer, 1.0µl of 10mM dNTP, 1.0µl of 5c upper primer that lands upstream of the 5’UTR, 1µl of rev 5c repair primer, 0.5µl Pfu DNA polymerase and 40.9µl dH2O for a total reaction volume of 50µl. The right PCR reaction contained 0.6µl of template DNA (5’UTR-CTB), 5.0µl of 10x Pfu buffer, 1.0µl of 10mM dNTP, 1.0µl CTB lower primer that lands on the 3’ end of the CTB gene, 1.0µl for 5c repair primer, 0.5µl Pfu DNA polymerase, and 40.9µl of dH2O for a total reaction volume of 50µl. The PCR reactions were carried out using the Perkin Elmer Gene Amp PCR system. The amplification was performed as follows: 94°C for 5 min. followed by 25 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 45s, followed by a 7min. elongation step at 72°C.

The final PCR products were then mixed together for the following PCR in order to splice the two products together. The PCR reaction utilized 5.0µl of both the R-5cr-PCR and the L-5cr-PCR as DNA templates, 5.0µl of Pfu 10x buffer, 1.0µl of 10mM dNTP, 1.0µl of 5’UTR upper primer, 1.0µl of CTB lower, 0.5µl Pfu DNA polymerase and 31.5µl dH2O for a total reaction volume of 50µl. 25 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 45s, this was preceded by a 94°C denaturation and followed by 72°C for 5min for extension. This PCR reaction sliced the two initial PCR products together.
**PCR Product ligation into PCR2.1 Topo cloning vector**

The final PCR product was then cloned into the PCR 2.1 vector with the TOPO cloning kit (Invitrogen). The ligation reaction was performed in sterile 1.7µl microcentrifuge tubes as follows: 4.0µl of PCR product, 1.0µl of 10x ligation buffer, 0.5µl of TOPO PCR 2.1 vector, 1.0µl of T4 DNA polymerase and 3.5µl dH2O. Then the ligation mixture was incubated overnight at 14°C.

**Transformation into One Shot competent cells**

The microcentrifuge tubes which contained the ligation reaction was centrifuged briefly and placed on ice. 50 µl of competent cells were thawed and 2µl of the ligation reaction was added to the vial containing competent cells. The mixtures were mixed and incubated on ice for 30 min. followed by heat shock treatment for 30s at 42°C in a water bath and immediately relocated to ice for 2min. 250µl of SOC was added to the sample and then placed in an incubator at 37°C for 1hr at 225RPM in a rotary shaker. Following incubation the sample was placed at 4°C. The sample was then spread out on two LB plates one with 50µl, the other with 200µl, which contained 20µl of X-Gal, 20µl of IPTG, and 50µg/ml of Kanamycin. The plates were placed in a 37°C incubator overnight. Fifteen white colonies were then isolated the following day and grown in 15ml tubes containing 5ml LB and 100µg/ml ampicillin. The samples were then placed in a rotary shaker/incubator for overnight growth at 37°C and 225RPM.
**Mini-Prep plasmid isolation of PCR 2.1 by Alkaline Lysis Method**

The following day 1.5ml of each of the fifteen cultures was transferred to 1.7ml microcentrifuge tubes and centrifuged for 1 min at 13,000rpm and 4°C in an Eppendorf 5810R centrifuge. After centrifugation the supernatant was discarded and 150µl of 4°C stored solution 1 (50mM glucose, 25mM Tris Cl (pH 8.0), 10mM EDTA (pH 8.0) and 100µg/ml RNase freshly added) was added. The pellet was then resuspended by vortexing followed by addition of 150µl of freshly prepared solution 2 (100µl 2N NaOH, 100µl of 10% SDS and800µl dH₂O) then mixed by inverting centrifuge tube 6-8 times, followed by addition of 150µl solution 3 (60ml 5M potassium acetate, 11.5ml glacial acetic acid, and 28.5ml dH₂O) then mixed gently by inverting 6-8 times. The mixture was then centrifuged at 13,000 RPM for 10 minutes at 4°C. The supernatant was then transferred to a fresh 1.7ml centrifuge tube, without transferring any white floculant material which consists of bacterial chromosomal DNA, SDS, and membrane proteins. Then 900µl of ice cold 95% ethanol was added to the supernatant and pipetted up and down. The supernatant was centrifuged at 13,000 RPM at 4°C for 10 minutes. Following the spin the supernatant was discarded, being careful not to dislodge the pellet which contained the plasmid DNA. Four hundred µl of ice cold 70% ethanol was added and centrifuged for 5 minutes. The ethanol was then discarded and the pellet was dried in the speedvac on medium setting for 5 minutes. The dried pellet was resuspended in TE (pH 8.0). Plasmid concentration and quality of the isolated DNA was determined by spectrophotometer. DNA was then stored at -20°C. The plasmid DNA was the run on a 1% agarose gel for 40 minutes at 80 volts to visualize the plasmid DNA.
Sequencing

The sequencing reaction was performed by the University of Central Florida. The primers used for sequencing were M13 forward and reverse. The sequence data showed that clone number 9 was the one that contained the correct inframe sequence.

Cloning 5CF9 into the pLD Vector

The clones 5CF9, 5CP frameshift and 5CPTP frameshift were then digested with SalI (NEB) for 1 hour at 37°C in separate reactions. This was followed by using the PCR purification kit (Qiagen) to get pure digested plasmid DNA. The plasmid DNA was subsequently digested with SmaI for 1 hour at 25°C, this digested fragment contained the 5’UTR-CTB. The digest was performed on all three of the previously described clones. Then the plasmid DNA was run on a 1% agarose gel followed by using the gel extraction kit (Quiagen) to excise the ~520 bp fragment from the 5CF9 clone and the remaining vector after the ~520 frameshift fragment was excised from the other two clones. The 520 bp fragment was then ligated into the frameshift fragment removed pLD-CtV vectors 5CP frameshift and 5CPTP frameshift which contain human proinsulin and codon optimized proinsulin.

Preparation of Ultra Competent Cells by the Rubidium Chloride Method (Kumar and Daniell 2004)

Ultra competent cells were prepared utilizing the rubidium chloride method. E. coli XL1-Blue MRF<sup>ab</sup> (Stratagene), a disabled non pathogenic tetracycline resistant strain, which has a history of safe laboratory use due to its inability to survive in the environment, was used to prepare the
ultra competent cells. The E. coli glycerol stock was streaked on a LB agar plate containing 12.5 µg/ml tetracycline and incubated at 37°C overnight. A single isolated colony was picked and grown in 5ml of Psi broth containing 12.5 µg/ml tetracycline and incubated at 37°C for 12-16 hours in a rotary shaker set at 225 RPM. One ml of the overnight culture was inoculated in 100ml of Psi broth and was incubated at 37°C for two hours in a shaker set at 225 RPM. The O.D. was checked at 550 nm after two hours and subsequently after each half hour. The culture was allowed to grow until it reached an optical density of 0.48. The culture was then placed on ice for fifteen minutes. The cells were the pelleted by centrifugation at 3000g/5000rpm for five minutes in a sorval centrifuge. The supernatant was removed and the pellet was resuspended in 40 ml of ice cold TFB-I solution. Once again the cells were pelleted at 3000g/5000rpm for five minutes. The supernatant was discarded and the cells resuspended in 4 ml of TFB-II solution and immediately placed on ice for fifteen minutes. This suspension was then divided into 100µl aliquots then quick freeze in liquid nitrogen and stored at -80°C.

**Transformation of Ultra Competent Cells**

One hundred µl aliquots of competent cells were removed from the -80°C freezer and immediately thawed on ice and transferred to a 14ml falcon tube. Approximately 100ng (1µl) of ligated DNA was added to the competent cells resting on ice and mixed gently by tapping. The mixture was incubated on ice for thirty minutes with gentle swirling after the first fifteen minutes. The mixture was then incubated at 42°C for forty-five seconds and immediately placed on ice for two minutes. Nine hundred µl of TB broth was added to the cells and incubated for forty five minutes at 37°C in a shaker rotating at 225 rpm. The cells were then pelleted by
centrifuging at 13,000rpm for one minute. The supernatant was discarded leaving one hundred µl followed by resuspension of the cells. Two samples of 25 µl and 75 µl of the suspension was the equally distributed on agar plates with both ampicillin and spectinomycin for selection. This was followed the next day by picking colonies and growing in 50 ml TB broth with the appropriate selection agent. This was then followed by kit midi prepping (Bio-Rad) the plasmid DNA the next day in preparation for particle bombardment.

**Particle Bombardment of pLD-5’UTR-CTB-Human Proinsulin and pLD-5’UTR-CTB-Codon Optimized Proinsulin**

**Preparation of Gold Particles**

50 mg of gold particles (0.6µm) and 1ml of 70% ethanol was placed in a 1.7ml microcentrifuge tube. The mixture was vortexed for 4 minutes and then incubated for 15 minutes at room temperature. The mixture was then quick pulsed in a centrifuge in order to pellet gold particles. Supernatant was then discarded and 1ml of H₂O was added to the particles and vortexed. The particles were allowed to settle for 1 minute and once again pulse centrifuged for a few seconds, the supernatant was then discarded. The previous step was repeated three times. Fifty percent glycerol was then added to the gold particles to a concentration of 60 mg/ml, and the particles were then stored at -20°C (Kumar and Daniell 2004).
Tobacco Tissue Culture Media

The regeneration media of plants (RMOP) contained one packet of Murashige Skoog basal salt mixture, thirty grams of sucrose, 100mg myoinositol, 1ml of benzylaminopurine (BAP: 1mg/ml), 100µl of α-naphtalene acetic acid (NAA: 1mg/ml), 1ml of thiamine hydrochloride (1mg/ml), and topped off to 1 liter with water. This media was used for regeneration (shoot formation) and selection of transgenic plants following particle bombardment. The pH was adjusted to 5.8 with 1N KOH. Six grams per liter of phytagar was added to the media and autoclaved followed by cooling down then adding 5ml of spectinomycin (100mg/l) and pouring into deep Petri dishes. The MS0 (Murashige Skoog zero vitamins) media which is used to promote rooting was prepared with the following ingredients: 30g sucrose, one packet of MS basal salt mixture, and topped off to one liter with water. The pH was set at 5.8 with 1N KOH and six grams per liter of phytagar were added before autoclaving. After cooling down 5ml of spectinomycin (100mg/L) was added then the media was poured into jars (Kumar and Daniell, 2004).

