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Multigene Metabolic Engineering Via The Chloroplast Genome

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MULTIGENE METABOLIC ENGINEERING VIA THE CHLOROPLAST GENOME

by

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ABSTRACT

The vast majority of valuable agronomic traits are encoded polygenetically. Chloroplast genetic engineering offers an alternate approach to multigene engineering by allowing the insertion of entire pathways in a single transformation event, while being an environmentally friendly approach.

Stable integration into the chloroplast genome and transcription of the *pha*A gene coding for β-ketothiolase was confirmed by Southern and northern blots. Coomassie-stained gel and western blots confirmed hyperexpression of β-ketothiolase in leaves and anthers, with high enzyme activity. The transgenic lines were normal except for the male sterile phenotype, lacking pollen. Scanning electron microscopy revealed a collapsed morphology of the pollen grains. Transgenic lines followed an accelerated anther developmental pattern, affecting their development and maturation, resulting in aberrant tissue patterns. Abnormal thickening of the outer wall, enlarged endothecium and vacuolation, decreased the inner space of the locules, affecting pollen grain and resulted in the irregular shape and collapsed phenotype. Reversibility of the male sterility phenotype was achieved by exposing the plants to continuous illumination, producing viable pollen and copious amounts of seeds. This is the first report of engineered cytoplasmic male sterility and offers a new tool for transgene containment for both nuclear and organelle genomes.

Detailed characterization of transcriptional, posttranscriptional and translational processes of heterologous operons expressed via the chloroplast genome is reported here. Northern blot analyses performed on chloroplast transgenic lines harboring seven different

heterologous operons, revealed that in most cases, only polycistronic mRNA was produced or polycistrons were the most abundant form and that they were not processed into monocistrons. Despite such lack of processing, abundant foreign protein accumulation was detected in these transgenic lines. Interestingly, a stable secondary structure formed from a heterologous bacterial intergenic sequence was recognized and efficiently processed, indicating that the chloroplast posttranscriptional machinery can indeed recognize sequences that are not of chloroplast origin, retaining its prokaryotic ancestral features. Processed and unprocessed heterologous polycistrons were quite stable even in the absence of 3'UTRs and were efficiently translated. Unlike native 5' UTRs, heterologous secondary structures or 5'UTRs showed efficient translational enhancement independent of any cellular control. Finally, we observed abundant read-through transcription in the presence of chloroplast 3'UTRs. Such read-through transcripts were efficiently processed at introns present within native operons. Addressing questions about polycistrons, as well as the sequences required for their processing and transcript stability are essential for future approaches in metabolic engineering.

Finally, we have shown phytoremediation of mercury by engineering the *mer* operon via the chloroplast genome under the regulation of chloroplast native and heterologous 5'UTRs. These transgenenic plants hyperexpress were able to translate MerA and MerB enzymes to levels detectable by coomassie stained gel. The knowledge acquired from these studies offer guidelines for engineering multigene pathways via the chloroplast genome.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

GENERAL INTRODUCTION

In the past, most plant genetic engineering approaches involved the insertion of single genes to confer new traits of defined and will understood characteristics. Traits such as herbicide resistance, disease resistance or insect resistance improved the value of our crop plants, increasing yield and decreasing cost. However, the potential of single gene genetic engineering is limited because a majority of the agronomic traits are multigenic and complex. Advancement in the understanding of complex metabolic pathways as well as the improvement in plant genetic engineering methodologies are shifting the focus of the field from single gene engineering to multigene engineering for the expression of metabolic pathways.

Plant genetic engineering is achieved by nuclear transformation or by chloroplast (plastid) transformation. Typically, plant nuclear genes are transcribed monocistronically, representing a major disadvantage when multiple genes are engineered into the nuclear genome for the expression of metabolic pathways. The expression of foreign genes via the nuclear genome can vary greatly from plant to plant due to position effect and gene silencing (Fagard and Vaucheret, 2000). In multigene engineering, having to choose the transgenic line with the best expression among many transgenic lines for the successive insertion of a second gene, or to backcross two single gene homozygous nuclear transgenic lines for the insertion of two genes in a single plant can be a very arduous task, this multipliesthe task when even more number of genes need to be inserted for the completion of a pathway. Additionally, possible segregation of transgenes during subsequent generation may disrupt the proper function of the genetically engineered pathway (Yin et al., 2004). Nevertheless, few multigene pathways have been

1

engineered using nuclear genetic engineering. Sequential sexual crossing allowed the introduction of several genes in a single plant for the expression of IgA antibodies (Ma et al., 1995) and the production of polyhydroxybutyrate (Nawrath et al., 1994; Bohmert et al., 2000). Similarly, three genes were introduced into rice to generate the biosynthetic pathway for β-carotene expression (Ye et a., 2000), this effort required seven years for completion. Recently, sequential transformation of *Arabidopsis thaliana* with three genes resulted in the production of polyunsaturated omega-3 and omega-6 fatty acids in plants (Baoxiu et al., 2004). A better way to engineer multigenes in plants is through chloroplast transformation.

Chloroplast Genetic Engineering

The technology of chloroplast transformation was concived in 1987 when Daniell and McFadden were able to transform isolated chloroplasts. Their '*in organello*' expression system was able to efficiently and in a prolonged manner transcribe, translate, and express foreign genes (Daniell et al. 1987, 1986, 1983; Daniell and Rebeiz, 1982). In 1986 Daniell et al. transformed the cyanobacterium *Anacystis nidulant* with the *E. coli* expression vector pBR322. One year later they expressed the small and large subunist of ribulose bisphophate carboxylase/oxygenase and the selectable marker chloroamphenicol acetyltransferase (CAT) in isolated etioplasts. From these preliminary studies they developed the concept of chloroplast genetic engineering (Daniell and McFadden, 1988 U.S. Patents; Daniell, World Patent, 1999). The discovery of a new transformation method called gene gun, or biolistic method, (Sanford et al. 1986) allowed chloroplast transformation without the need of chloroplast isolation and protoplast. The gene gun

method overcame the problems encountered with other methods, such as the DNA microinjection and polyethylene glycol treatment (Daniell et al., 1999), because it allows the direct targeting of DNA into the cells and into the chloroplast by means of sub-micron size particles of idle gold or tungsten. Chloroplast genetic engineering was accomplished in several phases. The transient expression of foreign genes in chloroplast was first shown in dicots with the expression of chloroamphenicol acetyl transferase (Daniell et al. 1990) and β-glucoronidase (Ye et al. 1990). These studies were followed by the first reports of transient expression in monocots (Daniell et al. 1991), where albino plants derived from anther culture were used to demonstrate sub-cellular localization of transgene product, this was the first report that unequivocally demonstrate expression of a foreign gene in chloroplast (Daniell et al., 1991). The use of the gene gun as a gene delivery method increases chloroplast transformation's efficiency 200-fold. Unique to chloroplast transformation was the development of a foreign gene expression system, which used autonomously replicating chloroplast expression vectors (Daniell et al. 1990). Stable integration of a selectable marker gene into the tobacco chloroplast genome of a higher plant (Svab and Maliga, 1993) was also accomplished using the gene gun.

Chloroplast transformation would not be possible without the development of a suitable selection method to propel the advancement of chloroplast transformation technlogy. The translational machinery of plastids is of prokaryotic origing, containing 70S ribosomes, although the plant cell is eukaryotic and contains 80S ribosomes. The 70S ribosomes are sensitive to antibiotics such as spectinomycin and streptomycin. These antibiotics block the translocation of peptidyl-tRNA from the A-site to the P-site by inhibiting the elongation process in the ribosome, which enables the protein synthesis (Brink et al., 1994). Spontaneous mutations in the coding

sequences of the 16S rRNA can confer resistance to spectinomycin and streptomycin (Svab and Maliga, 1991). Resistance to these antibiotics can also result from the insertion of the *E. coli* chimeric *aad*A gene into the chloroplast genome (Goldshmid-Clermomt, 1991). The *aad*A gene, codes for the enzyme amynoglycoside 3'- adenyl transferase which transfers the adenyl moiety of ATP to spectinomycin, inactivating this antibiotic (Daniell et al., 2001a). Thus the *aadA* gene can be used as a selectable marker for chloroplast transformation. This selection has been successful in regeneration of transgenic chloroplast that can resist up to 500 µg/ml of spectinomycin. The antibiotic selection allows for the visual selection of transformed cells that proliferate from a bleached callus and allow the transformed chloroplast and the cell to proliferate and grow. In past years, the fear of using antibiotic resistance genes for selection of transgenic crops pushed the scientific community to find a non-antibiotic selection (Daniell, 1999). A new environmentally friendly and non-antibiotic selection system has been discovered in which the betaine aldehyde dehydrogenase (BADH) was used and this gene conferd resistance to the plant toxic betaine aldehyde chloride (BA) (Daniell et al., 2001a, b) This system is completely safe given that *BADH* is a plant gene that does not confer resistance to antibiotics, while allowing for the effective selection of the transformed chloroplast (Daniell et al.2001). Other selectable markers and reporter genes such as green fluorescent proteins (*gfp* gene), aminoglysoside phosphotransferase (*aphA*-6 gene), neomycin (*npt*II gene), β-glucoronidase (*uid*A) and chloroamphenicol acetyl transferase (*cat* gene) have been used in the selection of chloroplast transformants in different species.

Only recently has the applicability of chloroplast genetic engineering for the expression of traits of agronomic and commercial value been demonstrated. High expression levels of

protein have been achieved by the expression of the foreign genes in the chloroplast of higher plants. For example, chloroplast transgenic plants expressing the *cry*IAc gene were able to kill B.t. toxin resistant insects, becoming the first report of chloroplast transformation expressing this traits (McBride et al. 1995). Later, expression of the *cry*IIA gene via chloroplast transformation produced plants that were able to kill insects that were up tp 40,000 fold resistant to B.t. (Kota et al. 1999). Finally, the introduction of the native *cry*2Aa2 operon into tobacco chloroplast lead to the formation of cuboidal crystals and the highest ever accumulation of a foreign protein in plants, 46.1% total soluble protein was observed (De Cosa et al., 2001). The hyper expression of proteins in chloroplast is due to several factors which include the high copy number of chloroplast genomes per cell (gene dosage), which can be as many as 10,000 copies per cell because mature tobacco plants can have up to 100 genomes per chloroplast and 100 chloroplast per cell (Bendich, 1987). Additionally, expression of foreign proteins in chloroplast can be enhanced by the use of chloroplast 5' regulatory secuences. Finally, proteins expressed in the chloroplast are protected from the cytoplasmic environment, including nuclear encoded proteases. Additionally, the integration of transgene into the chloroplast occurs by site-specific homologous recombination (Daniell et al., 2000), which allows the integration of the foreign gene without position effect (Bogarad, 2000) or gene silencing. The chloroplast transformation vectors are designed to have homologous recombination sequences (homologous flanking sequences) that direct the transgenes efficiently into specific regions of the chloroplast genome. Homologous recombination in chloroplasts is facilitated by a chloroplast-encoded recombinase encoded by the *rec*A gene (Bock and Hagemann, 2000).

Chloroplast genetic engineering has been used for the production of biopharmaceuticals including vaccines and human therapeutic peptides. In this approach the chloroplast transgenic plants serve as bioreactors for production of large quantitities of the desired biopharmaceutical. Plants are suitable for production of biopharmaceuticals because they use sunlight as their source of energy, which makes it cheap, and procedures for large-scale agricultural production are already in place. A protein such as human somatotropin (*hst* gene), which is used in the treatment of hypopituitary dwarfism in children, was produced via chloroplast transformation to up to 7% tsp (Staub et al., 2000). Recently, human serum albumin (*hsa* gene), which is extensively used in treatments of cirrhocis, burns and blood volume disorders was expressed in chloroplast to levels of up to 11.1% tps (Fernandez-San Millan et al., 2003). In addition to the previous reports, chloroplast transformation has been successufuly used for the expression of vaccines. The integration of the native colera toxin B gene (*ctx*B) into tobacco chloroplasts led to expression of CTB to 4.1% tsp (Daniell et al., 2001c). Additonally, it was observed that CTB was in its pentameric functional form, indicating that there is proper disulfide bond formation in chloroplasts. This important observation opened the door for the expression of complex multimeric proteins that need to be assembled in order be functional. Recently, a plant vaccine against *Bacillus anthracis* was produced via chloroplast genetic engineering by the integration and expression of the protective antigene gene (*pa*), this plants expressed PA to concentrations of 78 μg/mL (Daniell et al., 2004). With the observed expression levels, 600 million doses of vaccine per acre, free of contaminants could be produced. Recent investigations have shown that chloroplast expression is possible in chloroplast of other useful crops besides that of tobacco (Sidorov et al. 1999; Daniell, 1999). Kumar et al., (2004) demonstrated that carrots could be

transformed, selected, and effectively regenerated. In this report, homoplasmy of the carrot plastid genomes was achieved, as well as abundant expression of the foreign gene in chromoplasts of the carrot root. They reported that foreign gene expression in chromoplast was about 70% the expression in leaf. This indicates that chloroplast transformation can be applied to express foreign genes in plant plastids of different species. Additionally, this work opened the door for the production of edible vaccines that will not have to be purified from the plant, and with n increased shelf life without the requirements of current vaccines for refrigeration.

Multigene Engineering via Chloroplast Transformation

The majority of the aforementioned examples demonstrated the applicability of chloroplast transformation for the expression of simple traits of agricultural and commercial value requiring the integration of a single gene for the expression of the new trait. The vast majority of agronomic traits are quantitative and controlled polygenetically. Plant genetic engineering is shifting gears for the production of more complex traits, often requiring the use of multigenes for their completion and proper functioning.

A suitable approach to engineering multigene pathways is to do it via the chloroplast genome. Chloroplast genetic engineering possesses many advantages that make it a suitable place to engineer multigene pathways. Among the most important advantages of chloroplast engineering is the ability of chloroplast to effectively transcribe operons and translate polycistronic mRNA. Integration of multigene operons into the chloroplast genome occurs by homologous recombination, which allows the targeting of several genes in a single

transformation event, thus removing the possibility of detrimental position effect. Additionally, chloroplast genetic engineering offers increased transgene containment due to the maternal inheritance of chloroplasts. So far, no size limitation for foreign operons has been reported in chloroplast. Because the chloroplast is the site of photosynthesis, carbohydrate and fatty acid biosynthesis, it is the logical place to genetically engineer different metabolic pathways. One example of carbohydrate engeneering by chloroplast genetic engineering was the production of the osmoprotectant trehalose in tobacco chloroplast (Lee et al., 2003). This osmoprotective sugar has been proposed as a safe preservative for foods and therapeutics, and was recently approved by the Food and Drug Administration (FDA) and the European Union regulation system (Schiraldi et al., 2002). The chloroplast has also been engineered with the *acc*D gene, the expression of this gene increased the levels of the plastid encoded acetyl-CoA carboxylase subunit and consequently increase the levels of fatty acid biosynthesis in chloroplast of the transgenic plants (Madoka, et al., 2002). Zhang et al (2001) demonstrated the possibility of increasing amino acid content in plants by introducing a feedback insensitive mutant version of the antranilate synthase α-subunits into the chloroplast genome. The expression of this deregulated enzyme increased the levels of tryptophan.

Multigenes have been only recently engineered via the chloroplast genome to produce new metabolic traits. The *Bt cry*2Aa2 operon was used as a model system to test the feasibility of multigene operon expression in genetically engineered chloroplast (De Cosa et al., 2001). In this report, the expression of the operon containing selectable marker *aad*A, *orf*1, *orf*2 (chaperone) and *cry*2Aa2 gene lead to the formation and accumulation of CRY2AaA crystals in the chloroplast to 46.1% total soluble protein. Later, targeting of an operon containing the three

bacterial genes for the biosynthetic pathway of polyhydroxybutyrate resulted in the expression of the biopolymer in plastids (Lossl et al., 2003). More recently, a multigene operon comprising a selectable marker and the *mer*A and *mer*B genes coding for the phytoremediation pathway of organic-mercury lead to the detoxification of phenylmercuric acetate to the far less toxic elemental mercury, and conferred resistant to high concentration of organomercurial to the transgenic plants (Ruiz et al., 2003). These examples support the applicability of multigene engineering for the expression of metabolic pathways via the chloroplast genome. Chloroplast transformation has also conferred resistance to herbicides (Daniell et al., 1998), conferred drought tolerance (Lee et al., 2001), and phytoremediation capabilities (Ruiz et al., 2003).