Bombardment Procedure

The particle bombardment was performed as previously described by Daniell (1987). For particle bombardment a sterile environment is required. Therefore all the equipment necessary was thus sterilized prior to use. The stopping screens, macrocarrier holders, forceps, Whatman filter paper, and kimwipes were all sterilized by autoclaving. The macrocarriers and the rupture disks were sterilized by immersion into 95% ethanol overnight followed by drying in the hood. Fifty µl of
gold particles placed in a microcentrifuge tube followed by ten µl of DNA (1µg/ µl). Fifty µl of 2.5M CaCl$_2$, 20 µl of 0.1M spermidine-free base was added sequentially to the mixture in order to ensure tight binding of the DNA to the gold particles. Vortexing was done following the addition of each component to ensure proper mixing of components and binding of DNA to the gold particles. The mixture was then vortexed for twenty minutes at 4°C. Two hundred µl of absolute ethanol was added to the vortexed mixture at room temperature and followed by a quick centrifugation at 3000rpm for thirty seconds, the supernatant was then removed and this wash procedure was repeated four more times. The gold particles were then resuspended in thirty µl of absolute ethanol. The gold particles bound to DNA were then placed on ice to be used within the next two hours. From aseptically grown tobacco plants Nicotiana tabacum circum variety Petit Havana, dark green leaves were removed from the young plant growing in MS0 media within Mason jars were placed on thin Petri dishes (100 x 15) which contained RMOP media without a selection agent and on top of a Whatman filter paper. The leaves were placed abaxial side up. The gene gun utilized was a Bio-Rad PDS-1000/He device which was sterilized with 70% ethanol prior to use. The macro carriers were placed on the macrocarrier holders. The gold particle mixture sitting on ice was vortexed and 5 µl of gold mixture were placed on the topside of the macrocarrier. Vortexing in between each addition of gold mixture is essential to prevent the particles from settling to the bottom. The rupture disk, stopping screens and macrocarrier holders containing the macrocarriers along with a single leaf were set in place and secured to proceed with the bombardment. The gene gun and the vacuum pump were turned on and the helium tank was set at the open position and the valve turned so that the pressure reached 1350psi. The vacuum within the gene gun was allowed to reach 28psi and held briefly and then fired, the fire switch was held until the sound of the rupture disk being penetrated was heard.
(1100psi). Following the shot the vacuum was released and the Petri dish containing the leaf removed and covered. After all samples were bombarded they were covered in aluminum foil for forty-eight hours to allow for recovery.

**Tissue Regeneration and Selection**

Following two days of incubation in the dark the leaves were transferred to deep Petri dishes (100 x 25) which contained the shoot inducing media RMOP along with 500 µg/ml of spectinomycin for selection. After approximately four-six weeks the shoots that appeared were cut into 5mm² pieces and transferred to freshly made RMOP/Spectinomycin plates in order to go through another round of selection. Following the cuttings DNA was isolated from the shoots and PCR analysis performed in order to screen out mutants that may have arisen and also to confirm integration. After another four-weeks on secondary selection, the shoots were transferred to Mason jars which contained MS0 plus 500 mg/l spectinomycin this was the third selection (Kumar et al., 2004).

**Extraction of DNA from Plants**

In order to isolate DNA from plants the Qiagen DNeasy plant kit was used. One-hundred mg of plant material was weighed out placed in a microcentrifuge tube, frozen with liquid nitrogen the ground up using a micropestle followed by the addition of buffer AP1 and 4 µl of RNase A (stock 100 mg/ml). The mixture was incubated at 65°C for ten minutes mixing every two-three minutes by inverting the tube. Then 130 µl of AP2 was added to the lysate, vortexed then incubated for
five minutes on ice. This was followed by centrifugation at 13,000rpm for five minutes and the supernatant was then transferred to the Qiashredder spin column sitting in a two ml collection tube which was then centrifuged at 13,000rpm for two minutes. The flow through was then transferred to a 1.7ml centrifuge tube and 1.5 volumes of buffer AP3/E were added to the lysate and mixed immediately. Six-hundred fifty µl of the mixture was applied to the DNeasy mini spin column and centrifuged for one minute at 8000rpm. The flow through was discarded and the collection tube reused to repeat the previous step with the remaining sample. The collection tube and supernatant was then discarded and the column placed in the supplied 2 ml collection tube. Five-hundred µl of buffer AW was added to the column and centrifuged for one minute at 8000rpm. The flow through discarded and the collection tube reused. The DNeasy column was washed again with 500 µl of buffer AW followed by centrifugation for two minutes at 13,000rpm followed by discarding supernatant. Once again the column was centrifuged for 2 minutes in order to speed dry the membrane. The column was then transferred to a new 1.7ml centrifuge tube and then 100 µl of preheated (65°C) buffer AE was added directly into the DNeasy mini spin column membrane. The column was allowed to sit for five minutes before centrifugation for one minute in order to elute the DNA. The DNA was then stored at -20°C until needed.

Confirmation of Integration by PCR

In order to confirm integration into the chloroplast genome and also to screen out mutants, PCR was performed using the primer pairs 3P (5’-AAAACCCGTCTCTAGTTCGGATTGC-3’) -3M (5’-CCGCGTTGTTTCATCAAGCCCTACG-3’) which confirms the left flank. In order to
confirm the integration of the right flank which contains the gene of interest the PCR primers 5P
(5’-CTGTAGAAGTCACCATTGTTGTGC-3’) 2M (5’-TGACTGCCCACCTGAGAGCGGACA-3’) were utilized. A 50 µl PCR reaction consisted of
the following: 100ng of plant DNA, 5 µl of 10x buffer, 4 µl of 2.5mM dNTP, 1 µl of each primer
(final concentration 0.5 µM), 0.5 µl Taq DNA polymerase and up to 50 µl with water.
Amplification was performed under the following conditions: denaturation for five minutes at
94°C, followed by 25 cycles using the temperature sequence: 94°C for 1 minute, 65°C for 1
minute and 72°C for 1.5 minutes (3P3M) 2.0 minutes (5P2M) followed by a 7 minute extension
at 72°C. Five µl of the PCR product was then run on a 1% agarose gel for confirmation. A
positive control consisted of a previously confirmed plant DNA and the negative control
consisted of wild type plant DNA.

Detection of Properly Expressed Proteins

Protein Extraction from E. coli

E. coli XL1-Blue cells that contain the tetracycline resistance gene within their genome was
transformed with pLD-5’UTR-CTB-human Proinsulin (5CP) and pLD-5’UTR-CTB-codon
optimized proinsulin (5CPTP) were grown in 5 ml of TB with ampicillin (100µg/ µl) and
tetracycline (50 µg/ µl) at 37°C for 16 hours. Untransformed E. coli cells were also grown to
serve as a negative control. The buffer and gels used in this study were made from the protocols
in SDS-PAGE buffer System (Laemmli, 1970). Eight hundred µl of cultured cells were
centrifuged for one minute at 13,000 rpm. The supernatant was discarded from the pelleted E.
coli which was then washed with one ml of 1x Phosphate-Buffered Saline (PBS : 140mM NaCl, 2.7mM KCl, 4mM Na₂HPO₄, 1.8mM KH₂PO₄, pH 7.2). The pellet was resuspended in PBS followed by centrifugation at 13,000rpm for one minute. Supernatant was then discarded. Fifty µl of 1x PBS was added and mixed in order to resuspend the pellet. A mixture containing 425µl of 2x loading buffer, called SDS reducing buffer (1.25ml 0.5M Tris-HCl, pH 6.8, 2.0ml of 10% SDS, 0.2ml of 0.5% bromophenol blue, 2.5ml glycerol, with sdH₂O up to 9.5ml, and 25µl of β-mercaptoethanol was prepared. Equal amounts of sample and loading buffer were taken and boiled for five minutes then 15µl was immediately loaded onto 13% polyacrylamide gels.

**Protein Extraction from Plant Samples**

In order to determine if CTB and proinsulin is being translated within the chloroplast transgenic tobacco plants, extracts from the Petit Havana plants were taken. The steps performed were described previously in Daniell et al., (2004). One-hundred mg of tobacco leaf tissue was weighed out and ground with a mortar and pestle to a fine powder then placed inside a 1.7 ml microcentrifuge tube. Two-hundred µl of extraction buffer which consists of 100mM NaCl, 10mM EDTA, 200mM Tris-HCl-pH8, 0.05% Tween 20, 0.1% SDS, 14mM BME, 400mM sucrose, and 2mM PMSF was added to the samples and mixed for 5 minutes with a micropestle. The samples were then centrifuged at 13,000rpm for 10 minutes in order to obtain a supernatant which contained soluble proteins. Ten µl of the soluble extract was then mixed with 10 µl of the sample loading buffer which contained BME. The samples were then boiled for 5 minutes and loaded onto a 13% SDS-PAGE gel.
In order to detect the presence of CTB and proinsulin within the E. coli crude extracts and the crude plant extracts of which had been transformed with the two constructs 5CP and 5CPTP the samples were run on an SDS-PAGE gel which were made with the following: 30% Bis-Acrylamide (Bio-Rad) solution in the ratio 37:5:1, the resolving gel contained the resolving buffer (5M Tris-HCl, pH 8.8), the stacking gel contained the stacking buffer (0.5M Tris-HCl pH 6.8) which was used at the top of the gel in order to improve resolution, 10X electrode buffer (30.3g Tris Base, 144g glycine, 10g SDS, topped off to 1L with water, then stored at 4°C), 10% SDS, N,N,N,N’-Tetra-methyl-ethylene diamine (TEMED Bio-Rad), and 20% Ammonium Persulfate (APS) made fresh. The 13% resolving gel was made with the following ingredients: 4.3ml of 30% Bis-Acrylamide, 2.5ml of resolving buffer, 3.1ml deionized water and 100 µl of 10% SDS in a 14ml falcon tube. Immediately before casting the gel 40 µl of 20% APS and 10 µl of TEMED was added and then the entire mixture was added between the two vertical thin glass plates (Mini-Protean 3 gel system Bio-Rad) leaving approximately 1.5cm at the top to allow for the stacking gel. After polymerization (~15 minutes) the 4% stacking gel was added which contained the following: 1.3 ml 30% Bis-Acrylamide, 2.5 ml of stacking buffer, 6.1 ml deionized water, 100 µl 10% SDS and immediately before pouring 40 µl 20% APS and 10 µl TEMED. The 4% stacking gel is poured on top of the resolving gel then the comb is inserted. Allowing 15 minutes for polymerization the comb is removed forming wells and the gel inside the glass plates is placed into a vertical PAGE apparatus then 1x Electrode buffer is added. Twenty µl of the extract which contains the SDS-reducing buffer immediately following boiling was loaded with the following: untransformed E.coli (control), transformed E.coli which contain 5CP and 5CPTP,
CTB protein standard (for CTB probed membranes), and the transgenic tobacco plants which contain 5CP and 5CPTP. The gel was run at 50 volts until the samples left the stacking portion then ran at 80 volts until the dye reached the bottom of the gel which was approximately 2.5 hours.