Rationale and Approach

In plant and animal cells, nuclear mRNAs are translated monocistronically. This poses a serious problem when engineering multiple genes in plants (Bogarad, 2000). Therefore, in order to express the polyhydroxybutyrate polymer or Guy's 13 antibody, single genes were first introduced into individual transgenic plants, these plants were then back-crossed to reconstitute the entire pathway (Nawrath et al. 1994; Ma et al. 1995). Similarly, in a seven-year effort, Ye et al introduced a set of three genes for a short biosynthetic pathway that resulted in β-carotene expression in rice. In contrast, most chloroplast genes of higher plants are co-transcribed (Bogarad, 2000). Multiple steps of chloroplast mRNA processing are involved in the formation of mature mRNAs. Expression of polycistrons via the chloroplast genome provides a unique opportunity to express entire pathways in a single transformation event. Additionally, chloroplast

genetic engineering is an environmentally friendly approach resulting in containment of foreign genes and hyper-expression (Daniell, 1997, 1999). The objective of this investigation is to develop recombinant DNA vectors harboring multigene constructs that can be expressed polycistronically via chloroplast genetic engineering, producing new metabolic traits. Because there are only a few studies reporting foreign multigene operons in chloroplast, not much information is available in the molecular regulation and modification of these operons. In order to further study these processes, we proposed the creation of chloroplast transgenic lines expressing multigene cassettes for the expression of β-ketothiolase. Additionally, we will engineer the degradative pathway for organomercurials via chloroplast transfomation. The plants harbouring these multigene constructs will be characterized at the DNA, RNA and protein levels to understand the new characteristics imparted by these operons. Finally, this work will focus in the characterization of posttranscriptional modifications of foreign operons in chlroplasts. For this purpose, multiple plant lines engineered with foreign operons will be employed and characterized for RNA stability, posttranscriptional modifications, and translation. The extent of this research will provide useful insite into genetic engineering and expression of multigene operons via the chloroplast genome, and may facilitate the construction of future multigene operons.

ENGINEERING MALE STERILITY VIA THE CHLOROPLAST GENOME

Introduction

PHB synthesis takes place by the consecutive metabolic action of β-ketothiolase (*pha*A gene), acetoacetyl-CoA reductase (*pha*B) and PHB synthase (*pha*C). Poirier et al., (1992) reported the expression of PHB in plants for the first time by expressing the *pha*B and *pha*C genes in the cytosol via nuclear transformation; taking advantage of available cytosolic acetoacetyl-CoA. This approach yielded very low levels of PHB; but severe pleiotropic effects were observed in the transgenic plants. In an attempt to increase the PHB yield in plants, Nawrath et al (1994) introduced the *phb*A, *phb*B and *phb*C genes in individual nuclear *Arabidopsis* transgenic lines and reconstructed the entire pathway, targeting all enzymes to the plastids. This approach resulted in PHB expression up to 14% leaf dry weight, and no pleiotropic effects. This suggested that the depletion of metabolites from essential metabolic pathways in the cytoplasm might have caused the pleiotropic effects, and that by targeting the enzymes to chloroplast, which is a compartment with high flux through acetyl-CoA, the adverse effects were overcome. When expression of optimized gene constructs, PHB yield increased up to 40% leaf dry weight, but this was accompanied by severe growth reduction and chlorosis (Bohmert et al., 2000), indicating that targeting the PHB pathway to the chloroplast can result in pleiotropic effects, at higher concentrations of polymer synthesis (Bohmert et al., 2002). Lossl et al. (2003) reported the expression of PHB in tobacco by expressing *pha*A, *pha*B and *pha*C via plastid transformation. The expression of PHB resulted in severe growth reduction and authors

concluded that in tobacco significant levels of PHB could only be achieved if a sufficient pool of acetyl-CoA precursor is generated (Lossl et al., 2003). Additionally, they observed that when the transgenic plants were grown autotrophycally, PHB levels significantly decreased which overcame the stunted phenotype, but male sterility was still observed. It was not known whether the polymer or other metabolic factors were responsible for the male sterile phenotype (Lossl et al., 2003).

In an attempt to address the role of *pha*A expression in the pleiotropic effects observed in transgenic plants expressing PHB, Bohmert et al., expressed the *phb*A gene constitutively and under inducible promoters via the nuclear genome (Bohmert et al., 2002). Constitutive expression of the *phb*A gene led to a significant decrease in transformation efficiency, inhibiting the recovery of transgenic lines and prevented analysis of plants expressing the β-ketothiolase gene (Bohmert et al., 2002). Such toxic effect exerted by *phb*A expression was speculated to be the result of PHB biosynthesis intermediates or its derivatives, the depletion of the acetyl-CoA pool, or of interaction of the β-ketothiolase with other proteins or substrates (Bohmert et al., 2002).

While investigating the *phb* operon, we proposed to address the specific role of β-kethiolase (*pha*A gene) that result in pleiotropic effects in transgenic plants by expressing the polydroxybutyrate pathway. Therefore, we hyperexpressed the β-ketothiolase gene by inserting the transgene into the chloroplast genome under the regulation of the *psb*A promoter and 5' untranslated region (5'UTR), and assessed the effects of *pha*A expression in the chloroplast transgenic plants at the molecular and physiological levels.

Because transgenes that are engineered into our annual crops could be introgressed into

wild crops, persist in the environment and have negative ecological consequences, it may be necessary to engineer a male sterility system that is 100% effective (Stewart et al., 2003). Several natural gene containment systems available today, rely on nuclear and mitochondrial mutations that occur spontaneously and render the plant male sterile (Kriete et al., 1996), but such naturally occurring systems are not available for most crops used in agriculture. In currently available cytoplasmic male sterile lines, nuclear genome controls various restoration factors (often controlled by multiple loci), which are not fully understood. Also cytoplasmic male sterility has been associated with such diseases as southern corn blight (CMS-T) and cold susceptibility (CMS Ogura). Male-sterility systems have been created by different mechanisms and most of these affect tapetum and pollen development (Kriet et al., 1996; Yui et al., 2003; Zheng et al., 2003). Unfortunately, additional severe phenotypic alterations observed in male sterile lines precluded their use in agriculture (Hernould et al., 1999; Napoli et al., 1999; Goetz et al., 2001).

The engineering of a cytoplasmic male sterility system has been proposed as a feasible way to address concerns with today's male sterility systems, but this has been elusive so far (Perez-Prat and van Lookeren, 2002). In this study, the *pha*A gene is constitutively expressed, and fully regenerated transgenic plants are recovered. Therefore, evaluation of the specific effect of β-ketothiolase in the transgenic lines was possible for the first time. Detailed characterization revealed that severe pleiotropic effects such as stunted phenotype and chlorosis observed during polyhydroxybutyrate expression (Bohmert et al., 2000; Lossl et al., 2003) were not observed during *pha*A expression via chloroplast transformation, with the exception of complete (100%) male sterility. Such elimination of pleiotropic effects was not surprising because the chloroplast genetic engineering system has been able to overcome the toxic effects (stunted growth,

13

chlorosis and infertility) associated with nuclear expression of the trehalose (Lee et al., 2003), xylanase (Leelavanthi et al., 2003) and cholera toxin B subunit (Daniell et al., 2002) gene, producing totally normal plants that were healthy and fertile. In this report, we characterize the male sterile phenotype generated by the expression of β-ketothiolase in chloroplast transgenic plants and demonstrate the first engineered system for cytoplasmic male sterility in plants.

Materials and Methods

Chloroplast Vector Construction

Native Genes Primer Design and Gene Amplification

Plasmid DNA (pJKD 1425) from *Acinetobacter sp* (accession number) coding for the complete polyhydroxybutirate metabolic pathway and containing the functional *pha*A gene (β-ketothiolase) was kindly provided by Metabolix (Cambridge, MA).Isolation and amplification of the *pha*A gene from the native plasmid was performed by polymerase chain reaction (PCR) with the utilization of *pha*A specific 5' and 3' flanking DNA primers. All primers were designed using the QUICKPRI program of the DNASTAR software. *Nde*I and *Not*I restriction sites were engineered in the 5' and 3' primers, respectively, to allow directional cloning into the suitable vectors. The 5' and 3' primers had 15 bases of homology with the 5' and 3' ends of the *pha*A gene, respectively. Either primer contain 10 bases, which are upstream of the *Nde*I restriction site in the 5'primer or downstream of the *Not*I restriction site in the 3' primer to facilitate subsequent

restriction digestions, these regions base-pare with the regions flanking the *pha*A gene in the native plasmid DNA.

The primers were diluted to 100 µM to make the stock solution and from these working solutions were produce by further dilution to 10 µM, the solutions were kept at -20°C. The PCR reaction for the amplification of the *pha*A gene was as follows: 1 µL of DNA (100-200 ng), 5 µL of 10X buffer, 1 µL of 10 mM dNTP (200-250 µM), 1 µL of each of 10 µM forward an reverse primers, 0.5 µL Pfu Turbo polymerase (2.5 U) (Stratagene, La Jolla, CA) and 40.5 µL dH$_2$O to a total volume of 50 µL. The polymerase chain reaction was performed using the Gene Amp PCR system (Perkin-Elmer) according to the manufacturers instructions. The amplification was carried out during 30 cycles with a program that alternated temperatures and time in the following way: denaturation at 94°C for 1 min, primers anneling at 65°C for 1 min, and gene extension at 72°C for 2 min (for the *pha*A). The 30 cycles were preceded by denaturation of the PCR reaction for 5 min at 94°C and were followed by a 72°C final extension time. The PCR products were cloned immediately after the PCR run ended.

pCR2.1-5'UTR-*pha*A Vector Construction

The PCR product (*pha*A gene) was cloned into the vector pCR2.1-5'UTR*psb*A, which contained the functional *psb*A gene promoter and 5' regulatory sequence, by directional cloning after *Nde*I and *Not*I restriction digestion of the PCR product and vector. The digestion reactions for the PCR amplified *pha*A and the pCR2.1-5'UTR*psb*A were performed as follow: 5 µL of

vector DNA (0.25 µg) or 5 µL of *pha*A (1.0 µg), 1.0 µL of *Nde*I, 1.0 µL *Not*I, 2µl of 10X

reaction buffer compatible with *Nde*I/*Not*I, and 11 µL of sterile Milli Q water were added for a

final volume of 20 µL. The restriction digestions were incubated at 37°C for 2 hours. The

reactions were run in a 0.8% agarose gel and the bands of interest were cut out and eluted from

the gel for later ligation.

DNA Band Elution from the Agarose Gel

A commercial gel extraction kit (Qiagen gel extraction kit) and protocol was use to

extract the DNA bands from the agarose gel, the bands were projected by placing the gel on a

flat UV transiluminator. They were then cut out with a razor blade and placed into a 1.5

microcentrifuge tube. The two pieces of gel containing the bands of interest plus the tube were

weighed, knowing previously the weight of the tube to determine the volume of the gel. Three

volumes of buffer QG (for example: 900 ml to 300 mg of gel) were added to the tube. The

sample was incubated at 50°C for 10 minutes to melt the agarose gel. To help the melting

procedure, the sample was vortexed about 3 times. After the gel had melt one volume of

isopropanol was added and then mixed by vortex. The mixture was added to a QIA quick spin

column and centrifuged for 1 minute at 13,000 rpm. The flow through was discarded and 750 µl

of buffer PE were added to the column. The column was centrifuged for 1 minute at 13,000 rpm.

The flow through was discarded and the column was centrifuged for an additional minute at

maximum speed to remove any trace of isopropanol. The column was transferred to a sterile

micro centrifuge tube and 50 µL of EB buffer (elution buffer) were added to the column to elute

the DNA and then the column was centrifuged for 1 minute at maximum speed. Flow through was used in subsequent steps.

Ligation Reaction

The sample obtained from the gel elution was concentrated in a speed-vac until the volume reached 15 µl. Then, 1µl of T4 DNA ligase and 4 µl of 5X T4 DNA ligase buffer were added to the 15 µl of DNA to complete the reaction mixture (final volume of 20 µL). The reaction mixture was incubated over night at 14°C to allow ligation to take place. Followed, ligated vector containing the insert was cloned into plasmid receptive ultra competent *E. coli* cells.

Ultra Competent Cells Preparation

100 µL of Cells from a overnight culture of *E. coli* XL1 Blue MRF'[ab] Tc growing on LB liquid media was inoculated in 50 mL of SOB media at 37°C and shaken at 275 rpm until the optical density was 600 nm (this is approximately 3 hours). Following, the culture was incubated on ice for 10 minutes and then spun at 2,500 x g for 10 minutes at 4°C. The pellet was resuspended on 20 mL of ice-cold transformation buffer (TB: 10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, adjust pH to 6.7 with KOH before adding the $MnCl_2$, and filter sterilize). The mixture was put on ice for 10 more minutes and then centrifuged at 2,500 x g for 10 minutes at 4°C. The cells pellet was re-suspended in 4 ml of ice cold TB and DMSO was

added to a final concentration of 7% (about 280 µL in 4 mL of sample). The mixture was placed on ice for 10 minutes and then aliquoted into 200 µL and stored into the Deep Freezer (-80°C).

E. coli **Transformation With The Plasmid pCR2.1-5'UTR-*pha*A**

In a 15 mL falcon tube, 100 µL of ultra competent cells and 5 µL of DNA (pCR2.1-5'UTR-phaA) from the ligation reaction were added and gently mixed. The mixture was placed on ice for 30 minutes and was shaken about 3 times. Followed, the transformation mixture containing cell and DNA was heat-shock treated for exactly 45 seconds at 42 °C without agitation. The tube was inmediately returned to the ice and incubated there for 2 more minutes. Later, 900 µL of LB (Luria Bertani) medium was added and the transformed cells were allowed to grow at 37 °C for 1 hour at 225 rpm in a rotary shaker. After the 1-hour incubation, 100 µL of cells were spread onto a LB plate containing 100 µg/mL of ampicillin. The rest of the 900 µL were centrifuged at 8,000 rpm and the pellet was re-suspended in only 100 µL of LB medium and plated onto the solid LB medium. The plated cells were incubated overnight at 37°C. The next day the cells that were growing on the plate were selected and inoculate in LB broth with 100 µg/mL of ampicillin to perform plasmid isolation and restriction analysis to confirm that the *pha*A gene was cloned into the pCR2.1-5'UTR.

Mini Plasmid Isolation Of pCR2.1-5'UTR-*pha*A

From an overnight culture of the selected colonies, 1.5 ml were transferred to a micro centrifuge tube and centrifuged for 1 min at 8,000 rpm at room temperature in the Fisher Marathon Micro H centrifuge. The supernatant was discarded and 200 µL of solution I (50 mM glucose; 10 mM EDTA; 25 mM Tris, pH 8.0), and 2 µl of RNase A were added to the pellet and vortexed to resuepend. 200 µL of solution II (100 µl of 2 N NaOH; 100 µL of 10% SDS; 800 µL sterile water) was added to the tube and mixed by inverting five to six times, and then incubated on ice for 5 minutes. Solution III (60 mL of 5 M NaOAc; 11.5 mL glacial acetic acid; 28.5 mL sterile water) was added and then mixed by inverting 5-6 times and then incubated for 15 min on ice. After incubation, the samples were centrifuged for 5 minutes at 12,000 rpm at 4°C in a Savant µ Speed Fuge. The supernatants were transferred to fresh sterile tubes and 2 volumes of 95% ethanol were added followed by mixing by inverting 5 to 6 times, then the samples were incubated on ice for 10 min. The samples were centrifuged at 12,000 rpm at 4°C for 15 min. The supernatants were discarded and the pellets were dried in a Savant DNA speed-vac on medium setting for 5 minutes. The dried pellets were re-suspended in 50 µl of TE (Tris-EDTA) buffer and 4 µl of each of the samples were run on a 0.8% agarose gel for 40 minutes at 60 volts to confirm the successful isolation of plasmid.

Verification of *pha*A Gene Integration into pCR2.1-5'UTR Vector

The plasmid DNA isolated from the different bacterial clones were subjected to restriction digestion with *Nde*I and *Not*I to cut out the *pha*A gene from the pcR2.1-5'UTR vector and positive identify the integration of the *pha*A gene. The digestion reactions were performed as explained previously in the vector construction section. The digested samples were resolved in 8% agarose gel stained with Ethidium bromide. One of the clones characterized positive for the integration of *pha*A was used for Midi-prep plasmid isolation and further studies.

Midi-prep of Plasmids pCR2.1-5'UTR-*pha*A

A midi plasmid isolation (Bio-Rad Quantum Prep midiprep kit) was performed from an overnight culture of the *E. coli* clone containing the pCR2.1-5'UTR-phaA vector. Fifty mL of culture was transferred from the 250 mL Erlenmeyer flask to a 50 mL conical tube and spun down for 5 minutes at 5,000 rpm. The supernatant was discarded and 5 mL of Cell Resuspension Solution was added to the pellet to resuspend it by vortexing. Followed, 5 mL of Cell Lysis Solution was added to the sample, and the sample was mixed by inverting the tube 6-8 times. 5 mL of Neutralization Solution was added to the sample and was mixed by inverting 6-8 times. The sample was centrifuged for 10 minutes at 8,000 rpm and the supernatant was poured carefully into a new 50 mL tube to which 1 mL of the Quantum Prep matrix was added and then swirled for 30 seconds. The sample was centrifuged for 2 minutes at 8,000 rpm and the supernatant was discarded. Followed, 10 mL of wash buffer was added to the matrix pellet and

the pellet was resuspended by shaking. The sample was centrifuged for 2 minutes at 8,000 rpm, the supernatant was discarded and the pellet was re-suspended in 600 μL of wash buffer by vortexing. Re-suspended sample was transferred to a provided spin column (and collection tube) and centrifuged for 30 seconds at 12,000 x g. Flow through was discarded and 500 μL of wash buffer was added to the column and centrifuged for 30 seconds at 12,000 x g. The flow through was discarded and the column was spun for an extra 2 minutes at maximum speed to remove any residual wash buffer. The spin column was transferred to a sterile 2 ml microcentrifuge tube and 600 μL of TE buffer were added to the matrix. The column was spun down for 2 minutes at maximum speed and the flow through plasmid DNA was recovered and kept at -20°C for further studies.

*Pha*A Gene Sequencing

The pCR2.1-5'UTR-*pha*A vector and the vector specific M13 forward and M13 reverse primers, which flank the multiple cloning site of the pCR2.1 vector, along with an internal primer specific for the *pha*A gene were used to sequence the entire 5'UTR *psb*A and the *pha*A gene. Three independent reactions with each of the primers were performed. Before setting up the sequencing reactions the ng grams of DNA needed for the reaction were calculated accordingly to the size of the vector plus insert, the size is approximately 5.0 kb what required 165 ng of DNA. To achieve the required DNA amount in the reaction, 1.65 μL of plasmid DNA from a stock solution of 100 μg/μL was added into a PCR tube and the volume was broght to 5 μL with sterile water. Followed, the sample was heat diluted in the termocycles at 96°C for 1

minute and then allowed to cool to room temperature. Then, 1 µL of primer from a 5 µM stock solution and 4 µL fo DTCS quick start master mix were added for a total sample volume of 10 µL. The samples were put into the thermocycler and were amplified with the next sequencing PCR program for 30 cycles: 96°C for 20 seconds (denaturation), 50°C for 20 seconds (annealing temperature), 60°C for 4 minutes (extension); and a final hold at 4°C.

After the PCR the samples were purified by ethanol precipitation by first adding 2 µL of 50:50 mixture of NaOac and EDTA, followed by 0.5 µL of glycogen into the 10 µL PCR reactions. Then, 60 µL of ice cold 95% EtOH were added, mixed and immediately centrifugated at 14,000 rpm at 4°C for 15 minutes. The supernatant was removed and the pellet was rinsed with 400 µL ice cold 70% EtOH. The sample was centrifugated at 14,000 rpm at 4°C for 4 minutes. The supernatant was removed and the pellet was vacuum dried for 10 minutes. Finally, the sample was resuspended in 20 µL of sample loading solution and stored at 4°C until was time to use. The DNA sequencing was performed in a Beckman Coulter sequencer at the UCF sequencing facility. Finnally, electrophoregram was compared with gene bank sequences by using the BLAST 2 Sequences program from the NCBI website.

pLDR-5'UTR-*pha*A Vector Construction

The vector pCR2.1-5'UTR-*pha*A and the chloroplast transformation vector pLD-ctv were restriction digested with *Nde*I and *Not*I, the samples were resolved in a 0.8% agarose gel an the DNA fragment corresponding to the digested pLD-ctv vector and to the 5'UTR-*pha*A construct

were eluted from the gel. Followed, these samples were used for ligation and subsequent *E. coli* transformation and confirmation of integration of the 5'UTR-*pha*A insert into the pLD-ctv vector. These procedures were performed essentially as described earlier in this chapter in the Materials and Methods section.

Chloroplast Transformation and Selection of Transgenic plants

Preparation of Tobacco Tissue Culture Media

MS Medium

The Murashige and Skoog (MS) medium is used at different stages of tobacco tissue culture, and is essential for seed germination, plant propagation and root induction after the second round of regenerative selection after bombardment. The medium was prepared by mixing in a 1-L beaker, 4.3-g packet of MS salt mixture (Invitrogen) and 30 g of sucrose, adjusting the volume to 900 mL with sterile Milli Q water and adjusting the pH to 5.8 with 1N KOH. Finally, the volume was broght to 1000 mL and 6 g of Phytagar for a final concentration of 0.6% was added. The medium was autoclaved for 20 minutes at 121°C. After autoclaving, the medium was allowed to cool to 40°C before antibiotics were added. The plant medium was poured into deep Petri dishes, jars or Magenta boxes.

RMOP Medium

RMOP medium or regeneratin medium is used for the induction of shoots from tobacco leaves after the bombardment procedure. The medium is essentially made as explained for MS medium with the exception that the following phytohormones and nutrient need to be added: 100 mg of *myo*-inositol, 1 mL of a 100 mg/mL thiamine-HCl solution, 1 mL of a 100 mg/mL 6-benzylaminopurine (BAP) solution, and 100 μL of a 100 mg/mL naphtaleneacetic acid (NAA) solution

Spectinomycin Solution

Spectinomycin is the antibiotic normally used for selection of chloroplast transformants in tobacco and it is prepared by dissolving 1 g spectinomycin into 10 mL of sterile Milli Q water at a final concentration of 100 mg/mL. The solutions have to be filter-sterilized under aseptic conditions and stored at -20°C.

<u>Preparation for Bombardment</u>

Preparation of Tobacco Tissues for Bombarment

The leaf materials used for chloroplast transformation were obtained by either germinating untransformed tobacco seeds in MS medium or by micropropagation through nodal

sections from an existing untransformed tobacco plant growing in MS medium. The leaves used in the bombardments were green leaves with no damages or defects with an approximate size of 2 in. × 1 in.

Preparation of Consumables for Bombardment

The consumables to be used during bombardment were prepared in advanced to the day of bombardment. The macrocarrier holder, stopping screens, filter paper and Kimwipes were autoclaved and dried in the heat oven. Consumables such as macrocarriers and rupture disks that are not autoclavable were sterilized by submerging them in 100% ethanol for 10 minutes and then allowed them to dry over sterile Kimwipes in the laminal flow hood.

Preparation of Gold Particles Suspensions

The gold particles to be used for bombardment were make in advance and stored at -20°C were they are good for up to 6 months. 50 mg of gold particles (0.6 µM) were put into a 1.5-mL centrifuge tube to which 1 mL of 100% ethanol (molecular grade) was added, and then it was mixes by vortex for 3 minutes. Followed, the gold particles were pelleted by centrifugation at maximun speed for 2-5 minutes in a benchtop microcentriguge. Then the supernatant was discarded and the pellet was resuspended in 70% ethanol by vortexing for 2 minutes. The suspension was incubated for 15 minutes at room temperature and the content was mixed 3 times during the incubation. The gold particles were pelleted by centrifugation at maximun speed for 3

25

minutes and the supernatant was removed. The particles were completely resuspendend in destilles 1 mL of sterile Milli Q water by vortex-mix for 1 minute. The gold particles were allowed to settle down for 1 minute and a final centrifugation at maximun speed for 2 minutes was carried out to pellet particles still in suspension. Finally, the supernatant was discarded and the previous 3 steps were repeated twice. After the final wash with water the pelleted gold particles were resuspended in 50% glycerol for a final gold concentration of 60 mg/mL. Gold particle suspension was stored at −20°C.

Particle Bombardment of Tobacco Leaves

DNA Coating of the Gold Particles

In a 1.5-mL centrifuge tube 50 µL of gold particles from the previously made stock suspension (60 ng/mL) were added, followed by 10 µL of plasmid DNA (in this case pLDR-5'UTR-phaA), and a 5 seconds vortex-mix. Then, the following chemical solutions were added in strick order and mixed by vortex for 5 seconds after each addition, first 50 µL of 2.5 M $CaCl_2$ (freshly made) and 20 µL of 0.1 M spermidine-free base. After the last chemical addition the suspension was vortexed for 20 minutes at 4°C. Followed, 200 µL of room temperature absolute ethanol were added to the mixture and were mixed by vortexing for 5 seconds, immediately the mixture was centrifuge for 30 seconds at 3,000 rpm to pellete the gold particles. The supernatant was removed and the ethanol wash was repeated four more times. After the final step the gold

particles were resuspended in 30 µL of 100% ethanol and the particles were kept on ice until ready to use (the gold particles were used within 2 hours).

Tobacco Leaf Samples for Bombardment

A single DNA-gold particle mixture (30 µL) is enough for 5 bombardments, for that reason 6 (5 for bombardment and 1 negative control) leaves from fully expanded, undamaged green leaves were obtained from a young wild type tobacco plant growing in MS medium in a glass jar. The collected leaves were placed adaxial on Petri dishes (100" x 25") containing RMOP medium with out antibiotic selection and to which sterile Whatman filter disk were previously placed on their surface. The Petri dishes containing leaves were cover until ready to bombard.

Macrocarrier Loading

The sterile macrocarriers were placed inside the macrocarrier holder with its concave side facing outward. The final insertion of each the macrocarrier was performed by using the macrocarrier insertion tool in a push and rotate motion. Followed, 5 µL of the gold carrying the plasmid DNA (microcarrier) were added to the center of the macrocarrier, and spread until the target area was completely covered. Before each addition of gold particles to the macrocarriers the gold particles were thoroughly resuspended to inhibit the formation of clump. Any remaining

gold particle after the loading of the five macrocarriers can be use for a second application into one or more of the macrocarriers.

Particle Bombardment

The inner chamber, the internal parts, the door and the outside of the gene gun were sterilized with 70% ethanol before bombardment. Followed, the helium tank valve was opened to the maximum position and the helium pressure was regulated by rotating the adjustment handle of the helium regulator clockwise until the gauge measured 1350 psi. Then, the vaccum pump and the gene gune were turned on. A rupture disk (1100 psi) was placed into the rupture disk-retaining cap and position it into the gene gun tightly followed by placing the stopping screen into microcarrier launch assembly. The macrocarrier holder containing the loaded macrocarrier was positioned into the microcarrier launch assembly and covered with the macrocarrier coverlid. The microcarrier launch assembly was placed into the gene gun. Then, target plate shelf was taken out of the gene gun and the cover of the Petri dish containing the leaf was removed. The Petri dish was placed on top of the target plate shelf making sure that the leaf covered the target area. The loaded target plate shelf was inseted into the gene gun and the gene gun door was closed. By pressing the vac/vent/hold switch to the vacuum position a 28 Hg in vacuum was achieved, then quickly the vac/vent/hold switch was changed to the hold position in a single and fast motion, finally, the Fire switch was holded to the fire position until the helium pressure gauge in the gene gun indicates 1,100 psi and the rupture disk bursted and a small explosion was herd. After this step, the fire switch was release and the pressure of the chamber was recostitute

by switching the vac/vent/hold switch to the vent position and wait until pressure in the vacuum gauge reads zero. The bombarded leaf was taken out, covered and sealed with parafilm. The gene gun consumables (rupture disk, stopping screen) were replaced for the subsequent bombardment.

Tobacco Tissue Regeneration and Selection

The Petri dishes containing the bombarded leaves were incubated in the dark for 48 hours at 27°C and after the 48 hours the leaves were cutted into small pieces of about 5 mm^2 each and placed in RMOP media plates (Petri dish 100" x 25") containing 500 µg/mL spectinomycin antibiotic for selection; this is called first round of selection. The bombarded side of the leaves was put in direct contact with the selection media (RMOP medium induces shoot formation). Then, the leaf tissues were incubatedat 27°C in a photoperiod of 16 hours light and 8 hours dark, the first putative transgenic shoots were observed after 4 to 6 weeks on selection. Once the putative transgenic shoots growing in first selection developed leaves and achieved a size of about 1 cm^2, they were placed in second round of selection. For the second round of selection, leaves from the primary shoot were cut in pieces of about 2 mm^2 and were placed on RMOP medium plates (Petri dishes 100" x 25") containing spectinomycin. Again the abaxial side of the leaf was placed in contact with the medium. Putative transgenic shoots took about 3 weeks to develop. Once the shoot developed into a normal plant with a size of 1 to 2 inches in the second round of selection, the plant was detached from the adjacent callus by cutting through the stem close to the callus and the chloroplast transgenic plant was transferred to a third round of selection. For the third round of selection the plants were grown in MS medium jars containing

spectinomycin. This step (third round of selection) induces the formation of roots and the proper development of the plant. The transgenic plants took around 4-8 weeks to fully develop. Then, they were moved to soil. To transfer the plant to soil, the plant was first gently pulled out from the media and the roots were washed thoroughly with tap water to remove any agar attached to the roots. Finally, the plants were grown in a high nutrient soil in a photoperiod of 16 hours light and 8 hours dark at 27°C in a growth chamber. Seeds were collected from T_0 generation transgenic plants. The material and methods used for the transformation of tobacco chloroplast have been previously published (Daniell, 1997; Daniell et al., 2004; Kumar and Daniell, 2004).

Characterization of Chloroplast Transgenic Plants

Plant DNA Extraction Procedure

The DNeasy™ Plant Mini Kit (QIAGEN, inc, Valencia, CA) was used for the isolation of total DNA from wild type and transgenic plants as follows; 100 mg of tissue sample were removed from the plant using aseptic techniques, placed into a microcentrifuge tube, and grinded by using a micro pestle in 400 µL of buffer AP1 and 4 µL of RNase A (stock solution 100 mg/ml). The mixture was incubated for 10 minutes at 65°C and mixed about 2-3 times during incubation by inverting the tube. 130 µL of buffer AP2 were added to the lysate, vortexed and incubated for 5 minutes on ice. Following, a centrifugation was done at maximum speed for 5 minutes and the supernatant was transferred to the supplied spin column (lilac) sitting in a 2 mL collection tube. The centrifugation was performed at full speed for 2 minutes. The flow through was transferred to a new tube and 1.5 volumes of buffer AP3 were added to the lysate and mixed

immediately. 650 μL of the mixture was applied to a DNeasy mini spin column (clear) and centrifuged for 1 minute at 8000 rpm. The flow through was discarded and the collection tube reused to repeat the previous step with the rest of the sample. The tube with the flow through was discarded and the column was placed in a supplied 2 mL tube. 500 μL of buffer AW were added to the column and then it was centrifuged for 1 minute at 8,000 rpm. The flow through was discarded, and the tube was reused. The Dneasy column was washed once again with 500 μL of AW buffer and buffer was removed by centrifuging for 2 minutes at maximum speed. The Column was transferred to a clean 1.5 mL tube and 100 μL of preheated (65°C) buffer AE were directly delivered into the Dneasy membrane. The membrane was incubated for 5 minutes at room temperature and then centrifuged at 8,000 rpm for 1 minute to elute the DNA. The DNA was kept at -20°C.

Confirmation of Chloroplast Integration by PCR

Isolated total plant DNA from untransformed and transgenic plants was used as the template for PCR reactions. The PCR primer pairs 3P-3M and 4P-4M were used to confirm the integration of the gene cassette into the chloroplast, essentially as described previously. Primer pair 5P-2M, 5P-phaA internal and 5P-3'phaA were used to confirm the presence of the *pha*A gene. PCR analysis was performed using the Gene Amp PCR System 2400 (Perkin Elmer, Chicago). The 50 μL PCR reaction was setted the following way: 1 μL of 100 ng/ul genomic DNA, 5 μL of 10X PCR reaction buffer, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 0.5 μL of Taq DNA polymerase and 41.5 μL of sterile Milli Q water. The PCR reaction

was carried for 30 cycles with a program that alternates temperature and time in the following manner: 94°C for 1 minute, 65°C for 1 minute, the elongation step is carried at 72°C for an amount of time that depends on the primer pair to be used (usually 1 min per 1,000 bases). Cycles were preceded by denaturation for 5 minutes at 94°C and followed by a final extension time of 5 minutes at 72°C. The PCR amplification products were detected by electrophoresis in a 0.8% agarose gel stained with ethidium bromide (EtBr).