**Transfer and Immunoblotting**

Following the separation of proteins on the SDS-PAGE gel, the separated proteins were transferred to a 0.2 µm Trans-blot nitrocellulose membrane. This was done by electroblotting, utilizing the Mini-Transfer Blot Module for one-hour at 80 volts in transfer buffer which consisted of 360ml 10x Electrode buffer, 360ml methanol, 0.18g of SDS, and 1080ml of water. Following the transfer to nitrocellulose membrane, the membrane was blocked with P-T-M which is made up of PBS (12mM Na$_2$PO$_4$, 3mM NaH$_2$PO$_4$-H$_2$O , 145mM NaCl pH 7.2), 0.5% Tween 20, and low fat powdered dry milk. This was followed by immunoblotting with anti-proinsulin monoclonal antibody at a 1:20,000 dilution and on a separate membrane anti-CTB polyclonal antibody at a 1:3,750 dilution in P-T-M for 2 hours. After the primary antibody blotting, the membrane was washed with water, then the secondary antibody was added, for the anti-insulin antibody membrane it was goat derived anti-mouse IgG antibody conjugated to horse radish peroxidase (HRP) and the anti-CTB membrane the secondary was mouse derived anti rabbit with Alkaline Phosphatase (AP) conjugated. The blots were then washed thrice with PBST (PBS with Tween 20) for 15 minutes each. Followed by a single wash with PBS for 10 minutes. Immediately following the final wash Lumiphos WB (Pierce) was added to the AP conjugate, and the SuperSignal West Pico (Pierce) which contains luminol enhancer and stable peroxide.
which was mixed together in a 1:1 ratio for the HRP conjugate and each was incubated at room temperature for ~5 minutes. The chemilluminescence was detected with a X-ray hyperfilm following exposure to the membrane at varying time intervals then developed in a film processor.

**Autoradiography**

The blots were placed within a film cassette and taken to the dark room which contained the film processor. Utilizing the red safe light the x-ray film was placed over the immunoblots and closed for a varying duration. Followed by, running the film through the film developer.
Southern Blot Analysis

Restriction Enzyme Digestion of Plant DNA

Total plant DNA from initial bombarded generation tobacco plants and wild type untransformed tobacco plants was extracted following the Qiagen DNeasy Plant Mini Kit protocol. Following extraction 2µg of total plant DNA was digested with AflIII (NEB) the digestion reaction consisted of: 2 µg plant DNA per sample, both transgenic and non-transgenic (control), 2 µl of buffer #3 (NEB), 2 µl of AflIII, and topped of to 20 µl with water. The reaction was placed in a 37°C incubator overnight.

Agarose Gel Electrophoresis

The total 20 µl reaction from transgenic lines, non-transgenic (negative control) and probe (positive control) DNA was loaded onto a 0.8% agarose gel. The positive control probe was generated by digestion of pLD 5CP with MfeI (NEB) and NotI (NEB) approximately 360 base pair (bp) for gene specific and the 810bp flanking sequence probe was generated by BamHI (NEB) and BglII (NEB) digestion of pUC-ctv plasmid. The agarose gel was run for 3 hours at 50 volts. Following the completion of the run the gel was reduced in size by removing the wells and cutting just above the running dye with a razor blade. The gel was then depurinated by immersion in depurination solution (0.25M HCl) for 15 minutes. The gel was then washed twice with water for 5 minutes each, and then transferred to transfer buffer (0.4N NaOH, 1M NaCl) which contains 16g of NaOH, 58.4g NaCl and filled up to 1L with water, for 20 minutes. Four
pieces of whatman filter paper and the nylon membrane was cut to the size of the gel. The nylon membrane was then presoaked in water then transfer buffer for 5 minutes. Two thick sponges and two filter papers were placed in a glass dish filled 2/3rds of the way with transfer buffer. The gel was the placed on top of these with the wells facing down. The bubbles were then removed. Parafilm was placed along the edges in order to prevent lateral transfer. Then the nylon membrane was placed on top of the gel followed by two more filter papers. A stack of paper towels cut to fit neatly over the nylon membrane was then added along with two small weights (~500g) in order to promote capillary action. The capillary transfer was allowed to sit overnight, the following day the membrane was washed twice with 2X SSC (3M NaCl, 0.3M sodium citrate, water, the pH was adjusted to 7.0 with 1N HCl and topped off with water to 1L) for 5 minutes each. The nylon paper was then allowed to dry on a whatman filter paper for ~5 minutes, then cross linked using the Bio-Rad GS Gene Cross Linker at the C3 setting (150mjoules).

Generating Flanking Sequence and Gene-Specific Probes

The flanking sequence probe was generated by digesting pUC-ctv plasmid which contains the chloroplast flanking site-directed regions \textit{trn}I and \textit{trn}A. The digestion was performed using the enzymes BglII and BamHI releasing the 810 bp fragment used for determination of homoplasy or heteroplasy. Following the 37°C overnight digestion the DNA was run on a 1.0% agarose gel and the 810bp fragment was cut out of the gel and purified using the Qiagen gel extraction kit. The gene specific probe was obtained by MfeI/NotI digestion of 5CP and incubated at 37°C
overnight and run on an agarose gel, and then the 350bp fragment was gel extracted utilizing the gel extraction Qiagen kit.

**Labeling the Probes**

Forty-five µl of probe DNA was denatured by incubation within a microcentrifuge tube for 5 minutes at 94°C. After 5 minutes the samples were placed on ice for 3 minutes and then pulse centrifuged. The denatured probe was then added to the Ready-To-Go DNA labeled beads (dCTP, Amersham Biosciences) which contained all the necessary enzymes and mixed by flicking. Five µl of $\alpha^{32}\text{P}$ (50 µCi) was added to the tube and the mixture was then incubated for 1 hour at 37°C.

The Quant G-50 Micro Columns (Amersham) were prepared by resuspending the resin by vortexing, the cap was then loosened and the bottom plug removed. The column was then placed in a microcentrifuge tube with the cap removed and centrifuged for 1 minute at 3,000 rpm. The collection tube was discarded along with the supernatant and the column was then transferred to a new tube. The DNA probe was added to the center of the resin and centrifuged at 3,000 rpm for 2 minutes, the column was then disposed of. The mixture was then aliquoted into 50 µl samples and 3ml of Opti-Fluor was added to each sample. The activity of the radiolabeled probe was measured using a Beckman LS 5000TD. The two samples including a blank which contained only 3ml Opti-Flour were placed inside the holder. The readings were taken by using the auto-read mode of the machine. The amount of probe to be used was determined by calculating the
amount of probe needed to yield $2.5 \times 10^6$ cpm/2ml. The amount of probe was determined by the following calculation: the reading value (502050) was equal to $0.50 \times 10^6$ cpm/µl, multiplied by 50 µl of total volume of sample for a total of $35 \times 10^6$ cpm/µl. Since 5ml of hybridization was used, $6.25 \times 10^6$ cpm was required, therefore $6.25 \times 10^6$ cpm divided by $0.7 \times 10^6$ cpm is equal to 12.5 µl of labeled probe needed.

**Prehybridization and Hybridization**

Quik-Hyb solution (Stratagene) was mixed and incubated at 68°C for 10 minutes. The blot was then placed into a hybridization bottle with the front of the membrane facing inwards then 5ml of the Quik-Hyb prehybridization was added in order to block the membrane. The bottle containing the membrane and solution was incubated at 68°C for 1 hour inside a Fisher Biotech Hybridization Incubator.

One-hundred µl of salmon sperm DNA was added to the labeled probe and heated for 5 minutes at 94°C. One ml of the Quik-Hyb solution was drawn out of the heated bottle and added to the labeled probe solution. The content was then placed back in the bottle containing the membrane and incubated at 68°C for 1 hour. Following the incubation the probe containing solution was discarded into the liquid radioactive waste container.

The membrane was then washed twice with 50 ml of solution one (2x SSC and 0.1% SDS) for 15 minutes at room temperature. The membrane was then washed twice again with preheated to 60°C solution two (0.1X SSC and 0.1% SDS) and incubated at 60°C in order to increase
stringency. The radiolabeled membrane was then placed in a film cassette and taken to the dark room where an x-ray hyperfilm was placed on top of the membrane and the film cassette closed. The cassette containing the blot was then place into a black bag that does not allow light to enter and placed in the -80°C freezer overnight. The following day the cassette was removed from the freezer and allowed to thaw for one hour then the film was removed inside the darkroom and put into the film processor for development.

CPL Materials and Methods

Construction of the CPL Chloroplast Transformation Vector

The E. coli ubiC gene (GenBank accession number M92628) was amplified from genomic DNA of strain W3110 (Campbell et al., 1978) using two PCR primers. Primer 1 (5’-CTA CTC ATT gaa ttc aca tgt CAC ACC CCG CGT TAA C-3’) introduces an AflIII site at the CPL start codon and an EcoRI site that is immediately upstream from the AflIII site. The underlined bases hybridize to the target gene, while the bold lower case letters indicate the two restriction sites. Primer 2 (5’-CAT CTT ACT ggc gcc gct TTA GTA CAA CGG TGA CGC C-3’) hybridizes at the other end of the gene and introduces a NotI site just past the CPL stop codon. The 100-µl PCR reactions contained ~100 ng of genomic DNA and both primers at a final concentration of 0.5 µM. The other reaction components were provided by the GeneAmp PCR Reagent Kit (Perkin Elmer) according to the manufacturer’s protocol. Amplification was carried out in a DNA Thermocycler 480 for 22 cycles, each comprising 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; there was a 7-min extension period at 72 °C after the last cycle. The PCR product
was cut with EcoRI and NotI, and the resulting fragment was ligated into pBluescript SK (+/-) (Stratagene) that had been digested with the same enzymes. The ligation reaction mixture was used to transform *E. coli* DH10B electrocompetent cells (GibcoBRL) using a BTX Tranfecter 100 (Biotechnologies and Experimental Research Inc.), and growth was selected on LB media that contained ampicillin (100 µg/ml). Transformants that contained plasmids with a CPL insert were identified by restriction digestion analysis after cleavage with EcoRI and NotI, and a representative plasmid with no PCR errors was selected for further manipulation. The plasmid containing CPL coding region was digested with AflIII and NotI and ligated into the plasmid pBSKs+HSA, that contains the *psbA* promoter/5’UTR which had been digested with NcoI and NotI. The resultant plasmid (PpsbA-CPL) was cut with EcoRI and NotI and cloned into similarly digested pLD-CtV to yield the final construct that was used for chloroplast transformation, pLDK.

**Particle Bombardment and Selection of Chloroplast Transformants**

Chloroplast transformants were obtained as previously described (Daniell, 1997). Nicotiana tabacum cv. Petit Havana leaves were bombarded with the Bio-Rad PDS-1000/He biolistic device. The bombarded leaves were then placed in the dark for 48h, followed by cutting leaf particles into .5 cm² and placed on RMOP media which contained 500 µg/ml spectinomycin for two rounds of selection in petri dishes. Shoots were then cut and transferred to jars which contained MSO media with 500 µg/ml spectinomycin to induce root formation followed by transfer to soil.
**Confirmation of Site-Specific Integration by PCR**

Plant DNA was extracted from transgenic and wild type tobacco using Qiagen DNeasy plant mini kit. The PCR primers, AAAACCCGTCCTCAGTTGATGAC (3P) and CCGCGTTGTTCATCAAGCTTACG (3M), were used to perform PCR on transgenic and wild type plant DNA as described previously (Daniell et al., 2004d; Kumar and Daniell, 2004). PCR was carried out in a Perkin Elmer Gene Amp PCR System 2400 under the following conditions, denaturation for 5 minutes at 94°C, followed by 25 cycles using the following temperature sequence: 94°C for one minute, 65°C for one minute and 72°C for 1.5 minutes. After PCR confirmation, the selected shoots were subjected to a second round of selection on RMOP with spectinomycin (500 µg/ml).

**Southern Blot Analysis to Demonstrate Site-Specific Integration and Homoplasmy. These steps were performed essentially as previously described (Daniell et al., 2004d).**

DNA from T₁ tobacco plants was extracted using Qiagen DNeasy plant mini kit. Plant DNA (2µg) was digested with AflIII and was separated on a 0.8% agarose gel at 50V for 3 hours. The gel was soaked in the depurination solution (0.25 N HCl) for 15 minutes, and then rinsed twice in water for 5 min. The gel was then soaked in the transfer buffer (0.4 N NaOH, 1 M NaCl) for 20 minutes and transferred overnight to a nylon membrane. Following transfer, the membrane was rinsed twice with 2X SSC for 5 min, placed on a filter paper and then cross-linked using the GS GeneLinker (Stratagene). The flanking sequence probe (0.81 Kb) was obtained by BamHI/BglIII digestion of pUC-ct plasmid, which contains the chloroplast flanking sequences trnl and trnA. The CPL specific probe (0.3 Kb) was obtained by SmaI/ BamHI digestion of
pLDK. Probes were labeled with dCTP $^{32}$P using Ready-To-Go™ DNA Labeling Beads (-dCTP, Amersham Biosciences) and purified using Quant™ G-50 Micro columns (Amersham Biosciences). The membrane was then prehybridized with QuikHyb™ (Stratagene) for 60 minutes at 68°C, followed by hybridization with radiolabeled probe for 1 hour at 68°C. The membrane was then washed twice with 2X SSC in 0.1% SDS at room temperature for 15 minutes, followed by two washes with 0.1X SSC in 0.1% SDS at 60°C for 15 minutes. The radiolabeled membrane was then be exposed to Hyperfilm (Amersham Pharmacia) and developed in a Konica SRX-101A.

**Analysis of pHBA Glucose Conjugates (Performed by DuPont)**

Leaf punches (50-150 mg FW) were used to monitor pHBA levels throughout development. Young, mature, and old tissues were used for this analysis as described in the text and figure legends. Unless otherwise indicated, all steps were conducted at room temperature. The tissue was placed in a Biopulverizer H tube that contained a ceramic bead (QBiogen (Carlsbad, CA)), and 1 ml of 50% (w/v) methanol was added. The tubes were agitated for 40 s using a FastPrep FP120 tissue disrupter (QBiogen) set at 5 m/s, and the samples were then placed on a rotary shaker (400 rpm) for 1 h. Debris was removed by centrifugation and a 50-µl aliquot of the supernatant was taken to dryness in a heated Speed-Vac. The residue was dissolved in 100 µl of 5 mM Tris-HCl (pH 8) for subsequent analysis of pHBA glucose conjugates. Alternatively, the leaf tissue was extracted with 1.0 ml of 5 mM Tris-HCl (pH 8.0) using the same procedure described above. Side-by-side experiments demonstrated that both approaches yield identical results. In some experiments, fully mature tobacco plants were analyzed for “whole-leaf” and
“whole-stalk” levels of pHBA glucose conjugates. For this type of analysis, all plant material above the ground was harvested, but total leaf and total stalk material were segregated and processed individually. Tissues were lyophilized to dryness and ground to a homogeneous powder in an electrically-driven mill. Five to twenty milligrams of the dry plant material was then extracted with either 1.0 ml of 50% (w/v) methanol or 1.0 ml of 5 mM Tris-HCl (pH 8.0) using the same procedure described above.

pHBA glucose conjugates were analyzed by HPLC using a C\textsubscript{18} column [Vydac 218TP54 (The Nest Group, Inc., Southborough, MA)] that was developed at 1.0 ml/min with a linear gradient (20 min) of 0-50% methanol /0.1% formic acid; the separation was performed at room temperature. Elution of the pHBA phenolic glucoside and pHBA glucose ester were monitored at 254 nm. Authentic standards (chemically synthesized and characterized at DuPont) were used to calibrate the HPLC runs, and extinction coefficients for both compounds were accurately determined under the conditions employed. Peak areas were integrated and values obtained with known amounts of the standards that were used to quantitate pHBA glucose conjugates. After accounting for the fraction of the extract that was injected, numbers were corrected to reflect total recovery from the leaf sample analyzed. This, coupled with individual measurements of the dry weight of the plant tissue analyzed (taken from the same leaf, on the same day), enabled the expression of pHBA glucose conjugates as a percentage of total dry weight.
Determination of CPL Enzyme Activity in Leaf and Stalk Cell-Free Extracts (Performed by DuPont)

Cell-free extracts were prepared at 0-4 °C. The lyophilized leaf and stalk powders described above were the starting materials for this procedure. Approximately 10 mg of the dry powder was transferred to a 1.5-ml polypropylene microfuge tube and 650 µl of a solution containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% β-mercaptoethanol, and 75 mg/ml polyvinylpolypyrrolidone was added. Following a 15-min rehydration period on ice, the sample was hand-homogenized with a small plastic pestle. Debris was removed by centrifugation (14,000 x g, 10 min) and the supernatant was filtered through a 0.22 µm Spin-X Centrifuge Tube Filter (Costar). An aliquot of the filtrate (~200µl) was then exchanged into Buffer Q (50 mM Tris-HCl pH 7.3, 0.3M NaCl, 5 mM MgCl2, 6% (w/v) glycerol, 5 mM dithiothreitol) using the following procedure. The sample was concentrated ~10-fold using a Microcon YM-10 concentrator (Millipore) and the retentate was diluted with 200 µl of Buffer Q. This step was repeated three times to yield the final preparations that were used to measure CPL enzyme activity.

CPL enzyme activity was measured spectrophotometrically using a continuous assay that is based on the increase in absorbance at 246 nm that occurs when chorismate is converted to pHBA. Reactions were carried out at 37 °C in a quartz cuvette that contained 90 mM Tris-HCl (pH 7.6), 0.2 M NaCl, 100 µM purified barium chorismate (Siebert et al., 1994). Assays were initiated with various amounts of cell-free extract. Initial rates of product formation were used to calculate CPL specific activities (expressed as pkats per mg of protein). An empirically derived molar extinction coefficient of 11,220 M⁻¹ for pHBA at 246 nm (determined under the same
conditions) was used for these calculations. Protein concentrations were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.
CHAPTER THREE: RESULTS

CTB-Pris Results

Vector Construction of pLD-5’UTR-CTB-Human Proinsulin (5CP) and pLD-5’UTR-CTB-Plastid Codon Optomized Proinsulin (5CPTP)