Southern Blot Analysis

Restriction Digestion of Plant DNA

Genomic DNA (2 μg) from T_0 and T_1 generation plants as well as wild type was digested with a suitable restriction enzyme. The restriction digestion produced DNA fragments of defined size; these fragments should have at least 2 kb difference between the transgenic fragment and the wild type fragment to allow good resolution. The restriction digestion was performed as follows: 2 μg plant total DNA, 2 μL of 10X reaction buffer, 1 μL BamHI restriction enzyme, and water to bring the volume to 20 μL. The reaction was placed a 37°C overnight.

Agarose Electrophoresis and DNA Transfer

The *Bam*HI restriction digested DNA from transgenic lines and wild type along with a suitable positive control and molecular weight marker were loaded into a 0.7% agarose gel (0.7

g agarose in 100 ml total volume) containing 5 µL of EtBr obtained from a 10 mg/mL stock solution. The gel was run for 2.5 hours at 55 volts. After the run was completed the DNA was transfer by capillarity to a nylon membrane as following explained. The unnecessary portions of the gel were removed and the upper right corner of the gel was removed to serve as an orientation mark. Followed the gel was depurinated by immersing it in 0.25 M HCL for 15 minutes and was shaked until the color of the sample dye changed to yellow. Then, the gel was washed twice for 5 minutes in distilled water and then equilibrated in transfer buffer (0.4 N NaOH and 1 M NaCl) for 20 minutes, the gel was always in agitation. Four pieces of Whatman paper and the nitrocellulose membrane were cut to fit the size of the gel. The upper right size of the membrane was cut to serve as an orientation mark. The membrane was briefly rinse in destilled water and equilibrated by immersing it in transfer buffer for 5 minutes in agitation. In a glass tray, a stack of two sponges was placed and enough transfer buffer was poured to cover the sponge that is in contact with the glass tray. Followed, two pieces of Whatman paper previously soaked in transfer buffer were placed on top of the sponges. Extra transfer buffer was poured onto the Whatman paper to soak and remove any air bubbles and the gel was placed facing down on the Whatman paper. Then, the pre-equilibrated nylon membrane was placed on top of the gel making sure that its cut corner is touching the cut corner of the gel, more transfer buffer was added remove any air bubbles. Two additional Whatman papers were placed on top of the membrane, followed by a stack of paper towels. A 500 g weight was placed on top of the transfer set up to accelerate the capillary transfer. The next day the membrane was removed from the transfer set up and was washd in 2X SSC (0.3 M NaCl and 0.03 M sodium citrate) for 5 minutes on agitation. The membrane was allowed to dry for 5 minutes onto a Whatman paper and then

cross-linked in the GS GeneLinker (Bio-Rad, Hercules, CA) at setting C3 (150 mjoules). The cross-linked membrane was covered with saran wrap and stored in a dry place until ready to use.

Generation of Probes

The flanking sequence probe (0.81 kb) was obtained by *Bgl*II/*Bam*HI restriction digestion of plasmid pUC-ct, which contains the chloroplast flanking sequence (*trn*I and *trn*A genes). The *pha*A probe (1.3 kb) was obtained by *Nde*I/*Not*I restriction digestion of plasmid pCR2.1-5'UTR-*pha*A. The digestion reaction was setted up by adding; 1 µg of DNA, 2 µL of 10 X buffer and 1 µL of each of the restriction enzymes, finally the volume was broght to 20 µL with destilled water. The digestion reactions were performed overnight at 37°C. The restriction digested probes were purified by agarose gel electrophoresis followed by gel elution accordingly to the QIAquick gel extration kit (QIAGEN inc, Valencia, CA) protocol and the final DNA elution step was performed with 50 µl of distilled water.

Probe Labeling

Fourty five µL of probe DNA (50-250 ng) was denature by incubating the DNA for 5 minutes at 94°C and then immediately placing the tube on ice for 3 minutes followed by a pulse centrifugation to bring droplets down. The denatured probe was added to the Ready-To-Go Labeling Beads tube (Amersham Biosciences, USA) and content was mixed by flicking. 5 µL (50 µCi) of $\alpha^{32}P$ (dCTP) were added to the reaction mix and then was incubated for 1 hour at

37°C. Unbound radio labeled nucleotide were removed by passing the probe mixture through the ProbeQuant G-50 Micro Colums (Amersham Pharmacia Biotech Inc, USA) according to the vendor's instructions. The radiolabeled probe and other radioactive materials were stored in a -20°C freezer according to the institutional radiation safety guidelines.

Prehybridization, Hybridization and Membrane Washing

The Quick-Hyb solution (Stratagene, USA) was pre-warmed for 10 minutes at 68°C. Followed, the blot was placed into a hybridization bottle with the top face of the blot facing in toward the solution and then 5 mL of the preheated Quick-Hyb solution was added. The bottle with the membrane inside was incubated for 1 hour at 68°C in a hybridization incubator (Fisher Biotech, USA). Fifteen minutes before the end of the prehybrydization step, 100 µL salmon sperm DNA (10 mg/mL) were added to 5 µL of the radiolabeled probe and were incubated at 94°C for 5 minutes (this step denatures the DNA). Followed, the hybridization step was performed by the addition of the probe and samon sperm DNA to the membrane, which was incubate for 1 hour at 68°C. After the 1 hour incubation the radioactive hybridization solution was discarded into the liquid radioactive waste container. Followed, the membrane was wash twice, for 15 minutes each wash, with 50 mL of 2X SSC / 0.1% SDS buffer solution. These two washes are performed at room temperature. The final two washes were performed for 15 minutes at 60°C (to increase stringency) with 0.1X SSC / 0.1% SDS buffer solution.

Autoradiography

The radio labeled membrane was raped in saran wrap, and then placed into a film-developing cassette (contain the intensifying screen) and was taken to the dark room. Under safe light (red light) the x-ray film was placed on top of the blot and was incubate (blot and film) at – 70°C for the appropriate exposure time. Then, the exposed x-ray film was developed in the Mini-Medical Series x-ray film processor (AFP Imaging, Elmsford, NY).

Northern Blot Analysis

RNA Extraction

Total plant RNA from untransformed and chloroplast transgenic plants, was isolated by using the RNeasy Mini Kit (Qiagen, Valencia, CA) and protocol. All the instruments were washed with DEPC water (1ml DEPC in 999ml of water to kill Rnases) and then autoclaved to remove the RNases. 100 mg of leaf material were taken from a plant growing in a glass jar constaining MS medium. The leaf material was freezed liquid nitrogen and then grinded thoroughly with a mortar and pestle. The powder was transferred to a 1.5 mL RNase free tube that was previously cooled in liquid nitrogen. Followed, 450 μL of buffer RLT (to which βME was added previously) was added, then vortexed and placed at 56°C for 3 minutes. The lysate was transferred into a QIAshredder spin column (lilac) that was placed into a 2 mL collection tube and centrifuged for two minutes at maximum speed. The flow-through fraction (~225 μL)

was transferred to a new tube and 0.5 volumes of ethanol were added and mixed by pipetting. The mixture (~650 µL) was transferred to an Rneasy mini column (pink) that was placed in a 2 mL collection tube. It was then centrifuged for 15 seconds at 10,000 rpm and the flow-through was discarded. Followed, 700 µL of buffer RW1 were added to the column and then centrifuged for 15 seconds at 10,000 rpm to wash the column. The flow-through and collection tube were discarded. The Rneasy column was transferred to a new collection tube and 500 µL of buffer RPE were added to the column, followed by a centrifugation step for 15 seconds at 10,000rpm. The flow-through was discarded and the column was washed again with 500 µL of RPE buffer and centrifuged as explained in the previous step. Then, the column was transferred to a Rnase free new 2 mL tube and centrifuged for 1 minute at maximum speed to remove any residual buffer. The column was transferred to a new 1.5 Rnase free tube and the RNA was eluted from the membrane by applying 50 µL of RNase-free water directly onto the Rneasy membrane. The tube plus the column were centrifuged for 1 minute at 10,000 rpm. Two µL of the eluted RNA was used to measure consentration and the rest was kept at −80 °C until ready to use.

RNA Agarose/Formaldehyde Gel

One hundred mL of 1.2% agarose gel was made by adding 1.2 g of agarose into a RNase free bottle, followed by the addition of 87 mL of DEPC water. The agarose was melted in the microwave. The liquid agarose was cooled down until the flask reached approximately 60°C. Then, 10 mL of MOPS buffer (200 mM MOPS, 50 mM Sodium acetate, 10 mM EDTA and adjusted pH to 7 with NaOH), 3 ml of 37% formaldehyde and 5 µL of ethidium bromide were

added to the liquid agarose, mixed and poured into the casting tray. The gel was allowed to solidify for 30 minutes. Followed, the gel running chamber was filled with running buffer (1X MOPS). The samples were prepared by adding 5 µL total RNA (for a final concentration of 2.5 µg) and 15 µL of RNA sample buffer into a 1.5 mL. Then the samples were mixed and incubated at 65°C for 15 minutes. The RNA ladder was prepared by adding 7 µL of RNA ladder and 7 µL of loading buffer, then mixed and incubated for 15 minutes at 65°C. Once the samples and ladder were loaded, the gel was run at 55 volts for 2.5 hours.

RNA Transfer

After the completion of the gel run, a picture of the RNA gel was taken and the RNA was blotted to a nitrocellulose membrane by capillarity transfer as followed explained. The gel was placed in a tray with RNase free water and gently shaken for 15 minutes. This step was repetead once. Followed, a long wet wick (made of Whatman paper) was placed onto the island that had been previously placed into a tray containing 20X SSC. A tube was rolled over the long wick to flush away any bubbles. The gel was placed face down on the long wick. The nitrocellulose membrane and two Whatman filter paper pieces were cut to perfectly fit the gel. The nitrocellulose membrane was soaked into DEPC water and then immediately into 20X SSC. The membrane was placed on top of the gel and bubbles were removed by rolling a tube over the membrane. Followed, the two filter papers were soaked into 20X SSC and placed on top of the nitrocellulose membrane, again bubbles were removed. A stack of paper towels was placed on top of the filter papers, and a cover and weight were placed on top of the paper towels. The next

morning, the membrane was removed from the setup, and the lanes were marked with a pencil. Following, the membrane was rinsed for a few seconds in a tray containing 2X SSC and then allowed to air dry on top of a Whatman paper. The gel was checked under the UV light to verify that the transfer was complete. Followed, the membrane was cross-linked using the Bio-Rad GS Gene Cross Linker at setting C3 (150 mjoules). The membrane with the Whatman paper was wrapped in saran wrap and stored in a dry place until use.

The *pha*A probe generation, labeling reaction, prehybridization/hybridization, membrane washing steps, and autoradiography were performed essentially as explained above in the Southern-Blot section.

Western-Blot Analysis

Protein samples were obtained from 100 mg of leaf material from wild type and transgenic lines by grinding the tissue to a fine powder in liquid nitrogen, subsequent homogenization in 200 μL plant protein extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl, 0.05% (w/v) Tween-20, 0.1% (w/v) SDS, 14 mM β-mercaptoethanol (BME), 400 mM sucrose and 2 mM phenylmethylsulfonyl fluoride) and a centrifugation step at $15.7 \times g$ for 1 minute to remove solids. Protein concentrations were determined by Bradford assay (Bio-Rad Protein Assay) with bovine serum albumin as the protein standard. Proteins were resolved by electrophoresis in a 12% (v/v) SDS-PAGE (Laemmli, 1970) and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked for 1 hr with PTM buffer: 1 x PBS (phosphate buffer solution), 0.05% (v/v) Tween-20 and 3% (w/v) non-fat

dry milk. The membrane was probed for 2 hrs with rabbit anti-β-kethiolase antibody (Metabolix, Cambridge, MA) in a dilution of 1:1,000, then rinsed with water twice and probed with alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit, Sigma) for 1.5 hrs in a 1:20,000 dilution. Finally, the membrane was washed 3 times for 10 minutes with PT buffer (1x PBS, 0.05% (v/v) Tween-20) and one time with 1 x PBS, followed by incubation in Lumi-phos WB (Pierce, Rockford, IL) reagent for the alkaline phosphatase reaction. Film exposure took place for 3 minutes.

β-Ketothiolase Activity Assay

Protein samples were obtained by grinding 1 g of leaf tissue to a fine powder in liquid nitrogen, followed by the addition of 2 ml ice cold β-ketothiolase extraction buffer (100 mM Tris-HCl pH 8.1, 50 mM $MgCl_2$, 5 mM βME) and homogenization. The homogenates were centrifuged for 10 minutes at 4°C at 5,000 g, and the supernatant was passed through PD-10 columns (Amersham, Arlington Heights, IL) containing Sephadex G-25 M for desalting, and elution was optimized for the recovery of proteins of size range 25 to 60 kDa. Protein concentration was determined by a Bradford assay. β-ketothiolase activity was measured spectrophotometrically at 304 nm in the thiolysis direction (breaking down acetoacetyl-CoA to acetyl-CoA) by monitoring the disappearance of acetoacetyl-CoA for 60 seconds, which in the presence of Mg ion forms a magnesium enolate with absorbance at 304 nm; this protocol is an adaptation of the protocol by Senior and Davis (1973). The reaction took place in a total volume of 1 ml containing 62.4 mM Tris-HCl pH 8.1, 50 mM $MgCl_2$, 62.5 μM CoA, 62.5 μM

Acetoacetyl-CoA (substrate was dissolved in sterile MilliQ water) and a volume of 50 mM phosphate buffer pH 4.7 equal to the volume of substrate used. The volume was brought 990 µl with sterile water. At the end 10 µl of plant extract (β-ketothiolase sample) were added, completing the 1 mL volume. The plant extract containing the β-ketothiolase was added at the end immediately before the sample reading. In this assay, the enzyme specific activity is given in units per mg of total plant protein and 1 unit is defined as the degradation of 1 µmol/min of acetoacetyl-CoA under standard reaction conditions.

Example:

Step 1: Assay for measuring acetoacetyl-CoA concentration for thiolase assay

a) Blank spectrophotometer at OD 304 with the following sample in a quartz cuvette:

31.2 µL from 2 M Tris-HCl, pH 8.1

500 µL from 100 mM $MgCl_2$

10 µL from 50 mM phosphate buffer, pH 4.7

458.8 µL H_2O

b) Measure at OD 304 of subtrate:

31.2 µL from 2 M Tris-HCl, pH 8.1

500 µL from 100 mM $MgCl_2$

10 µL substrate

10 µL from 50 mM phosphate buffer, pH 4.7

448.8 µL H_2O

c) Calculation of the concentration of acetoacetyl-CoA:

Absorbance 304 nm × (1÷extintion coefficient of sustrate) × (Moles÷L) × (1000 μL÷10 μL) ×

dilution factor = M/L

The extintion coefficient used for acetoacetyl-CoA was 16.9×10^3 $cm^{-1}M^{-1}$.

The dilution factor used was equal to 1.

Step 2: β-ketothiolase activity assay

a) Blank spectrophotometer with the following sample:

31.2 μL from 2 M Tris-HCl, pH 8.1

500 μL from 100 mM $MgCl_2$

62.5 μL from 1 mM CoA (final concentration 62.5 μM CoA)

Volume of 50 mM phosphate buffer, pH 4.7 is equal to the volume of substrate to be used

Bring to 1 mL total volume with water

b) Meassure slope due ti enzyme activity:

31.2 μL from 2 M Tris-HCl, pH 8.1

500 μL from 100 mM $MgCl_2$

62.5 μL from 1 mM CoA (final concentration 62.5 μM CoA)

62.5 μM acetoacetyl-CoA substrate (volume to be added is calculated from the empirical

substrate concentration)

Volume of 50 mM phosphate buffer, pH 4.7 is equal to the volume of substrate to be used

Bring to 990 mL with water

At the end at 10 μL of enzyme (plant lysate or purified enzyme)

c) Calculation of β-ketothiolase activity

i. Corrected Slope = slope of assay with enzyme – slope of assay without enzyme

ii. Corrected slope OD/sec $\times$ 60 sec/min $\times$ 1/Extintion coefficient substrate $\times$ mole/L $\times$ 10^6

μmol/mol $\times$ 0.001 L volume of enzyme assay = Y μmol/min = Y Units

iii. Specific activity (Units/mg) = (Z units/mL) ÷ by protein concentration of enzyme in mg/Ml

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed at the AMPAC facility at the University of Central Florida. Anthers and pollen samples were gold coated on a Sputter Coater (Emitech, Houston, TX) with a gold film thickness of 150 Amstroms. SEM pictures were produced using the scanning electron microscope model JSM-6400F (JEOL, Peabody, MA), and the x-ray energy dispersive spectrometer (Edax, Mahwah, NY) at an acceleration voltage of 6 kV.