The plastid codon optimized proinsulin was based on the highest translated product within the chloroplast, the psbA gene or D1 protein (photosystem II protein). It was hypothesized that the native human proinsulin sequence may be inadequate for expression (low AT: 38%). Therefore, the codon optimized plastid proinsulin gene was created by a method that utilized four overlapping oligos with a low annealing temperature (50°C). The products were then used as templates for high annealing temp (65°C) primers (Fig.1A), thus synthesizing the required 260 bp gene (Fig. 1B) (Prodromou and Pearl, 1992). The PCR product was then subsequently cloned into the PCR 2.1 vector and the sequence verified. The psbA 5’ untranslated region (UTR) was made by creating primers necessary to amplify the psbA 5’UTR from tobacco (Figure 1C), followed by cloning, and then sequence verification. The 5’UTR was then spliced together along with the cholera toxin B subunit (CTB) and codon optimized proinsulin/human proinsulin by a process that utilizes four primers and was termed by its creator, Splicing by Overlap Extension (Fig.1D) (SOEing, Horton et al., 1989). Thus, the construct now contained the 5’UTR, CTB, and a hinge region inserted by mutagenesis consisting of glycine, proline, glycine, and proline that was introduced to allow for proper folding of each protein with the reduction of steric hindrance, followed by plastid proinsulin, the final construct was termed 5CPTP. Following SalI/NotI digestion to release the fusion gene of interest, it was ligated into the pLD chloroplast
transformation vector which will be discussed more in depth later (Fig. 1E). The human proinsulin containing construct was developed in the same manner except that the primers for gene synthesis were the sequence to produce human proinsulin and this construct was termed 5CP. In the initially created constructs pLD-5CPTP and pLD-5CP it was discovered that a single base deletion causing a frameshift mutation was present within the Cholera toxin B subunit (CTB) gene. Therefore, it was necessary to correct this mutation within both the previously created constructs. This was done by PCR mutagenesis. Using the previous constructed 5CP as a template and newly made primers to amplify and correct the single base deletion, the 5’UTR-CTB portion was amplified by PCR. The PCR product once obtained was run on an agarose gel this was followed by excising the 520bp fragment with a razor blade and then using the Qiagen gel extraction kit to get the purified PCR product. The PCR product was then ligated into the Topo PCR 2.1 vector by adding dATP overhangs to the PCR product followed by a heat shock transformation and plating them on TB plates with IPTG, X-Gal and ampicillin as the selection agent. The transformed colonies were picked based on blue/white selection. The religated colonies would be blue and the colonies containing the insert would be viewed as white. After picking 10 white colonies and growing them overnight in an LB broth with kanamycin for selection, the following day they were mini-prepped by the alkaline lysis method. Restriction analysis was then done, this was performed by MfeI digestion, and this site was introduced near the mutation but did not change the translated product. Upon determination of which clones to sequence, they were sent out to determine which clone had the correct CTB sequence. Clone #9 was determined to be the corrected clone and then the plasmid from that clone was digested with SalI and SmaI, the same restriction enzymes were used on both pLD-5CP and pLD-5CPTP and the 5’UTR-CTB fragments were swapped out for the corrected ones by ligation/transform
(Fig. 1F). The 5CP and 5CPTP inserts were previously ligated into the chloroplast transformation vector, pLD which was developed previously by the Daniell laboratory (Daniell et al., 1998; Guda et al., 2000; Daniell et al., 2001b; DeCosa et al., 2001). The pLD vector contains the trnI and trnA flanking sequences utilized to facilitate homologous recombination into the inverted repeat region of the tobacco chloroplast genome (Fig: 1e). The 5CP and 5CPTP construct were expressed under the control of the psbA 5’UTR/promoter in order to achieve hyper-expression as previously demonstrated (Fernandez San-Millan et al., 2003; Dhingra et al., 2004). The 5’UTR is thought to enhance translation of proteins under its control (Eibl et al., 1999). The aadA gene confers resistance to spectinomycin in order to select for transformed shoots (Goldschmidt-Clermont 1991) and is regulated by the 16S rRNA promoter. The 3’UTR located at the 3’ end of the introduced gene confers transcript stability (Stern and Gruissem 1987). The pLD vector also possess the chloroplast origin of replication (autonomously replicating sequence) located within the trnI region which promotes replication of the plasmid following bombardment.
Figure 1 A: Codon Optimized and Human Proinsulin Gene Synthesis: Four overlapping long oligos with a low annealing temperature (50°C). The products are overlapping oligos with low annealing temperature (50°C). The products were then used as templates for high annealing temp (65°C) primers, thus synthesizing the required 260 bp gene (Prodromou and Pearl, 1992).
Figure 1 B: PCR Gene Synthesis of Human and Codon Optimized Proinsulin: Lane 1: (from bottom) MW marker, lane 2: PCR synthesized human proinsulin, lane 3: codon optimized plastid proinsulin. Demonstrating the correct fragment obtained by PCR gene synthesis for both plastid proinsulin and human proinsulin.

Figure 1 C: PCR’ing of the psbA 5’ UTR: Gel picture depicting the PCR product obtained that corresponds to the psbA 5’UTR gene.
Figure 1 D: Slicing by Overlap Extension: Process utilized to ligate 5’UTR-CTB-Plastid/Human proinsulin together. Protocol Provided by Horton et. al.,
Figure 1 E: Final Construct and Site of Integration into the Chloroplast Genome:

Depiction of ligation of 5’UTR-CTB-plastid/human proinsulin into the tobacco chloroplast transformation vector pLD and the site of integration into the tobacco chloroplast genome between the trnI and trnA genes located in the inverted repeat region.
### Figure 1 F: Following PCR Mutagenesis

The frameshift constructs below were digested with Sall/SmaI along with the repaired construct and re-ligated together in order to correct the deletion frameshift mutation present within the CTB gene.

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**E. coli Immunoblot Analysis**

E. coli crude extract from the clones that contained the 5’UTR-CTB-codon optimized proinsulin and the 5’UTR-CTB-human proinsulin were both subjected to SDS-PAGE along with untransformed E. coli that served as a control. Following immunoblot detection with the insulin antibody the correct size (~20kDa) CTB-Proinsulin fusion protein was detected in both of the clones (Fig. 2).
Figure 2: E. coli SDS-PAGE: E.coli crude extracts were taken from the clones that contained both the 5CPTP and 5CP constructs to determine fusion protein expression.
PCR Analysis to Screen for Chloroplast Transgenics

Chloroplast transgenic plants were obtained by particle bombardment as previously described (Daniell 1997; Daniell et al., 2004b; Kumar and Daniell 2004). Approximately, 10 shoots from each construct bombarded appeared after a period of 5-weeks. When the shoots appeared a portion of them were ground to a fine powder and plant DNA extracted using the Qiagen DNeasy plant mini kit. The two possible options that would allow the plants to grow on the selection media are: chloroplast transgenic and spontaneous mutants that tend to arise. The spontaneous mutants that arise are due to a mutation that arises at the level of the 16s rRNA gene. Nuclear transgenics would not express theaadA gene product due to the promoter and if they did, the expression levels would not be sufficient to be able to prosper in the high concentration of spectinomycin. Therefore, it is necessary to employ a PCR strategy in order to eliminate the unwanted shoots that arise. Two primers designated 3P and 3M were utilized in order to determine if site-specific integration had occurred via homologous recombination. The 3P primer lands within the flanking region of the native chloroplast genome, upstream of the site of integration. The other primer, 3M lands within the selectable marker geneaadA(Fig.3A). If proper integration within the tobacco chloroplast genome has occurred a 1.65kb band will be observed following PCR (Fig. 3B). Nuclear transformants and spontaneous mutants do not yield this PCR product because they lack the necessary combination of theaadA gene adjacent to chloroplast flanking sequence. A second pair of primers was used to confirm site specific integration of the two constructs. The primer 5P anneals to theaadA gene located upstream of the inserted gene and the 2M primer anneals to thetrnA gene located downstream of the inserted gene (Fig. 3A). The presence of a 2.35kb PCR product confirms site-specific integration of the gene of interest, adjacent to theaadA gene (Fig. 3C). Following PCR confirmation the shoots were once again cut into 5mm² and plated on RMOP containing plates 500 µg/ml spectinomycin for a second round of selection, this and the subsequent selection pressure is added in order to achieve homoplasmy. Following second selection, a third selection was conducted only this time the shoots were transferred to MSO rooting medium that contained 500 µg/ml spectinomycin in order to achieve a plant ready for planting into soil.
**Figure 3 A: 3P3M and 5P2M PCR Primers:** Photo illustration demonstrating the landing sites for the PCR primers 3P3M and 5P2M utilized to screen for chloroplast transgenics.
**Figure 3 B/C: PCRing of Human (5CP) and Codon Optimized (5CPTP) Proinsulin**

_T₀ transgenic tobacco plants: Fig 3B:_ PCR with 3P3M primers, lane 1 MW, lanes 2-4 (5CP), lanes 5-10 (5CPTP), Lane 11 untransformed, the correct 1.65 kb fragment is present. **Fig 3C:** 5P2M PCR, lane 1 MW, lanes 2-4 (5CP), lanes

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**Immunoblot Detection of Fusion Protein in Chloroplast Transgenic Tobacco Plants**

The tobacco plant crude extract was taken from the lines bombarded with 5’UTR-CTB-Human Proinsulin (5CP) and 5’UTR-CTB-Codon Optomized Plastid Proinsulin (5CPTP). Upon immunodetection it was seen that the expected ~20kDa fusion protein was present in the chloroplast transgenic tobacco plants bombarded with 5CP. Amazingly, the blot showed formation of monomers, dimmers, trimmers, tetramers, and pentamers which are expected to
form, but not after the addition of beta-mercaptoethanol (breaks disulfide bonds) and boiling for 5 minutes (Fig. 4A). This similar banding pattern was observed with immunodetection for both proinsulin monoclonal antibody and CTB polyclonal antibody (Fig. 4B). Why the disulfide bonds were unable to be broken is still unclear. The chloroplast transgenic lines that were bombarded with the 5CPTP construct, for unknown reasons no fusion proteins were detected with either proinsulin monoclonal antibody or CTB polyclonal antibody even though it could be seen in E.coli western blots. Therefore, further characterization was discontinued for the codon optimized chloroplast transgenic lines since initially it was thought that the low AT content (38%) of the human proinsulin gene would not be adequate for high level expression. But that was refuted when the human proinsulin immunoblots were observed, a huge amount of fusion protein was detected even after loading 7.5µl of crude extract was loaded. Quantification was then done by western blotting of the 5CP chloroplast transgenic tobacco plants. This was done by running three plant samples designated 5CP #13, #14 and M and a known quantity of purified CTB and reading them on a spot densitometer. The results revealed that 5CP-#13 contained approximately 270µg/100mg, 5CP-#14 358µg/100mg, and 5CP-M 364.8µg/100mg of plant tissue (Fig. 4B). This was a rough estimation and had to be performed since when ELISA’s were attempted on several occasions the controls always worked but no protein, for reasons still unknown could not be detected. It is also important to note that the extracts that were used for ELISA’s were also run on SDS-PAGE and the blots could be seen clearly. Also, GM1 binding assays that utilize ELISA were also unsuccessful.
Figure 4 A: Western blot analysis of 5CP and 5CPTP chloroplast transgenic, probed with proinsulin antibody: Lane 1 E.coli glycerol stock, lane 2 untransformed, lanes 3-5 5CP (13, 14, M), lanes 6-8 5CPTP (A-C) not shown since no protein expression was detected. Note dimers,
Figure 4 B: Western blot analysis of 5CP chloroplast transgenic, probed with CTB polyclonal antibody and Quantified Using a Spot Densitometer: Lane 1: 250ng of purified CTB, lane 2: 500ng, lane 3: 1000ng, lane 4: untransformed plant extract, lane 5: 5CP-#13 (358µg /100mg of plant tissue), lane 6: 5CP-#14 (270µg 100mg) and lane 7: 5CP-M (364.8µg/100mg). Note the presence of monomers, dimmers, trimers, tetramers and pentamers.
Southern Blot Analysis

The 5CP chloroplast transgenic tobacco plants were then subjected to Southern blot analysis in order to further confirm site-specific integration and to determine whether the chloroplast transgenics were homoplasmic or heteroplasmic. The gene specific probe (CTB-proinsulin) that was taken from the pLD-5CP vector by Smal/NotI digestion (Fig. 5A) (560 bp) bound to the proper transgenic plant fragment but not wild-type plant fragment when it was used as a probe (Fig. 5C) following digestion of transgenic plant DNA with AflIII (Fig. 5B), this indicates that the gene of interest was integrated into the correct region within the chloroplast genome and the untransformed plant DNA showed no such hybridization. Homoplasmy is defined as all the genomes within the chloroplast have all received the gene of interest, if not all are transgenic they are considered heteroplasmic. The flanking sequence probe which contain regions of the trnI and trnA genes was obtained by digestion of pUC-ct vector by BglII/BamHI digestion (Fig. 5A). Chloroplast transgenic and untransformed plant DNA was digested with AflIII (Fig. 5B). Upon hybridization with the flanking sequence probe, transformed chloroplasts should show ~6.4kb fragment and untransformed a 4.2 kb fragment. If the 4.2 kb fragment is not seen within the transgenic line this indicates that all the chloroplast genomes have been transformed and homoplasmy has been reached within our current limit of detection. Most of the lines tested showed only the 6.4kb fragment when hybridized to 1µg (Fig. 5D) or 2µg (Fig. 5E) of transgenic plant DNA, indicating that homoplasmy was achieved in the bombarded generation within the current levels of detection.
**Figure 5 A: Probes used for Southern blotting:** The pUC-ct vector was cleaved with BamHI/BglII to generate the 0.81kb flanking sequence probe. Gene specific probe (0.56kb) was obtained by SalI/NotI digestion of pLD-5CP vector.