Histological Analysis of Anthers

Anthers at relevant developmental stages were dissected from flower buds (all sepals and petals were removed to allowed better penetration of the fixative) and fixed in 3 % (v/v) glutaraldehyde in phosphate buffer for 12 hours at room temperature, applying a continuous vacuum for the first 3 hrs of incubation and degassing (by bringing the vacuum up and down slowly) for 10 min at 1 hr, 2hr and 3 hr. The volume of 3 % glutaldehyde solution used was 5 mL or 10 mL depending on the zise of the anters. The fixed anthers were dehydrated in an

ethanol series (5%, 10 % to 80 % in increments of 10%, 95 % and 100 %) for 30 min per gradient treatment, under constant vaccum. Samples were kept overnight in fresh 100 % ethanol with no vaccum application and were washed twice the next day for 1 hr in 100 % ethanol with no vaccum. The ethanol was removed from the tissue samples and then the samples were treated with a gradient (25 %, 50%, 75%) of Citro Solv clearing (Fisher, Pittsburgh, PA) reagent for 30 min per gradient treatment with no vaccum. Followed, the samples were treated with 100% Citro Solv for 1 hour with out vaccum application, this step was repeated twice. Without removing the Citro Solv 15 chips of Paraplast Plus (Fisher, Pittsburgh, PA) were added and left overnight at room temperature. Next day the solution was incubated 42°C to solubilized the remaining paraplast. Followed, 5 more chips were added and the solution was incubated at 42°C for 3 hours, this step was repeated twice. The paraffin/Citro Solv solution was removed and the tissue was embedded by adding 10 mL of molten paraffin to the tissue samples. The molten paraffin was changed every 8 to 12 hours for 3 days. Paraffin treated tissue was finally embedded into paraffin blocks by using the Leica EG 1160 paraffin embedding station (Leica, Solms). A metal blade microtome, model HM 315 (MICROM, Walldorf) was used for tissue embedded sectioning. Finally, tissue sections were put onto Superfrost/Plus microscope slides, which were placed in slide holders were they were allowed to driedout. Followed, slides were placed in an oven at 42°C to remove excess paraffin from the tissue section. Once most of the paraffin was melted and removed the slide containing the tissue sections were diped into 100% Citro solved for 15 minutes. Then, the slides were removed and inmerse into fresh 100% Citro Solv for 15 more minutes. Finally, the tissue sections were rehydrated with a decendent ethanol gradient (100%, 75%, 50%, 25%, 10% and water) for 10 minutes per gradient treatment. The tissue

sections were stained 0.05 % (w/v) toluidine blue. Tissue slides were observed under the Olympus BX60F5 light microscope and Olymppus U-CMAD-2 camera (Olympus, Melville, NY). Flower developmental stages were characterized following the procedure described by Koltunow et al. (1990).

Reversibility of Male Fertility

Two independent transgenic plants were moved to a separate growth chamber after the first indication of flower bud formation and were kept away from any contact with wild type and other transgenic lines; the flowers were covered with thin transparent plastic bags to inhibit any possibility of cross pollination. Bags were only removed to take pictures. Transgenic plants were kept under continuous illumination for 10 days with a photon flux density of 11,250 µEm-2 supplied throughout this period. The number of flowers developed was counted daily throughout these ten days, while newly formed flowers, senescent flowers, and fallen flowers were recorded. The development of fruit capsules and seeds were also counted. After the 10 days, a 16 hrs light/ 8 hrs dark photoperiod was reestablished, while the plants were kept from contact with any other plant for 20 days to allow maturation of the fruit capsules and to harvest seeds produced during continuous illumination.

Results

Chloroplast Transformation Vector

The *Acinetobacter sp* (accession: L37761) gene, *phaA* (1179 bp) coding for β-ketothiolase was amplified by PCR and cloned into the chloroplast transformation vector (pLD-ctv) to finally produce the pLDR-5'UTR-*pha*A vector (Fig 1). The vector contains homologous recombination sequences (flanking sequences), which allow site-specific integration by homologous recombination into the inverted repeat region of the chloroplast genome in between the *trn*I (tRNA Ile) and *trn*A (tRNA Ala) genes (Ruiz et al., 2003). This specific targeting mechanism allows high efficiency integration of the transgene construct containing the *aad*A (aminoglicoside 3'-adenylyltransferase) gene, which confers spectinomycin resistance and the *pha*A genes. The transcription of the *aad*A and *pha*A gene is driven by the constitutive action of the chloroplast 16S ribosomal RNA gene promoter (*Prrn*), which is found upstream of the *aad*A gene and should produce dicistrons (*aad*A-*pha*A). Additionally, the *psb*A gene promoter and 5' regulatory sequence (5' untranslated region; 5' UTR), which is known to enhance translation of foreign genes (Eibl et al., 1999; Fernandez-San Millan et al., 2003; Daniell et al., 2004a; Dhingra et al., 2004), was used upstream of the *pha*A gene and should produce *pha*A monocistrons. At the 3' end of the gene construct is the 3' *psb*A untranslated region (3'UTR), which is known to be involved in mRNA abundance and stability in chloroplast (Marchfelder and Binder, 2004).

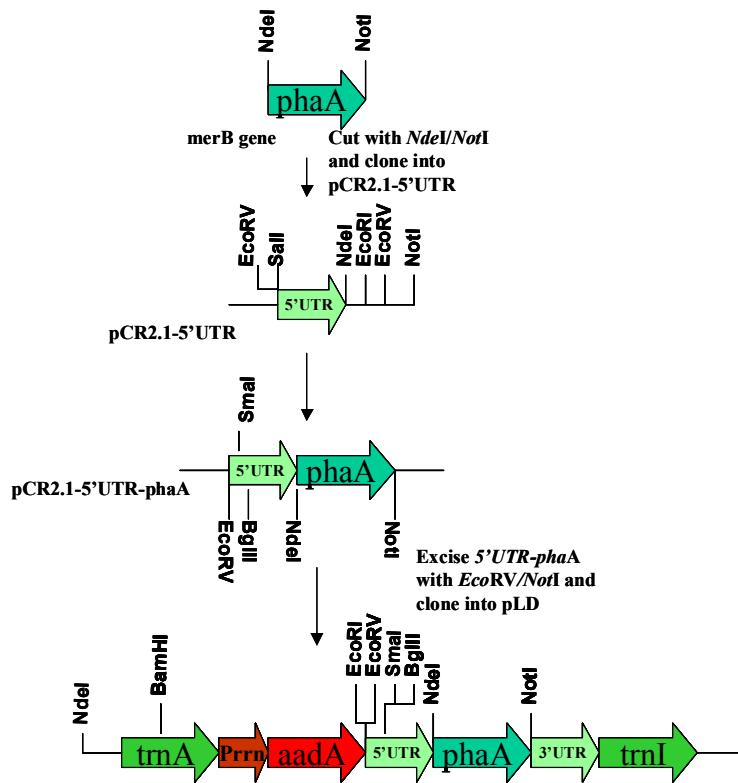**Figure 1: pLDR-5'UTR-*pha*A Vector Construction**
The map shows the specific steps and restriction enzymes used in the vector construction as well as additional restriction sites in the gene construct. PCR product *pha*A, was cloned inframe into the pCR2.1-5'UTR vector. The final step shows the integration of the 5'UTR-phaA gene construct into the pLD-ctv vector to for the functional pLDR-5'UTR-*pha*A vector.

Transformation and Selection of Chloroplast Transgenic Plants

Chloroplast transgenic plants were obtained through particle bombardment following the method described previously (Daniell, 1997; Daniell et al., 2004a; Kumar and Daniell, 2004a). More than 10 positive independent transgenic lines were obtained. Transgenic lines transformed with the pLDR-5'UR-*pha*A vector as explained in the Materials and Methods section were passed through a selection process. Three days after bombardment the bombarded leaf tissue was placed on regeneration media with 500 μg /mL spectinomycin antibiotic. After 3 to 4 weeks in selection the first putative transgenic shoots were observed (Fig 2A). The transgenic shoot is characterized by being green over a background of bleach leaf material (Fig2A). From the first selection the transgenic lines were move to second selection, were pieces of leaf material from the transgenic plant recovered from first selection were placed in media with 500 μg/mL spectinomycing for selection (Fig 2B). During this selection many more shoots identical (clones) to the parental primary transgenic shoot are generated (Fig 2B). Because the initial leaf tissue use in secondary selection is transgenic the callus does not bleach as much as in first selection, allowing faster regeneration of the transgenic clones. This step increases homoplasmy by selecting the transgenic chloroplast and genomes. Finally, once fully regenerated plants are recovered from second selection, these are transfer to third selection. Third selection is performed in MS medium amended with 500 μg/mL spectinomycin to induce rooting and the developments of a fully normal plant (Fig 2C). Finally, the transgenic plants were move to soil and allowed to grow to collect seeds. Transgenic seeds were recovered from the different

48

transgenic lines and were germinating in MS medium with 500 µg/mL spectinomycing to

demonstrate maternal inheritance of the genetic engineered traits.

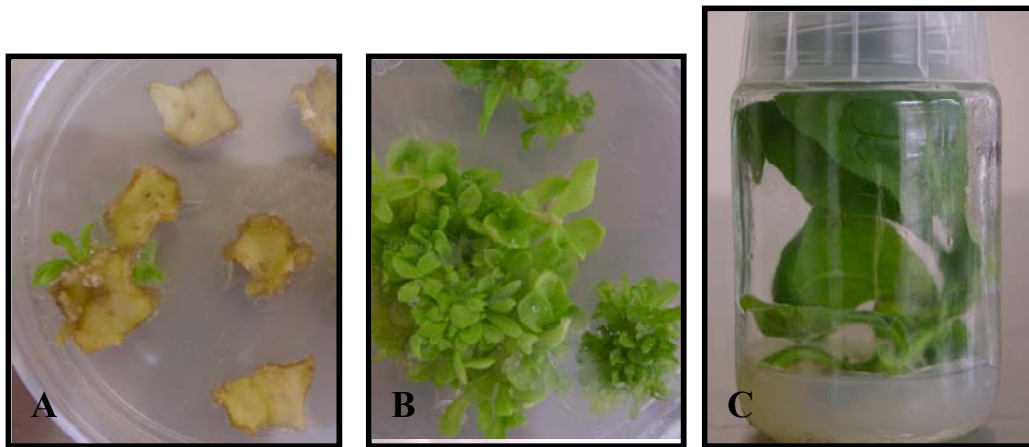**Figure 2: Selection of Chloroplast Transgenic Plants**
A, Transgenic plant shoot in first selection (RMOP medium with 500 µg/mL spectinomycing).
B, Second selection in RMOP medium with spectinomycin shows multiple transgenic shoot. C,
Third selection in MSO medium with spectinomycin.

Molecular Characterization of Chloroplast Transgenic Plants

In this report, several transgenic lines are characterized, confirming that independent chloroplast transgenic lines show little variation in foreign gene expression (Daniell et al., 2001). PCR based analysis with the primer pairs, 3P and 3M and 4P-4M were used to test the integration of the transgene construct into the chloroplast genome. The 3P and 4P primers land on the native chloroplast genome, upstream of the gene cassette, and the 3M and 4M primers land on the *aad*A gene, which is located within the gene cassette (Fig 3A). If site-specific integration has occurred, a PCR product of 1.65 kb should be obtained, such product was detected in transgenic lines (Fig 3C lanes 2, 3). Untransformed plants as well as mutant plants, which had undergone spontaneous mutation of the 16S rRNA gene and acquired resistance to spectinomycin, did not show any PCR product, indicating that these plants are negative for integration (Fig 3C lane 1). The integration of the *aad*A gene, as well as the *pha*A was further confirmed by the use of primer pairs 5P-2M, 5P-3'phaA, and 5P-phaAinternal, which produce PCR products of sizes 3.56 kb, 2.0 kb and 1.5 kb, respectively. These primers land at different location within the gene construct (see Fig 3A). Results revealed specific PCR size products in the transgenic lines (Fig 3C lanes 4-6), confirming the presence of the *pha*A gene.

The DNA from T_0 and T_1 generation transgenic plants as well as from wild type plant (wt) was extracted and used for Southern-blot analysis (Fig 3D-G). The flanking sequence probe of size 0.8 kb (Fig 3B), which hybridizes with the *trn*I and *trn*A genes, allows the detection of site-specific integration of the transgene cassette as well as the achievement of homoplasmy of the transgenic chloroplast genome. Additionally, the *pha*A probe (~1.2 kb) was used to confirm

the presence of the *pha*A gene. DNA from untransformed plants and transgenic lines were digested with *Bam*HI (Fig 3A and B) and probed with either a flanking sequence probe or a *pha*A probe, resulting in the detection of a 10 kb transgenic chloroplast fragment (Fig 3D-G lanes 2-4). The detection of a 7.1 kb fragment by the flanking sequence probe in the wild type indicated that these chloroplasts lacked the integration of the foreign genes (Fig 3D, F lane 1). The fact that no 7.1 kb size fragment (wt size) in the transgenic sample was observed indicated that homoplasmy had been achieved through the selection process even in T_0 (Fig 3D lanes 2-4) and was maintained in T_1 generation (Fig 3F lanes 2-4), confirming stable integration of foreign genes within all chloroplast genomes (to the limits of detection by Southern blots). The lack of signal in the wild type when screened with the *pha*A probe indicated the absence of *pha*A gene (Fig 3E,G lane 1). If any unexpected size fragment was observed in the transgenic samples when probing with the transgene, unspecific integration into another plant genome would be indicated (Daniell and Parkinson, 2003). This was not observed.

Transcript abundance and stability from chloroplast transgenic plants was studied by northern-blot analysis using the gene specific probe *pha*A on total plant RNA (Fig 3H). The chloroplast transgenic lines were expected to transcribe a 2,255 nt dicistron (*aad*A-*pha*A) from the upstream 16S promoter (P*rrn*), in addition to a 1,384 nt monocistron (*pha*A) transcribed from the *psb*A promoter located upstream from the *pha*A gene (Fig 3H lanes 2-3). The results showed both the monocistron and dicistron transcripts were abundant in the transgenic plants, probably because of the action of the *psb*A and P*rrn* promoters, which are strong promoters. Additionally, larger size transcripts were detected, with one of them correlating with the size of a transcript starting at the chloroplast native 16S promoter (P*rrn*) and terminating at the 3' UTR *psb*A; the

51

predicted size of this transcript is 4,723 nt. Other transcripts detected may be either read through (because 3' UTR does not terminate transcription efficiently in chloroplast) or processing products.
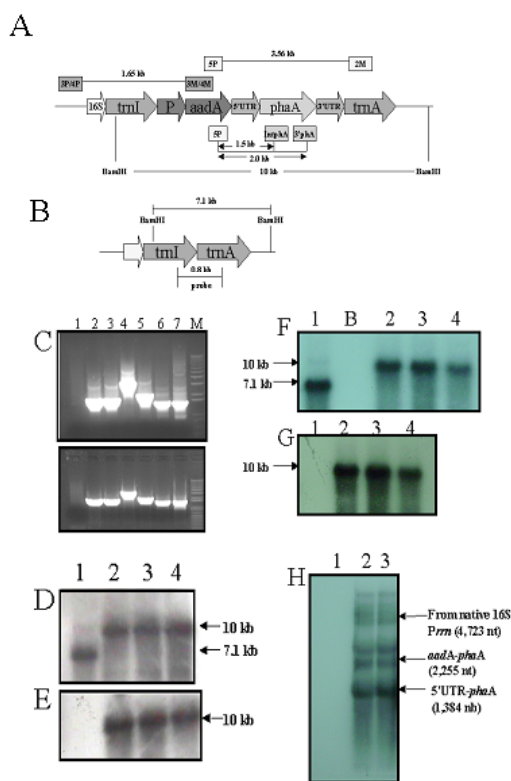
Figure 3: Molecular Characterization of Transgenic Lines

A, Schematic representation of the transformed chloroplast genome and the pLDR-5'UTR-*pha*A construct. Landing sites for the primer pairs and expected sizes of PCR products are shown. *Bam*HI restriction sites, DNA fragment produced after restriction digestion and the *pha*A probe, used in Southern-blot analyses are shown. B, The map shows the wild-type chloroplast genome, restriction digestion sites used for Southern-blot analysis, expected fragment size after digestion and the flanking sequence probe. C, PCR analysis of wild type and putative transformants. Top panel, transgenic line 4B; bottom panel is transgenic line 4A. 1: Untransformed wild type; 2: 3P-3M (1.65 kb); 3: 4P-4M (1.65 kb); 4: 5P-2M (3.56 kb); 5: 5P-3'phaA (2.0 kb); 6: 5P-phaAinternal (1.5 kb); 7: positive control (pLD-5'UTR-*phb*A plasmid DNA) 5P-phbAinternal (1.5 kb); M: marker. D and F, Southern-blot analysis of T_0 and T_1 generation transgenic lines, respectively, with the chloroplast flanking sequence probe; 10 kb fragment showing integration of transgenes observed, 7.1 kb fragment detected in wild type sample. E and G, transgenic T_0 and T_1 plants, respectively, and wild type plant probed with the *pha*A probe. 10 kb fragment observed in transgenic lines but not in wild type. H, northern-blot analysis using the *pha*A probe. Mononocistron and polycistrons containing the *pha*A gene observed in the transgenic lines. In figure 1D-H; 1: wild type; 2-4: chloroplast transgenic lines 4a, 4b, 4c, respectively.

53

β-Ketothiolase Expression and Activity

To confirm expression of β-ketothiolase in the chloroplast transgenic lines, untransformed and transformed plants were subjected to western-blot analysis by using anti-β-ketothiolase antibody. Chloroplast-synthesized β-ketothiolase treated with β-mercaptoethanol (BME) and boiled appeared mostly as monomeric forms (40.8 kDa), or in polymeric forms, which included the homotetrameric form (163 kDa) (Fig 4B lanes 2-6). No β-ketothiolase was detected in wild type samples (Fig 4B lane 1). The appearance of a distinct band at 40.8 kDa in the Coomassie-stained sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel (Fig 4A lanes 1-4) but not in the unstransformed sample (lane 5) suggests that the chloroplast transgenic plants were expressing high levels of β-ketothiolase (Fig 4A).

The activity of the chloroplast-expressed β-ketothiolase was measured in the thiolysis direction (breaking down acetoacetyl-CoA to acetyl-CoA) spectrophotometrically at 304 nm (Fig 4C). The chloroplast transgenic lines showed β-ketothiolase activities that were up to 30-fold higher than previous levels demonstrated from nuclear transgenic plants. No endogenous β-ketothiolase activity was detected in untransformed tobacco plants. The chloroplast transgenic lines showed levels of activity that reached 15.38 U/mg total plant protein and compared positively with the levels of activities obtained for our purified bacterial β-ketothiolase, 48.41 U/mg. The high levels of enzymatic activity correlated well with the high amounts of protein detected by the Coomassie-stained gel and western-blot analyses performed on total plant samples; these results suggested that the enzyme was in its biosynthetically active form

(tetramer). No adverse effects were observed in the transgenic lines hyperexpressing β-ketothiolase. Compartmentalization of proteins in chloroplasts has been shown to avoid pleiotropic effects, as previously reported for CTB (Daniell et al., 2001a), trehalose (Lee et al., 2003) and xylanase (Leelavathi et al., 2003).
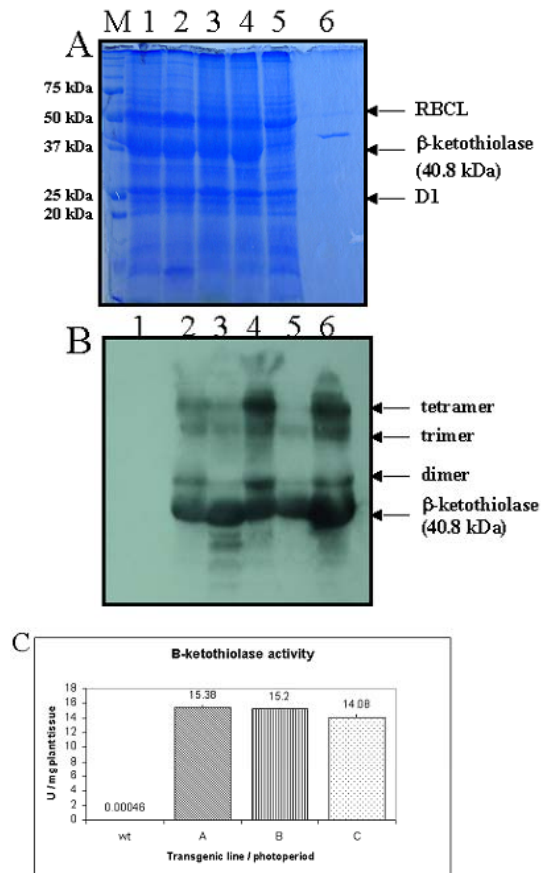
Figure 4: β-Ketothiolase Characterization in Transgenic Lines

A, Coomassie-stain shows abundant β-kethothiolase expression in transgenic plants, 15 μg total plant protein was used per lane. M: marker; 1-3: transgenic lines 4a, 4b, 4c, respectively; 4: 4a T_1 generation; 5: wild type; 6: bacterial purified β-ketothiolase (positive control). B, western-blot analysis: 10 μg total plant protein from transgenic lines and wild type were loaded per lane, anti-β-ketothiolase antibody was used. 40.8 kDa monomers as well as multimers can be observed in transgenic lines. 1: wild type; 2-4: transgenic lines 4a, 4b, 4c, respectively; 5-6: 4a and 4b T_1 generation, respectively. C, β-kethotiolase activity was measured spectrophotometrically at 304 nm. WT: wild type; A, B: transgenic lines 4A and 4B under continuous light for 5 days; C: transgenic line 4B under normal photoperiod (16hr light).

Male Sterility Characterization

From the 10 T_0 transgenic lines expressing β-ketothiolase, 100 % of the flowers produced by transgenic plants failed to develop fruit capsules and seeds, finally senescing and falling off (Fig 5, a-c). The male sterility phenotype was maintained in T_1 generation transgenic lines. T_0 (Fig 5, l) and T_1 (Fig 5, m) generation transgenic plants showed no difference in growth and development when compared to untransformed tobacco plants under the same growth conditions. Chlorophyll content analysis of three independent T_1 transgenic lines showed average chlorophyll content of 1.90 ± 0.12 mg/g fresh weight. The chlorophyll content of three wild type plants average, 1.92 ± 0.20 mg/g fresh weight. These results showed that the chlorophyll levels in the leaves of the transgenic lines expressing β-ketothiolase were similar to the levels in wild type tobacco plants, confirming that chloroplast biosynthetic functions and integrity of the thylakoid membranes, although β-ketothiolase was hyperexpressed. However, the chloroplast transgenic lines showed specific defects in anther development and failed to produce viable pollen. The anthers were characterized by the lack of pollen grains (Fig 5, h) or when pollen grains were formed, they were abnormal with a collapsed phenotype (Fig 6). Additionally, the stamens were shorter (Fig 5, f) than in wild type plants (Fig 5, g), adding a degree of severity to the male sterile phenotype. To investigate that plants were sterile due to lack of pollen or non-viable pollen, emasculated wild type flowers were pollinated with the pollen from the transgenic anthers, but these crosses failed to produce any seeds. The shorter stamens may be a consequence of the failure of the cells to elongate in the central and upper parts of the anther filament. To investigate the possibility of female infertility affecting the transgenic plants, they

57

were fertilized with pollen from wild type. The transgenic plants developed normal fruit capsules (Fig 5, j) and normal seeds that were able to germinate and develop normally (Fig 5, k). The T_1 seedlings grew well in the medium supplemented with 500 μg/ml spectinomycin and were identical to the parental line (T_0) and Southern blot analysis showed the presence of the gene construct (Fig 1F, G).

Scanning electron microscopy was performed on transgenic anthers as well as wild type anthers to further characterize male sterility in transgenic plants. The SEM revealed that the pollen grains in the transgenic anthers exhibited collapsed morphology and consisted of a heterogeneous population with respect to size and shape (Fig 6, d-f). Wild type anthers showed a homogenous population of pollen grains of uniform size and shape (Fig 6, a-c). The apparent lack of turgidity of the transgenic pollen may be produced by lack of intracellular material, resulting in the distorted and collapsed morphology. The aberrant pollen morphology observed under the SEM may account for the inability of the transgenic plant to produce seeds.
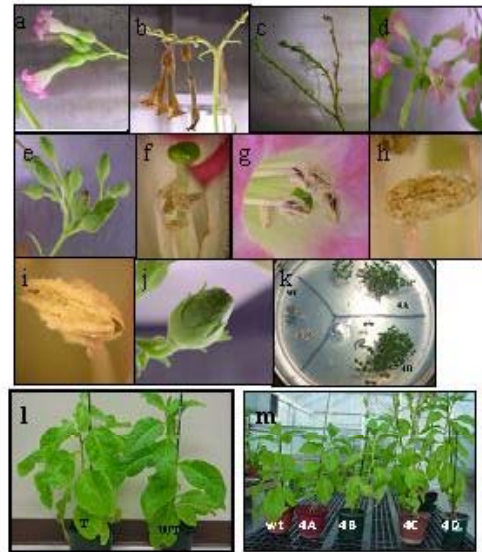
Figure 5: Characterization of male sterility, growth and development.
A-C, Flowers from transgenic lines; note the absence of fruit capsules and fallen flowers. D and E, wild type tobacco flowers and fruit capsules. F and G, comparison of stamens and stigma. Note shorter stamens in transgenic lines (F). H and I, comparison of mature anthers. Note abundant pollen in untransformed plant (I) and the lack of pollen in transformed anther (H). J, transgenic fruit capsule with seeds developed after pollination of transgenic stigma with wild type pollen. K, germination and growth of T_1 seedlings on medium with 500 µg/ml spectinomycin. wt: wild type; 4A: T1 transgenic line 4A; 4B: T_1 transgenic line 4B, obtained after pollination with WT pollen. L and M, comparison of growth and development. L, wild type and T_0 generation transgenic line (4A) grown for two months in soil. M, wild type and T_1 generation independent transgenic lines 4A, 4B, 4C and 4D 1.5 month after germination.
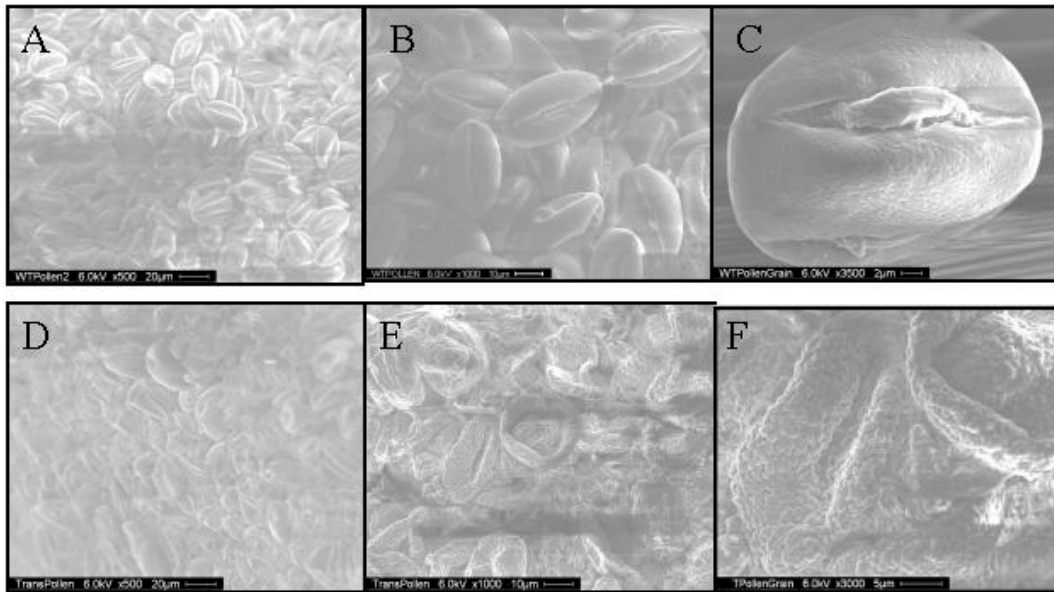
Figure 6: Scanning Electron Micrograph
SEM pictures of pollen grains in anthers of wild type (A-C) and transgenic (D-F) plants at different magnifications. Magnification: A, D: × 500; B, E: ×1000; C: ×3500; F: ×3000.

β-Ketothiolase Expression in Anthers

Plastids in anthers may be in low abundance when compared to the numbers in leaves, but they produce enough β-ketothiolase to affect pollen development in anthers. As shown in figure 7, flowers and specifically anthers were green during anther development (stage 1 of flower development is shown). This means that chloroplasts are present and are metabolically active. Northern-blot analysis of leaves showed that the mRNAs coding for β-ketothiolase are found in monocistronic and polycistronic form, the monocistron form being the most abundant transcript (Fig 1H). The same pattern was maintained in the *pha*A transcripts in flowers and anthers (Fig 7B). Northern-blot analysis of transgenic flowers and anthers showed that transcription of the *pha*A gene occurs in the flower and anther; this was expected because P*rrn* and *psb*A promoters are constitutive promoters, allowing transcription to occur in both photosynthetic as well as non-photosynthetically active plastids (Fig 7B). The translation of the *pha*A monocistron is under the light regulated *psb*A 5' regulatory sequence but because the flowers, as well as anthers are green, containing photosynthetically active chloroplasts, translation was quite efficient (Fig 7C). β-ketothiolase was detected by western-blot in both whole flowers and anthers from transgenic plants, confirming that the β-ketothiolase was present during anther development, and should play a role in the male sterility phenotype (Fig 7C).
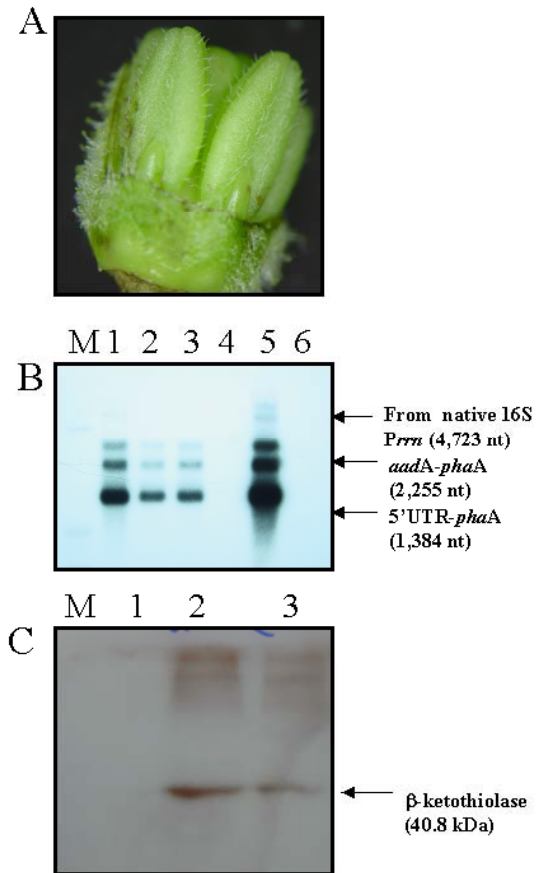
Figure 7: β-Ketothiolase Expression in Anthers
A, Pigmentation of anthers during flower development in transgenic plants, Stage 1 of flower development is shown. B, Northern-blot analysis of flower and anthers. Monocistrons and polycistron are observed. 3 μg of total plant RNA was loaded per lane and the *pha*A probe was used. M: marker; 1: transgenic flower; 2-3: transgenic anthers form line 4A and 4B; 4: wild type flower; 5: transgenic leaf line 4A; 6: wild type leaf. C, β-ketothiolase expression in transgenic flower and anther detected by western-blot analysis, lanes 2 and 3, respectively. RNA and protein samples used per lane were the product of the combined extraction from flowers or anthers from stage 1 and 3.