**Figure 5 B: Photo Illustration Depicting Wild Type and Chloroplast Transgenics.** Untransformed tobacco plant digested with AflIII and chloroplast transgenic tobacco plant digested with AflIII. The expected size for each is depicted along with the hybridization site for the flanking sequence probe and gene specific probe.
Figure 5 C: Southern Blot Hybridization with Gene Specific Probe: Lane 1: 5CP #13, lane 2: 5CP #14, lane 3: 5CP-M lane 4: 5CP-O, lane 5: 5CP-P, lane 6: untransformed wild type. Gene specific probe CTB-human proinsulin hybridized to 1µg of plant DNA from transgenic chloroplast lines and non-transgenic plant.
**Figure 5 D: Southern Blot Hybridization with Flanking Sequence Probe:** Lane 1: untransformed wild type, lane 2: 5CP #13, lane 3: 5CP #14, lane 4: 5CP-M, lane 5: 5CP-O, lane 6: 5CP-O. Flanking sequence probe hybridized to 1µg of plant DNA from transgenic chloroplast lines and non-transgenic plant. Note the absence of the untransformed fragment in the transgenic lines which indicates homoplasmy has been achieved within detectable limits.
Figure 5 E: Southern Blot Hybridization with Flanking Sequence Probe: Lane 1: untransformed wild type, lane 2: 5CP #13, lane 3: 5CP #14, lane 4: 5CP-M, lane 5: 5CP-O, lane 6: 5CP-O. Flanking sequence probe hybridized to 2µg of plant DNA from transgenic chloroplast lines and non-transgenic plant. Note the absence of the untransformed fragment in the transgenic lines which indicates homoplasmy has been achieved within detectable limits. Similar fragment pattern was seen in both the blots for 5CP-P, may be an indication of a possible gene rearrangement.
Plant Phenotype and Maternal Inheritance

The plants transformed with the CTB-human proinsulin construct demonstrated an altered phenotype when compared to the untransformed plant (Fig. 6A). Currently, there is no explanation, although nuclear transgenics expressing CTB did demonstrate a stunted phenotype (Daniell et al., 2001). The altered phenotype when compared with an untransformed plant, demonstrated a tendency to die at an early age upon transfer to soil, stunted growth, and a long delay in flowering. The seeds were removed following pod formation and grown on 500µg/ml spectinomycin. The wild type seeds were bleached out in appearance and the chloroplast transgenics were visualized as green, (Fig. 6B) indicating that the transgenes were present and inherited in a maternal fashion in the next generation.
Figure 6 A: Photograph depicting 5CP plants: Four-month-old transgenic plants (5CP #13-14) and 1 month-old Wild Type untransformed tobacco plant.
Figure 6 B: Photograph Depicting Maternal Inheritance: The seeds from 5CP #13 chloroplast transgenic and untransformed wild type were grown on 500 µg/ml spectinomycin. Note the green appearance maintained in the transgenic seeds indicating that the transgene was transferred to the next generation.
CPL Results

Vector Construction (Partially Performed at DuPont)

Tobacco chloroplast transformation vector pLD-CtV previously developed in the Daniell laboratory (Daniell et al., 1998; Guda et al., 2000; Daniell et al., 2001a; DeCosa et al., 2001) was used to integrate the *E. coli ubiC* gene between the *trnI* and *trnA* genes which act as the flanking sequences to facilitate homologous recombination into the inverted repeat region of the tobacco chloroplast genome. The chloroplast transformation vector pLDK, which contains the chorismate pyruvate-lyase gene (*ubiC*), was expressed under the control of the *psbA* 5’UTR/promoter in order to maximize expression. It has previously been shown that genes under the control of the *psbA* promoter/5’UTR achieve very high levels of expression (Fernandez San-Millan et al., 2003; Daniell et al., 2004e, c; Dhingra et al., 2004). The 5’UTR has been hypothesized to enhance translation of proteins under its control (Eibl et al., 1999). The *aadA* gene confers spectinomycin resistance for selection of transformed shoots (Goldschmidt-Clermont 1991). The *psbA* 3’UTR located at the 3’ end of the *ubiC* gene confers transcript stability (Fig. 1A, Stern and Gruissem 1987).

PCR Confirmation of Chloroplast Transgenics

Chloroplast transgenic plants were obtained by particle bombardment as previously described (Daniell 1997; Daniell et al., 2004d; Kumar and Daniell 2004). More than 20 independent shoots tested positive for stable integration of CPL. Each shoot was subjected to several rounds of selection with spectinomycin (500 µg/ml). Nuclear transformants usually do not survive this
high level of concentration of spectinomycin due to low levels of transgene expression and are therefore eliminated in the selection process. However, in order to identify undesirable spontaneous mutations that occur at the level of the 16S rRNA gene, a PCR strategy was employed. One pair of primers were designed to determine site-specific integration of CPL into the chloroplast genome by homologous recombination (Daniell et al., 2001b). The 3P primer lands on the native chloroplast genome, upstream of the site of integration. The second primer 3M lands on the \textit{aadA} gene (selectable marker) (Fig.1A). The presence of a 1.65 kb PCR product confirms site-specific integration of transgenes into the chloroplast genome (Fig 1B). Nuclear transformants and spontaneous mutants do not yield this PCR product because they lack the necessary combination of the \textit{aadA} gene adjacent to chloroplast flanking sequence. Following the second round of selection, shoots were transferred to MSO rooting medium that contained 500 µg/ml spectinomycin.
**Figure 1**: Schematic representation of gene cassette integrated into chloroplast genome:

The map shows (a) the gene cassette integrated into the chloroplast genome and PCR primers 3P3M (expected fragment 1.65kb) landing sites. These primers were used to screen out mutants and to confirm site-specific integration. Prrn: 16s RNA promoter; aadA: aminoglycoside 3’-adenylyl transferase; psbA 5’UTR; CPL: ubiC gene; psbA 3’UTR: terminator. Figure 1b. PCR analysis of untransformed wild type and transformants. (b) 3P3M PCR Lanes: 1 MW; 2: Untransformed; 3-4: T0 lines; 5-10: T1 lines. As expected untransformed does not show 1.65kb fragment. Transformed lines with 1.65kb fragment indicates site-directed integration. (c) 5P2M PCR lanes identical to (b).
Southern blotting was performed to confirm stable integration of transgenes into the chloroplast genome and to determine their homoplasmy or heteroplasmy. Upon achieving homoplasmy, all chloroplast genomes contain the integrated ubiC gene and are hence identical. In contrast, the presence of untransformed chloroplast genomes is a clear indication of heteroplasmy. Southern blots were probed with either a gene specific (ubiC) probe or a flanking sequence probe. The ubiC gene specific probe (0.3kb) was obtained by SmaI/BamHI digestion of the pLDK plasmid (Fig. 2A). The flanking sequence probe (0.81kb) was obtained by BamHI/BglII digestion of the pUC-ct plasmid, which contains the chloroplast flanking sequences trnl and trnA (Fig. 2A). The plant DNA was digested with AflIII (Fig. 2A). Upon hybridization with the flanking sequence probe transformed chloroplasts should exhibit a 6.3kb fragment and untransformed chloroplasts a 4.2kb fragment. If the 4.2kb fragment is not seen in the transgenic line, this is an indication that all chloroplast genomes have been transformed and homoplasmy has been achieved, within the limit of detection. All transgenic lines tested positive for site-specific integration when hybridized with the ubiC probe and untransformed lines showed no such fragment (Fig. 2C). All the lines from T1 whose seeds were germinated in the presence of spectinomycin showed only the 6.3kb fragment, indicating again that homoplasmy had been achieved and maintained in subsequent generations (Fig. 2D).
Figure 2: Figure 2a/b. Southern blot analysis to determine gene specific integration and homoplasmy.

(a) The 0.3kb CPL specific fragment and 0.81kb flanking sequence fragment that were used as probes for Southern blot analysis. (b) Untransformed tobacco plant DNA digested with AflIII (expected fragment size, 4.2kb). Transformed tobacco plant DNA digested with AflIII (expected fragment size, 6.3kb).
pHBA Accumulation (Performed by DuPont)

Shown in Figure 3 is the developmental time course for pHBA accumulation in leaves for a representative T₀ plant (line 4). Leaf punches obtained from the first or second leaf from the bottom of the plant were used for this analysis, and variation between replicates was typically less than 10%. Consistent with previous observations with nuclear-transformed tobacco plants expressing a chloroplast-targeted version of CPL (Siebert et al., 1996), virtually all of the pHBA accumulated in the vacuole as glucose conjugates, a phenolic glucoside and glucose ester. Both compounds contain a single glucose molecule attached by a 1-O-β-D linkage, and are readily converted to free pHBA by acid or base hydrolysis (Siebert et al., 1996). Also similar to nuclear-transformed tobacco plants expressing CPL, the phenolic glucoside was the predominant species in mature leaf tissue of the plastid-transformed plants, accounting for 60-80% of the total pHBA at all stages of development.