Anther Development and Male Sterility

Analysis of anther development revealed that the anthers of the transgenic lines followed an accelerated pace in their development and maturation resulting in aberrant tissue patterns (Fig 8). At stage –3 of flower development the WT plants showed a normal pattern of tissue development, where all major tissues were differentiated, the anther had acquired its characteristic shape, all tissues were interconnected and there was presence of callose depositions between microspore mother cells. This pattern was not followed in the transgenic lines, which at stage –3, showed characteristics of a more advanced stage. The transgenic anthers at stage –3, showed the microspores in tetrads, with stomium differentiation occurring and the tapetum was shrunken and broken. This pattern presented characteristics of more advanced stages of flower development, which included stages +1 and +2. Additionally, the transgenic anthers showed abnormal development of the epidermis and endothecium, probably resulting in the aberrant shape of anthers. The anthers of the transgenic lines at stage –2 were also advanced in the aberrant phenotype with the microspores already separated, a developmental step that should have been observed at stage +2. Again the tapetal layer was broken. We noticed that the transgenic anthers at stage –5, which is a very early stage of flower development, showed great similarities with stage –2 in the wild type plants, but aberrant pattern of tissue development could still be observed. At stage –1 in transgenic line the tapetum was shrunken and discontinuous and formation of pollen grains was evident at stage 1. These morphological changes observed in stage –1 and +1 should be observed at much later stages, +3 and +4. At early stages of floral development (stages -5 to +1), transgenic lines showed accelerated anther development, which

averaged +3 stages ahead from the wild type plants. At late stages of floral development, accelerated phenotype increased even more, at an average of 4 to 6 stages ahead of wild type. At stage +2 in the transgenic lines, cells adjacent to the stomium had degenerated and only remnants of the tapetum were observed. The thickening of the outer wall is accompanied by enlarged endothecium and vacuolation, which greatly decreased the inner space of the locules, crushing pollen grains and resulted in the irregular shape and collapsed phenotype. The developmental changes observed in the transgenic anthers at stage +2, although aberrant, were similar to the ones observed in wt at stage +6. In the wild type, abundant normal pollen grains were observed. Almost complete degradation of the connective tissue that separates the pollen sac occurred at stage +3 in transgenic anthers, while this occurred at stage +9 in wild type plants. Finally, both wild type and transgenic anthers were bilocular, connective tissue was absent but the major difference was that abundant pollen was present in the WT (stage +11) but not in transgenic anthers (transgenic, stage +9). Additionally, the pollen grains formed in transgenic anthers were collapsed. The data presented here allows us to understand the effect of β-ketothiolase during anther development in the transgenic lines.

Anther development is a very complex process involving the coordination of several genes and the specific development and maturation of several tissues and cells (Yui et al., 2003); any defect in these well-coordinated processes may lead to dysfunctional pollen. Many male-sterility systems produced by mutations or nuclear expressions of foreign proteins have shown to interfere with the function or differentiation of tapetum, indicating that this tissue is essential for the production of viable pollen (Goetz et al., 2001). Here we observed that the tapetum of the transgenic lines was severely impaired. The tapetum is critical for the development of pollen by

secreting essential substances such as proteins (Yui et al., 2003), carbohydrates (Goetz et al., 2001) and lipids (Zheng et al., 2003) into the locules. Developing microspores and the surrounding tapetal cells have been shown to be particularly active in lipid metabolism (Zheng et al., 2003). The precise differentiation and maturation of tapetum with respect to microspore development is of major importance for the successful production of pollen. Here we observed complete dysfunction in the anther tissue differentiation patterns, which may be caused by an alteration in chloroplast fatty acid metabolism in the transgenic lines expressing the *pha*A gene, affecting the development of pollen grains.
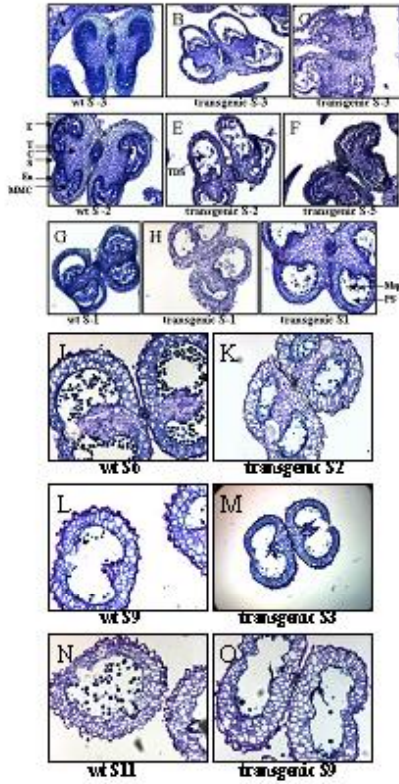
Figure 8: Analysis of Anther Development
Bright-field photographs of wild type (wt) and transgenic anthers at different developmental stages (S). Anthers at the designated stages were fixed, embedded with paraffin, and sliced into 5 and 10 μm transverse sections. The fixed sections were stained with toluidine blue and visualized under the light microscope at a magnifications of ×100. C, connective tissue; E, epidermis; En, endothecium; MMC, microspore mother cells; Msp, microspores; PS, pollen sac; S, stomium; T, tapetum.

Reversibility of Male Fertility

To test whether depletion of the acetyl-CoA pool destined for *de novo* fatty acid biosynthesis in chloroplast by β-ketothiolase is the cause of the male sterility phenotype, we decided to explore if a continuous light could revert male fertility of the chloroplast transgenic lines. It has been shown that the intermediates of fatty acid biosynthesis change during the transition to darkness in leaves and chloroplasts in a manner consistent with control at the levels of ACCase (Post-Beittenmiller et al., 1991). Recent reports have shown light-dependent regulation of ACCase by the redox status of the plastid whereby the enzyme is more active under the reducing conditions observed in light (Page et al., 1994; Kozaki et al., 2000). Therefore, two independent transgenic lines 4A and 4B were exposed to continuous light for a period of 10 days. These plants had been previously characterized and shown to be 100% male sterile, unable to produce any fruit capsules or seeds. These plants were isolated from all other plants (transgenic and wild types) in a growth chamber at the first indication of flower bud development (before any flower was opened), and the flowers were covered with transparent plastic bags to avoid of cross-pollination. We observed that from a total of 20 flowers produced during the 10 days of illumination by the two transgenic plants, 4 flowers were able to produce pollen (Fig 9A), normal length anther filaments (Fig 9A), developed fruit capsules (Fig 9B) and produced seeds (Fig 9C). Transgenic line 4A produced 3 viable flowers during the 10 day continuous light photoperiod, all of them being produced between days 8 and 10. Transgenic line 4B produced one viable flower at day 9 and was able to produce additional viable flowers three

days after the 10 days assay was completed. The seeds recovered (Fig 9C) from the reverted male fertility study were able to germinate in a medium with 500 µg/mL spectinomycin (Fig 9D), indicating that these seedlings were transgenic and contained the transgene; wild type tobacco seedlings were bleached (Fig 9E). These findings support the hypothesis that an increase in ACCase activity outcompetes, at least partially, the removal of acetyl-CoA by β-ketothiolase. Additionally, this line of evidence shows that male sterility is caused by the effect of β-ketothiolase expression in the chloroplast and not by any other unknown process.
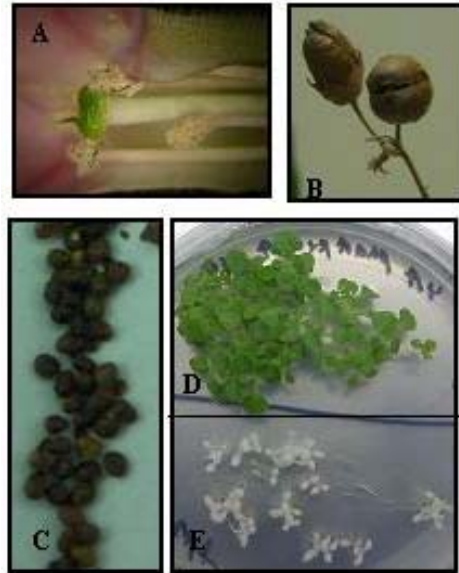
Figure 9: Reversibility of male fertility after 10 days under continuous light.
A, transgenic flower after 9 days in continuous light. A, note normal length of the anther filaments and pollen grains.. B, fully developed fruit capsules containing seeds from transgenic lines. C, abundant seeds from transgenic fruit capsule. D, seedlings produced via the reversibility to male fertility. Transgenic seeds germinated and grown green in medium supplied with 500 μg/mL spectinomycin. E, bleached wild type tobacco seedlings.

Discussion

We have shown that the hyperexpression of β-kethothiolase via chloroplast transformation resulted in normal growth and pigmentation, even when the activity of the enzyme was very high. Succesful expression of β-ketothiolase in transgenic plants showed that this enzyme could be safely expressed in the chloroplast and suggested that the complete PHB pathway needs to be expressed in order to cause the stunted phenotype. Although no growth reduction was observed in the chloroplast transgenic lines expressing β-ketothiolase, 100% male sterility was observed. This was interesting and worthy of further investigation because of the importance of the production of male sterile lines in gene containment and hybrid seed production (Perez-Prat and van Lookeren, 2002). Because the expression of β-ketothiolase did not disrupt growth and normal development, with the exception of the lack of pollen formation, the expression of β-ketothiolase may be used as a mechanism to generate a male sterility system, producing 100% infertility that can be applied to different plant species. Concerns related to constitutive expression of *pha*A can be easily overcome by restricting *pha*A expression to the anthers, where pollen formation occurs. Such transgenic plant systems expressing a chloroplast targeted T7 RNA polymerase via the nuclear genome, regulating the expression of a chloroplast integrated transgene under the g10 T7 promoter has been reported in the literature (McBride et al., 1994; Magee and Kavanagh, 2002). A similar approach in which the T7 RNA polymerase gene regulated by an anther specific promoter could be used to specifically induce *pha*A expression in anther plastids for transgene containment or hybrid seed production.

Here we demonstrate that it is possible to revert the chloroplast transgenic lines to fertility by continuous light exposure. This supports a mechanism of action in which β-ketothiolase depleted the pool of acetyl-CoA in the chloroplast, but by increasing acetyl-CoA carboxylase (ACCase) activity by continuous light (Post-Beittenmiller et al., 2001; Kozaki et al., 2000; Page et al., 1994), ACCase was able to compete more effectively for acetyl-CoA, thereby increasing the levels of plastidic fatty acid biosynthesis. Developing microspores and surrounding tapetal cells have been shown to be particularly active in lipid metabolism (Yui et al., 2003), which is especially needed for the formation of the exine pollen wall from sporopollenin (Wang et al., 2001; Ariizumi et al., 2003, 2004). Support for the normal growth and development observed in the chloroplast transgenic lines expressing *pha*A also comes from studies where a mutant plant with 10% activity of acyl-CoA synthase showed normal content of lipids in leaves and normal growth (Schnurr et al., 2002), indicating that under normal growth conditions, even severely impaired plants in fatty acid biosynthesis were able to grow normally. Alternatively, the formation of acetoacetyl-CoA or β-ketothiolase itself may have a detrimental effect on chloroplast metabolism. This view is hard to support from our results, because neither β-ketothiolase expression, nor acetoacetyl-CoA was affected by continuous illumination.

Chloroplasts genetic engineering approach offers a number of attractive advantages, including high-level transgene expression (Daniell and Dhingra et al., 2002a), multi-gene engineering in a single transformation event (DeCosa et al., 2001; Daniell and Dhingra, 2002; Ruiz et al., 2003; Lossl et al., 2003), transgene containment via maternal inheritance (Daniell et al., 2002b), lack of gene silencing (Ruiz et al., 2003),lack of position effect due to site specific transgene integration and lack of pleiotropic effects due to sub-cellular compartmentalization of

transgene products (Daniell et al., 2001a; Lee et al., 2003). Genetically engineered cytoplasmic male sterile via the chloroplast genome may be used for the safe expression of foreign genes via the nuclear genome and in those rare cases where plastids genomes are transferred paternally (Hagemann, 2004) or biparentally (Smith et al., 1986; Wang et al., 2004). Recently, plastid transformation was demonstrated in carrot (Kumar et al., 2004b), showing hyperexpression of the transgene in non-green plastids to levels of up to 75% the expression in leaf chloroplast. Additionally, plastid transformation of recalcitrant crops such as cotton (Kumar et al., 2004c) and soybean (Dufourmantel et al., 2004) indicates that chloroplast genetic engineering has overcome major hurdles and allows the application of the cytoplasmic male sterility system to commercially important crops.

In currently available cytoplasmic male sterile lines, nuclear genome controls various restoration factors and such factors are often located at multiple loci and are poorly understood. Also, CMS has been associated with rare diseases (Wise et al., 1999). In the case of β-ketothiolase induced male sterility, fertility can be obtained by the use of regulatory or inducible elements instead of constitutive expression; this would also increase the applicability of the cytoplasmic male sterility system for the production of hybrid crops. Also, a major disadvantage of nuclear-engineered male sterility is the possibility of seed production in the field, that could spread introduced traits. In contrast, if male sterility is engineered via the plastid genome, such a situation could be averted because of maternal inheritance of transgenes, which will not allow the male sterility trait to be diluted out. Thus, this is a novel approach for creating male sterile transgenic plants, which may help advance the field of plant biotechnology through effective transgene containment.

CHARACTERIZATION OF HETEROLOGOUS MULTIGENE OPERONS IN TRANSGENIC CHLOROPLASTS: TRANSCRIPTION, PROCESSING AND TRANSLATION

Introduction

Plastid genes in higher plants are mainly organized as operons, of which more than sixty have been described in tobacco chloroplast genome (Sugita and Sugiura, 1996). These may group genes of related or unrelated functions, the former being the most common (Barkan, 1988; Rochaix, 1996). Most of these genes are transcribed into polycistronic precursors that may be later processed and modified to render the transcripts competent for translation (Eibl *et al.,* 1999; Barkan & Goldschmidt-Clermont, 2000; Monde et al., 2000b).

The processing mechanisms for translation regulation in chloroplast genes of higher plants are still largely unknown. The general consensus is that most primary transcripts require processing in order to be functional (Barkan, 1988; Zerges, 2000; Meierhoff *et al.,* 2003), and that post-transcriptional RNA processing of primary transcripts represents an important control of chloroplast gene expression (Hashimoto et al., 2003; Nickelsen, 2003; Marchfelder & Binder, 2004). However, it is believed that more than one pathway may be involved in transcript processing (Danon, 1997; Choquet and Wollman, 2002).

For example, several studies have shown that the regulation of gene expression in the chloroplast relies more on RNA stability than on transcriptional regulation (Deng and Gruissem, 1987; Jiao et al., 2004). This stability is mainly influenced by the presence of 5'UTRs (Eibl *et*

73

al., 1999; Zou *et al.,* 2003), nuclear factors (Lezhneva and Meurer, 2004) and 3' UTRs (Adams and Stern, 1990; Chen and Stern, 1991, Marchefelder and Binder, 2004), without which rapid degradation or low accumulation of primary transcripts has been observed. The role of plastid 3'UTR differs from the one 3' untranslated regions (UTR) have been known to have in prokaryotes as effective transcription terminators; a role that was observed to be ineffective in chloroplasts (Stern and Gruissem, 1987).

Translation has been also a crucial step in the regulation of gene expression, as in many cases protein levels in the chloroplast did not correlate with steady-state transcript abundance (Monde *et al.,* 2000b). Therefore, the transcription of native chloroplast operons and their post-transcriptional and translational patterns have been the target of several studies. These have led to observations in which intercistronic processing was shown to enhance translation in chloroplast operons, such as the maize *psb*B and *pet* clusters (Barkan, 1988; Barkan *et al.,* 1994). This translation enhancement may be aided by the formation of stable hairpin structures in the intergenic region of the polycistronic transcript, which contains the ribosome-binding site and the start codons, thus preventing translation unless this site is spliced (Barkan *et al.,* 1994; Barkan, 2004).

Intergenic secondary structures have also been observed in tobacco, which play a role in monocistron processing prior to translation (Hirose and Sugiura, 1997). However, species such as *Arabidopsis* (Meierhoff et al., 2003)*,* tobacco (Monde et al., 2000a) and spinach (Westhoff and Herrmann*,* 1988) have a different mechanism than maize for the translation of the same *pet*D gene. *In vivo* mRNA studies in tobacco revealed that the translation of the *pet*D gene depends upon the establishment of dicistrons and tricistrons of this gene. This alternative processing,

which produces monocistronic *pet*D, causes the degradation of the transcript, inhibiting translation (Meierhoff *et al.,* 2003; Tanaka *et al.,* 1987; Monde *et al.,* 2000a, b). In *Chlamydomonas,* nearly all chloroplast genes appear to be transcribed as monocistronic mRNAs, with translation being an essential regulatory step of gene expression (Rochaix *et al.,* 1989; Zerges and Rochaix, 1994). Thus, different species may experience various processing mechanisms for the same gene cluster. Other mechanisms have also been observed, such as alternative processing, which produces a start codon triggered by C to U editing (Hirose and Sugiura, 1997; del Campo *et al.,* 2002). These examples provide evidence of different modifications of primary transcripts for efficient translation.