Continuous Light Studies (Partially Performed at DuPont)

When grown under the normal light/dark cycle (16 h on/8 h off), the leaf content of pHBA glucose conjugates in the T₀ plant steadily increased over a three-month period to a maximum value of 15% dry weight (DW). However, there was further increase in product accumulation when the plant was shifted to continuous light. This phenomenon, which was also observed with other T₀ and T₁ plants (data not shown), supports the notion that the psbA 5’ UTR is stimulated by light (Eibl et al., 1999; Dhingra et al., 2004; Daniell et al., 2004b, e). Indeed, under conditions of continuous light the level of pHBA glucose conjugates in old leaf tissue routinely
approached ~25% DW. To put this in perspective, the latter value corresponds to a pHBA content of ~11.5% DW after correcting for the attached glucose moiety. More important, the leaf content of pHBA glucose conjugates in the plastid-transformed \( T_0 \) tobacco plants was about 50-fold higher than the best value reported for nuclear-transformed tobacco plants expressing a chloroplast-targeted version of CPL (Siebert et al., 1996).

Since product accumulation continued to increase over the span of several months it was anticipated that young leaves would have a lower content of pHBA glucose conjugates than old leaves, and this turned out to be the case. The oldest leaves at the bottom of the plant had five to six times more pHBA than the youngest leaves at the top, while samples obtained from mature green leaves half way up the stalk contained intermediate levels. Although this experiment was performed with a \( T_1 \) plant that was derived from line 4, all the plastid-transformed plants exhibited this trend. It is also interesting to note that product accumulation in the first- and second-generation line 4 plants was very similar when old leaf tissue was analyzed, reaching a maximum value of ~25% DW in both cases. This observation suggests that the line 4 \( T_0 \) plant was homoplasmic or nearly homoplasmic, and further support for this conclusion is shown in Table I. Regardless of the explanation, it is clear from the forgoing experiments that the \( E.\ coli \) ubi\( C \) gene integrated in the plastid genome was stably inherited in the second-generation plants.

Because the amount of pHBA in leaves not only depends on the age of the plant but also the age of the leaves, the leaf punch experiments described above provide no insight on perhaps the most important question from a biotechnology perspective: What is the total leaf content of pHBA in a fully-mature plastid-transformed tobacco plant? It is also of interest to know how much product
accumulates in the stalk when contemplating the commercial feasibility of a plant pHBA production platform. Surprisingly, there are no reports in the literature regarding pHBA levels in stem tissue for nuclear-transformed tobacco plants expressing either CPL or HCHL. To address these important questions, fully mature plastid-transformed tobacco plants were sacrificed and all biomass above the ground was harvested for analysis. Total leaf and total stalk material were segregated and the tissues were lyophilized to dryness and ground to a fine powder in an electrically-driven mill. Following this procedure, the homogeneous dry plant material was analyzed by HPLC for pHBA glucose conjugates.

The total leaf content of pHBA glucose conjugates for the three T₀ lines ranged from 10.9% DW to 15.2% DW. The mean value (±SE) was 13.15 ± 1.26% DW, which is roughly half the amount that was observed with leaf punches that were obtained from the oldest leaves on the plant. To determine the stability of the transgene and long-term effects on the health of the plant, four second-generation plants were subjected to the same analysis. Like the parental lines, the T₁ plants were not adversely affected by pHBA overproduction and were phenotypically indistinguishable from non-transformed control plants (Fig. 3). The two line 4 offspring had slightly lower levels of product accumulation than the T₀ plant, but these differences are probably not significant. On the other hand, there was a 50-70% increase in the total leaf content of pHBA glucose conjugates for the two line 3 descendants. The most logical explanation for this discrepancy is that the line 3 T₀ plant was heteroplasmic, while the T₁ plants were homoplasmic. Indeed, a “mixed population” of chloroplast genomes in T₀ plants is frequently
observed with plastid transformation (Guda et al., 2000; Daniell et al., 2004e), but homoplasmy is almost always achieved by the second generation.

The data also reveals the dramatic difference between leaf and stalk content of pHBA, and all the first- and second-generation plants exhibited the same trend. The mean value for pHBA glucose conjugates in total stalk material representing fully-mature plants was 2.3% DW ± 0.36% DW for the T₀ lines and 2.5% DW ± 0.05% DW for the T₁ lines. Thus, product accumulation in the stalk was 5- to 8-fold lower than corresponding leaf levels. Another notable difference between leaf and stalk production of pHBA was the glucose conjugate profile. In stem tissue, almost the entire compound was converted to the phenolic glucoside and only trace amounts of the glucose ester were detected (<5%).
Figure 3: Photo depicting transgenic plants.
pHBA Glucose Conjugate Studies (Performed by DuPont)

In *Arabidopsis*, UDP-glucosyltransferases are members of a multigene family that consists of at least 107 distinct open reading frames (Li et al., 2001; Ross et al., 2002). Moreover, recent *in vitro* experiments with purified recombinant proteins have shown that eight of these ORFs code for polypeptides that are able to glucosylate pHBA (Lim et al., 2002). Three of the enzymes only catalyze the formation of the phenolic glucoside, while the others exclusively attach glucose to the aromatic carboxyl group to form the glucose ester. These observations – coupled with the almost complete absence of the pHBA glucose ester in tobacco stem tissue and to a lesser degree leaf tissue – have very important implications for a plant-based production platform. Since the compound of interest is free pHBA and removing the glucose could be an expensive step in downstream processing, it was important to determine which glucose conjugate is easiest to hydrolyze.

There was a relative susceptibility of the pHBA phenolic glucoside and glucose ester to acid hydrolysis after 48 h at 60 °C. From this data it is clear that the glucose ester is the most acid labile species. Quantitative conversion of this compound to free pHBA occurred with as little as 0.1 N HCl, while a 5-fold higher concentration of acid was required for complete hydrolysis of the phenolic glucoside. Since both glucose conjugates and free pHBA have limited water solubility at acid pH, alkaline hydrolysis was also examined. Again, the phenolic glucoside was relatively stable under the conditions employed and complete hydrolysis required >0.5% NaOH. In marked contrast, 0.1 N NaOH was sufficient to release all of the pHBA from
the glucose ester. Based on these observations, as well as economic and environmental considerations, we conclude that the glucose ester is the conjugate of choice to make in plants, if it is feasible to alter the \textit{in vivo} partitioning of pHBA through metabolic engineering with an appropriate UDP-glucosyltransferase.
CHAPTER FOUR: DISCUSSION

CTB-Pris Discussion

Previous studies to express CTB-proinsulin fusion protein in plants was done in potato plants (Arakawa et al., 1998). Their expression level in the nuclear transgenic potato plants was 0.1% tsp. The low expression levels lead to having to feed NOD mice large amounts of raw potatoes. Here we have expressed CTB-proinsulin fusion protein within transgenic tobacco chloroplasts.

In order to make the expression of this recombinant protein available on an industrial scale, much higher levels of expression were required than the nuclear transgenics could achieve. The expression levels of CTB-Proinsulin fusion protein in chloroplast transgenic tobacco plants ranged between 270µg/100 mg and 364.8µg/100 mg of plant tissue, which equates to approximately 30% tsp. The hyper-expression of CTB-Proinsulin should make this fusion protein abundantly available for animal studies in NOD mice. The ability to achieve these levels was dependent on chloroplast transformation technology and was much more suitable than nuclear transformation for several reasons. First, the direct expression of CTB-Proinsulin within transgenic chloroplasts eliminates the possibility of detrimental effects that may occur in the cytoplasm. Secondly, the expression of the foreign gene within chloroplasts provides a safeguard for transgene containment due to maternal inheritance of the plastid genome (Daniell, 2002). Finally, because of the high copy number of plastid DNA and the complete absence of gene silencing at the transcriptional (DeCosa et al., 2001) and the translational (Lee et al., 2003) level which is often observed in nuclear transformation, plastid transformation results in much higher levels of gene expression than nuclear transformation.
Previous studies have demonstrated the protective effects of oral administration of CTB-proinsulin fusion protein against autoimmune diabetes (Bergerot et al., 1997). The data indicated that the suppression of the disease was mediated by regulatory Th2-cells and the ability of CD4\(^+\) T-cells to suppress the adoptive transfer of diabetes was explained in terms of general bystander suppression mediated by Th2 anti-inflammatory cytokines. Since T-cell regulation is a major player in mucosal immunity, oral administration of an autoantigen can be used successfully to treat autoimmune diseases in animal models with the generation of active T-cell suppression. In the NOD mouse model it has been previously demonstrated that both insulin (Bergerot et al., 1994) and CTB-proinsulin (Bergerot et al., 1997) administration induced T-cells that were able to counteract the migration of autoreactive T-cells and thus the destruction of \(\beta\)-cells.

Mucosal autoantigen administration represents a potential way to establish tolerance towards autoantigens and the prevention of autoimmune diseases. However, mucosal tolerance at present still has its limitations at present, with potential dangers that are not well understood. The obvious challenge is to determine to what extent orally administered autoantigen proinsulin elicits cytotoxic T-lymphocytes (CTLs) that are involved in \(\beta\)-cell destruction and how such CTL can be controlled. It is already established that insulin is the primary target antigen for \(\beta\)-cell destructive CTLs in NOD mice. In order for the prevention of type 1 diabetes by oral delivery to be safe and effective, researchers must find ways to avoid the induction of CTLs and other harmful immune mediators. This must be accomplished before autoantigens are administered orally to individuals predisposed to developing type 1 diabetes.
The current study demonstrates the expression CTB-Proinsulin within transgenic chloroplasts and this was verified and quantified by western blot analysis with both anti-proinsulin and anti-CTB antibodies. PCR analysis confirmed site specific integration of the transgene. This was further verified by Southern blot analysis using a gene specific probe for hybridization. Homoplasmia was demonstrated by Southern blot analysis utilizing the trnI/trnA flanking sequence probe for hybridization in the T₀ plants tested. Although the plants exhibited a stunted growth phenotype, they were able to flower and set seed. The seeds were separated from the pods and grown on 500µg/ml spectinomycin to demonstrate the transmission of the transgene to the following generation. Animal studies have been performed by the Dr. Raheleh Ahangari and Dr. Mohtashem Samsam of the Daniell lab, they are still in the preliminary stages and the results look promising. They have thus far demonstrated that the CTB-Proinsulin producing plants suppress insulitis and clinical symptoms of diabetes in NOD mice. They will continue to perform studies in order to accumulate data which could possibly explain how and what is exactly occurring to induce tolerance/bystander suppression.

In conclusion, treatment with antigen specific therapy holds the promise of focusing the protective immune response while being able to limit the pathologic and metabolic consequences for these types of therapies. Its use may have limitations as to the situations in which the immune response has not fully progressed to clinical disease. With the methods to diagnose type-1 diabetes at an early stage and other autoimmune disorders before disease manifestation, offers the best test of whether this type of approach can be successfully utilized for the prevention and thus the development of type 1 diabetes.
CPL Discussion

Previous attempts to elevate pHBA levels in green plants using the *E. coli ubiC* gene have relied on nuclear-transformation for expression of the foreign protein. Using this approach in tobacco, pHBA glucose conjugates accumulated in leaf tissue to a maximum value of 0.52% DW, which is at least a 1000-fold increase over non-transformed control plants (Siebert et al., 1996). Although these results are quite impressive from a metabolic engineering perspective, a commercially-viable, plant-based production platform for pHBA will require much higher levels of product accumulation. The potential advantages of using plastid-transformation for CPL-mediated pHBA production in plants are manifold and obvious. First, direct expression of foreign genes in the plastid genome provides an additional safeguard for transgene containment (Daniell, 2002), since it is widely believed that plastid DNA is maternally inherited in most plant species. Second, CPL’s substrate chorimate is largely, if not entirely, synthesized in chloroplasts and other types of plastids (Sommer and Heide, 1998 and references therein). Third, using nuclear-transformation it is necessary to target CPL to plastids by fusing a chloroplast transit peptide to its N-terminus. As already noted, this manipulation is potentially associated with numerous complications that could result in sub-optimal levels of CPL enzyme activity in the compartment of interest, thus setting an artificial upper limit to pHBA production in plants that is easily circumvented with plastid transformation. Finally, because of the high copy number of plastid DNA and complete absence of gene silencing mechanisms that are routinely encountered when nuclear transformation is employed (DeCosa et al., 2001; Lee et al., 2003), plastid transformation results in much higher levels of transgene expression than nuclear transformation.
To test the hypothesis that pHBA production in nuclear-transformed plants is currently limited by CPL enzyme activity in the compartment of interest, not chorismate per se, the unmodified *E. coli ubiC* gene was integrated into the tobacco plastid genome. Thus, the foreign protein was directly expressed in the intracellular organelle where its substrate is synthesized. Using this approach we were able to achieve levels of pHBA glucose conjugates in “old” leaf tissue that exceeded 25% of the total dry weight when the plants were grown in continuous light. This is a 50-fold increase over the maximum value reported for nuclear-transformed tobacco plants expressing the same enzyme (Siebert et al., 1996). More important, the highest CPL enzyme activity that was measured in leaf tissue extracts that were prepared from the plastid-transformed plants was 50,783 pkat/mg of protein; the latter value was obtained from line 3-B, a T₁ plant. Based on the molecular mass of CPL (~18,800 Da) and the turnover number of the purified recombinant protein determined with the enzyme assay that was used in the present study (~2.8 sec⁻¹), the CPL content of the leaf tissue extract was approximately 35% of the total soluble protein. In sharp contrast, the highest CPL enzyme activity reported for nuclear-transformed tobacco plants is only 208 pkat/mg of protein (Siebert et al., 1996), which is more than two orders of magnitude lower than levels achieved in the present study.

It should be emphasized that the CPL-enzyme activity noted above is the average value for total pooled leaf material from a fully-mature, plastid-transformed plant that was obtained from two independently prepared cell-free extracts that differed by <5%. The total leaf content of pHBA glucose conjugates in the same plant was 18.3% DW (Table I). Given the extraordinarily high levels of CPL enzyme activity that were achieved in the present study with plastid transformation, it is quite possible that the latter value represents the upper limit of pHBA
production in tobacco leaf at the whole-plant level, although additional studies are necessary to test this hypothesis. Nevertheless, one clear-cut conclusion from our experiments is that CPL is a much better catalyst than HCHL for plant pHBA production when the enzyme is expressed at high levels directly in the plastid compartment.

The substrate for HCHL is pHCA-CoA, a key intermediate in the phenylpropanoid pathway. The highest leaf content of pHBA glucose conjugates reported for HCHL-expressing tobacco was only 2.9% DW and the transgenic plants were extremely sick due to a severe depletion of phenylpropanoids (Mayer et al., 2001). As an apparent compensatory mechanism there was a significant increase in transcripts for phenylalanine ammonia-lyase (PAL) and several other enzymes in the phenylpropanoid pathway, including those involved in the synthesis of pHCA-CoA (Mayer et al., 2001). Elevated PAL enzyme activity also accompanied HCHL expression in sugarcane (McQualter et., 2004), but the observed 10-fold increase was not sufficient to prevent an almost complete disappearance of leaf chlorogenic acid. Although PAL catalyzes the first step in phenylpropanoid biosynthesis and plays a major role in regulating carbon flow into the pathway (Bate et al., 1994), additional flux control points occur at various downstream branches (Howles et al., 1996). Superimposed on regulation of the individual pathway enzymes is the allocation of chorismate to phenylalanine biosynthesis and partitioning of phenylalanine to PAL; chorismate and phenylalanine are branch point intermediates that are substrates for multiple enzymes. Inability to recruit carbon from the shikimate pathway to replenish phenylalanine and/or downstream phenylpropanoid intermediates in response to rapid consumption of pHCA-CoA could explain HCHL’s adverse effects and why this enzyme becomes substrate-limited at much lower levels of pHBA accumulation than CPL.
In striking contrast to the metabolic chaos described above, our plastid-transformed, CPL-expressing plants were healthy and robust, and exhibited no discernible negative phenotype despite the fact that more carbon was converted to pHBA. This result is a clear indication that flux through the shikimate pathway was able to keep pace the massive diversion of chorismate to pHBA, and still provide enough carbon for downstream intermediates that are essential for plant growth and development, including pHCA-CoA (HCHL’s substrate) and other phenylpropanoids. In bacteria, regulation of carbon flow into the shikimate pathway is largely under transcriptional control and feedback inhibition of the first enzyme in the pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP); the aromatic amino acids mediate both processes. Although there is no evidence for a feedback-sensitive DAHP in plants, a recent study suggests that reduced thioredoxin plays a role in activation of this enzyme (Entus et al., 2002). Generally speaking, it is believed that all of the plant shikimate pathway enzymes are principally regulated at the transcriptional level, and are rapidly induced by wounding, elicitors, and various environmental assaults (Schimid and Amrhein, 1994). Given these observations, it would be very interesting to know if any of the shikimate pathway enzymes and/or phenylpropanoid pathway enzymes were elevated in our transgenic tobacco plants, and if so, to what extent? Indeed, expressing CPL in plastids to create an artificial metabolic sink for chorismate and measuring transcript levels or enzyme activities could provide new insight on the regulation and complex interactions between these two pathways.

The total stalk content of pHBA was at least five times lower than the total leaf content in plastid-transformed plants, and the present study provides no explanation for this unexpected
observation. Phenylpropanoid biosynthesis in stem tissue is a high-flux pathway that is largely devoted to lignin production, and lignin accounts for ~20% of the total dry weight of the stalk. Since all phenylpropanoids are ultimately derived from chorismate, including monolignols, it is reasonable to assume that substrate availability should not be the limiting factor for stalk accumulation of pHBA. The average CPL specific activity in cell-free extracts prepared from total stalk material obtained from fully-mature line 3-B was only 8,378 pkat/mg of protein, which is 6-fold lower the corresponding leaf value. However, this result does not necessarily indicate that the process is limited by catalyst. Additional experiments using different promoters and 5’UTRs that might function more effectively in non-photosynthetic plastids are necessary to see if it is possible to achieve higher stalk levels of pHBA with plastid transformation. In this context, we have recently utilized several regulatory sequences for foreign gene expression in non-green plastids. For example, the 5’UTR of the T7 gene 10 and 3’UTR of the rps16 gene facilitated 75% transgene expression in non-green edible parts of carrots containing chromoplasts (grown underground in the dark) and 48% in proplastids, compared to the 100% value in leaf chloroplasts (Kumar et al., 2004a). Similarly, expression of the aphA-6 gene regulated by the T7 gene 10 5’UTR, capable of efficient translation in the dark in proplastids present in non-green tissues, greatly facilitated stable transformation of the cotton chloroplast genome (Kumar et al., 2004b).

Another challenge for a commercially-viable, plant-based production platform is to control the partitioning of pHBA glucose conjugates. As already indicated, the phenolic glucoside and glucose ester are both formed in the cytosol by distinct UDP-glucosyltransferases and are subsequently transported into the vacuole by different carriers. The glucose ester, however, is
exquisitely sensitive to acid and base hydrolysis, and this could have a significant impact on the cost of downstream processing in the recovery and purification of polymer-grade pHBA. The ease of hydrolysis of this compound is undoubtedly related to the fact that it is a β-acetal ester. Indeed, it has been shown that the glucose ester of sinapic acid - a structurally similar compound that is formed in cruciferous plants - has a high free energy of hydrolysis (Mock and Strack, 1993), which allows it to serve as an acyl donor in the enzyme reaction that is catalyzed by sinapoylglucose:malate sinapoyltransferase (Strack, 1982). By co-expressing CPL and a UDP-glucosyltransferase that only attaches glucose to the aromatic carboxyl group, it might be possible to partition all or most of the pHBA to the glucose ester.

In summary, pHBA is the major monomer in liquid crystal polymers (LCPs). These thermotropic polyesters have excellent properties, including high strength/stiffness, low melt viscosity, property retention at elevated temperatures, environmental resistance and low gas permeability (Figuly et al., 1996). Although LCPs could be used for a variety of new applications they are currently too expensive for widespread use, largely due to the cost of ingredients. pHBA is also the chemical precursor for parabens (short-chain alkyl esters of pHBA, “generally regarded as safe”) that are commonly used as preservatives in food and cosmetics. The Kolbe-Schmitt process (Erickson, 1982) is currently used to synthesize pHBA, this high-temperature, high-pressure carboxylation reaction is relatively expensive. Using plants as a production platform for pHBA via metabolic engineering of the plastid genome is an attractive alternative to petrochemical synthesis, since it is an environmentally sustainable process that is less dependent on non-renewable resources. Exploiting plants to produce this compound might also lower the cost of manufacture of LCPs and allow them to expand into other niches.
LIST OF REFERENCES

CTB-Proinsulin References


CPL References


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