Traditionally, plant genetic engineering had involved the introduction of single genes through nuclear transformation. In the past decade, the introduction of multiple genes has also been successful through nuclear transformation, allowing the incorporation of complete metabolic pathways (Ma *et al.,* 1995; Nawrath *et al.,* 1994; Ye *et al.,* 2000). However, this was the result of a long process of integration of individual transgenes followed by breeding to reconstruct the desired pathways. Additionally, transgene segregation from nuclear transformed plants may be possible in subsequent generations, which may result in loss of function of the introduced pathway. Furthermore, plant nuclear genes are typically transcribed monocistronically, which requires separate promoter sequences for each of the introduced genes. Expression of foreign genes may also be influenced by position effects and gene silencing, causing levels of gene expression to vary among independent transgenic lines (Daniel and Dhingra, 2002).

On the other hand, plant genetic engineering through chloroplast transformation presents several additional advantages over nuclear transformation, such as their ability to efficiently transcribe and translate operons (DeCosa *et al.,* 2001; Lossl *et al.,* 2003; Ruiz *et al.,* 2003), as well as to confer hyperexpression capability (Daniell *et al.,* 2004c). In addition, chloroplasts are able to accumulate foreign proteins that are toxic in the cytoplasm, such as cholera toxin B subunit (Daniell *et al.,* 2001), trehalose (Lee *et al.,* 2003), and xylanase (Leelavathi *et al.,* 2003), without any deleterious effect, due to compartmentalization of transgene products (Bogorad, 2000). Concerns about position effect are also eliminated because of site-specific integration of transgenes via homologous recombination of chloroplast DNA flanking sequences (Daniell *et al.,* 2002) and, because chloroplasts are maternally inherited in most crops, the risk of outcrossing transgenes to related species through pollen is minimized (Daniell, 2002). Transformation of plastids in non-green tissues, such as carrots, offer promising options for oral delivery of vaccine antigens (Kumar *et al.,* 2004a).

As foreign genes are engineered into operons, the resulting transcript differs from the native operons by lacking native intergenic sequences that can form stable secondary structures. These sequences are removed during cloning or by PCR amplification of the heterologous genes. The effect of such modifications in transcription and translation has not yet been investigated for heterologous operons harboring multiple genes. Therefore, the purpose of this study is to examine how foreign operons engineered via the chloroplast genome are transcribed, processed and translated. The results obtained provide sufficient evidence to suggest that engineered polycistrons in chloroplast transgenic lines are efficiently translated and that processing into monocistrons is not required to obtain hyperexpression of transgenes. Additionally, this study

provides insight into the role of bacterial intergenic spacer sequences in post-transcriptional modifications and translation, as well as the role of 3' UTR in transcript stability. Addressing questions about polycistron translation as well as the sequences required for processing and transcript stability are essential for future approaches using metabolic engineering.

Materials and Methods

Chloroplast Transformation, Selection and Characterization of Transgenic Plants

The chloroplast transformation, selection and characterization of the transgenic lines used in this study have been previously reported (Daniell et al., 2001; De Cosa et al., 2001; Lee et al., 2003; Fernandez-San Millan et al., 2003) with the exception of the *ctb-gfp* transgenic lines. Sterile tobacco leaves were bombarded using the Bio-Rad PDS-1000/He biolistic device as described previously (Daniell, 1997; Daniell et al., 2004a, b).

Chloroplast Expression Vector Carrying the *hsa* Gene

The pLDA-sdHSA vector was constructed by inserting the *hsa* gene (1.8 kb) into *Eco*RI/*Not*I sites of the multiple cloning site of the chloroplast transformation vector (pLD-ctv). This construct contained the *hsa* and a ribosome binding site sequence (ggagg) upstream of the gene. For the pLDA-5'UTR-*hsa* vector, the promoter and 5'UTR (205 bp) from *psb*A gene were amplified by PCR from tobacco chloroplast DNA and then sequenced. The subsequent in-frame

cloning of the promoter/5'UTR upstream and *hsa* gene into pLD-ctv vector by EcoRI/NotI digestion produced the functional gene cassette.

Chloroplast Expression Vector Carrying the *ctx*B Gene

A ribosome binding site (GGAGG) was engineered five bases upstream of the start codon of the *ctx*B gene. The PCR product was then cloned into pCR2.1 vector (Invitrogen) and subsequently cloned into the chloroplast transformation vector (pLD-ctv) after the sequencing of the open reading frame. The pLD vector carrying the *ctx*B gene was used for successive transformation of tobacco chloroplast genome according to the published protocol.

Chloroplast Expression Vector Carrying the *tps*1 Gene

The yeast trehalose phosphate synthase (*tps*1) gene was inserted into the *Xba*I site of the universal chloroplast expression (pCt) vector between the *aad*A selection marker gene for spectinomycin resistance and the *psb*A terminator to form the final pCt-*tps*1 vector.

Chloroplast Expression Vector Carrying the *Cry*2Aa2 Operon

The *cry*2Aa2 operon was inserted into the universal chloroplast expression vector, pLD ctv, to form the final shuttle vector pLD-BD *Cry*2Aa2 operon (De Cosa *et al.,* 2001). This vector contains the 16S ribosomal RNA (rRNA) promoter (P*rrn*) upstream of the *aad*A gene

(aminoglycoside 3'-adenylyltransferase) for spectinomycin resistance, the three genes of the *cry*2Aa2 operon, and the *psb*A terminator from the 3' region of the chloroplast photosystem II gene.

Plant Transformation

Tobacco leaves were transformed by particle bombardment (Bio-Rad PDS-1000He device), using 0.6 μm gold microcarriers coated with the pCt-TPS1 chloroplast expression vector, and delivered at 1,100 psi with a target distance of 9 cm (Daniell, 1997). The bombarded leaves were selected on RMOP medium containing 500 μg/ml spectinomycin to regenerate the transformants, as previously described (Kumar and Daniell, 2004; Daniell *et al.,* 2004a)

Northern-Blot Analysis

Total plant RNA from untransformed and T_1 chloroplast transgenic plants, was isolated by using the RNeasy Mini Kit (Qiagen, Valencia, CA) and protocol. Northern blot analyses were performed essentially as follows. Total RNA (1 mg) per plant sample was resolved in a 1.2% (w/v) agarose/formaldehyde gel at 55 V for 2.5 h. The RNA was transferred overnight to a nitrocellulose membrane by capillarity. The next day, the membrane was rinsed twice in 2 X SSC (0.3 M NaCl and 0.03 M sodium citrate), dried on Whatman paper, and then cross-linked in the GS Gene Linker (Bio-Rad, Hercules, CA) at setting C3 (150 njouls).

The probes used for northern blot analyses were obtained as follows: the *aad*A (aminoglycoside 3' adenylyl transferase) probe was obtained by *Bst*EII/*Xba*I restriction digestion of plasmid pUC19-16S/*aad*A; the *ctx*B (cholera toxin β-subunit) and *tps*1 (trehalose phosphate synthase) probes were obtained by *Xba*I restriction digestion of plasmids pSBL-CTB and pSBL-TPS1, respectively. The plasmid pCR2.1-5'UTR-PA was digested with *Hin*dIII and *Bam*HI to obtain the pa (anthrax protective antigen) probe, whereas the *Cry*2Aa2 (*Bacillus thuringiensis* insecticidal protein) probe was obtained by *Xba*I digestion of plasmid pSBL-ctv-*Cry*IIA. The *hsa* (human serum albumin) probe was obtained by *Eco*RV/*Not*I digestion of plasmid pCR2.1 ATG-*HSA*.

Probes were radio labeled with ^{32}P dCTP by using Ready Mix® and Quant G-50® micro columns for purification (Amersham, Arlington Heights, IL). Prehybridization and hybridization were performed using the Quick-Hyb® solution (Stratagene, La Jolla, Ca). The membrane was then washed twice for 15 min at room temperature in 2 X SSC with 0.1% (w/v) SDS, followed by two additional washes at 60°C (to increase the stringency) for 15 min with 0.1 X SSC with 0.1% (w/v) SDS. Radiolabeled blots were exposed to x-ray films and then developed in the Mini-Medical Series x-ray film processor (AFP Imaging, Elmsford, NY). Relative transcript levels were measured by spot densitometry (Alphaimager 3300, Alpha Innotech, San Leandro, CA) on radiograms from the different northern blot analyses.

Western-Blot Analyses

Protein samples were obtained from 100 mg of leaf material from wild-type and transgenic lines by grinding the tissue to a fine powder in liquid nitrogen. Subsequent homogenization in 200 ml plant protein extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl, 0.05% (w/v) Tween-20, 0.1% (w/v) SDS, 14 mM b-mercaptoethanol (BME), 400 mM sucrose and 2 mM phenylmethylsulfonyl fluoride) was performed, followed by a centrifugation step at 15.7 ´ g for 1 minute to remove solids. The *Bacillus thuringiensis Cry*2Aa2 protein was extracted from 100 mg of transgenic leaf material by adding 200 ml of 50 mM NaOH to solubilize the *Cry* protein from the crystals formed in the transgenic plants, and centrifuged at 10,000 x g for 1 minute to remove cell debris. Protein concentrations for the samples were determined by Bradford assay (Bio-Rad Protein Assay) with bovine serum albumin as the protein standard.

Proteins were resolved by electrophoresis in a 12% (v/v) SDS-PAGEgel, and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked for 1hr with PTM buffer: 1 x PBS (phosphate buffer solution), 0.05% (v/v) Tween-20 and 3% (w/v) non-fat dry milk. The membranes were probed with primary and secondary antibodies as follows: for 2 hrs with primary antibody, then rinsed with water twice and probed with secondary antibody for 1.5 hrs. Finally, the membranes were washed 3 times for 15 minutes with PT buffer (1 x PBS, 0.05% (v/v) Tween-20) and one time with 1 x PBS for 10 minutes, followed by incubation in Lumi-phos® WB (Pierce, Rockford, IL) reagent for the alkaline phosphatase reaction or SuperSignal (Pierce) reagent for horseradish peroxidase (HRP) reaction.

Film exposure took place for 1, 3, 5 or 10 minutes, depending on the strength of the signal of each blot. The antibodies used and their respective dilutions were the following: anti-*cry*2A (Envirologix, Portland, ME), dilution 1:3,000; anti-*ORF*2 (Moar et al., 1989), dilution 1:1,000; anti-*HSA* (Sigma, St. Louis, MO), dilution 1:3,000; anti-CTB (Sigma), dilution 1:2,500; anti-*PA* (Dr. Stephan Leppla, NIH), dilution 1:30,000. Secondary antibodies were used as follows: alkaline phosphatase conjugated anti-rabbit antibody (Sigma) was used to probe against every primary antibody with the exception of anti-PA which was probed with HRP conjugated anti-mouse antibody; dilutions of 1:5,000 anti-rabbit antibody were used for anti-*HSA*, anti-*ORF*2 and anti-*Cry*2A, for anti-*CTB* the dilution was 1:4,000. Anti-mouse antibody was used in a 1:5,000 dilution.

ELISA Quantification

The Human Albumin Quantitation Kit (Bethyl Laboratories) was used for ELISA quantification. Leaf material (100 mg) of the *aad*A-*orf*1-*orf*2-*hsa* transgenic line as ground in liquid nitrogen and resuspended in 700 ml of 50 mM NaOH. The leaf extracts were then diluted to fit in the linear range of the provided HSA standard. Absorbance was read at 450 nm. The DC protein assay (Bio-Rad) was used to determine total soluble protein concentration following the manufacturer's protocol.

Results

Multigene Engineering in the Chloroplast

Multigene engineering in the chloroplast was achieved by using several examples of foreign genes, promoters, and 5' and 3' regulatory sequences (Daniell *et al.,* 2004a; Kumar and Daniell, 2004). All the constructs analyzed contained the *aad*A (aminoglicoside 3'-adenylyltransferase) gene, which confers resistance to spectinomycin (Goldschmidt-Clermont, 1991), and are driven by the constitutive chloroplast 16S ribosomal RNA gene promoter (*Prrn*), located upstream of the *aad*A gene. The gene or genes of interest were inserted downstream of the *aad*A gene and were flanked at the 3' end by the *psb*A untranslated region (3'UTR), which is involved in mRNA abundance and stability in the chloroplast (Deng and Gruissem, 1987; Stern and Gruissem, 1987; Marchfelder and Binder, 2004). These cassettes were flanked at the 5' and 3' ends by sequences homologous to the tobacco chloroplast *trn*I (tRNA Ile) and *trn*A (tRNA Ala) genes, respectively, allowing site-specific integration by homologous recombination into the inverted repeat region of the chloroplast genome (Daniell et al., 1988). More than thirty genes have been successfully integrated and expressed at this transcriptionally active spacer region (Daniell et al., 2004a, b). Some of the foreign genes were engineered downstream of the *psb*A promoter and 5' regulatory sequence (5' untranslated region; 5'UTR), which enhances translation (Eibl et al., 1999; Fernandez-San Millan et al., 2003; Dhingra et al., 2004). The following foreign genes were inserted into the expression cassettes: *aad*A, human serum albumin (*hsa*), cholera toxin β subunit (*ctx*B) fusion with GFP (green fluorescent protein), anthrax

protective antigen (*pa*), *Bacillus thuringiensis* insecticidal protein (*cry*2Aa2), along with an associated chaperonin protein (*orf*2), and trehalose phosphate synthase (*tps*1). Additionally, transgenic lines engineered to express Cry insecticidal protein contained the entire *cry*2Aa2 operon.

Transcription and Translation of the *cry* Operon

The chloroplast transgenic lines transformed with the transgene cassette containing the *aad*A gene and the complete *cry*2Aa2 operon were used to study the transcriptional and translational patterns of a heterologous operon in transgenic chloroplasts. This operon comprises the *orf*1, *orf*2, and *cry*2Aa2 genes under the regulation of the *Prrn* promoter (Figure 10A). Several transcripts were anticipated based on transcription initiation at the engineered promoter (P*rrn* promoter) and the native 16S ribosomal RNA promoter (native P*rrn*) in transgenic lines (Figure 10A). Northern blot analyses of three independent lines harboring the *cry*2Aa2 operon revealed that the predicted 4,944 nt polycistron containing all four transgenes (*aad*A-*orf*1-*orf*2-*cry*2Aa2) was the most abundant transcript detected when the *cry*2Aa2 specific probe was used (Figure 10B, c). Interestingly, the *cry* specific probe also revealed a shorter transcript of about 2,400 nt; which was of the same size as the *cry*2Aa2 gene (Figure 10B, a), suggesting that this transcript should be the *cry*2aA2 monocistron. Densitometric analysis revealed that the *cry*2Aa2 monocistron and the *aad*A-*orf*1-*orf*2-*cry*2Aa2 polycistron were equally abundant (Figure 10C), indicating that processing in the intergenic region between *orf*2 and *cry*2Aa2 occurred in about 50 percent of the polycistrons transcribed from the engineered P*rrn* promoter (Figure 10A, B a, c). Another prominent 7,445 nt transcript was predicted to initiate from the native 16S P*rrn*

promoter (Figure 10B, e). A low intensity ~6,000 nt transcript detected (Figure 1B, d) may be produced by read-through of the transcript starting at the *Prrn* promoter and terminating downstream of the engineered 3' UTR. The low intensity ~3,500 nt transcript (Figure 10B, b) terminates at the same location as the 6,000 nt transcript, although it is smaller due to the processing between *orf*2 and *cry*2Aa2. Because this fragment only contained the *cry* gene and sequences downstream from the 3'UTR, it cannot be detected with the *aad*A probe. The read-through transcripts processed downstream of the 3'UTR represent an average of 27.31 ± 2.73 % of those produced in these transgenic lines (Figure 10B, b, d).

Northern blot analyses with the *aad*A specific probe confirmed the results observed with the *cry*2Aa2 probe. The predicted 4,944 nt polycistron that harbors the *aad*A gene plus the complete *cry* operon was detected as expected (Figure 10D, c). Surprisingly, the predicted 2,528 nt tricistron (Figure 10D, f) containing the *aad*A gene plus the *orf*1 and 2, which was expected to be produced by the processing (observed with the cry probe) between the *orf*2 and the *cry*2Aa2 genes, produced a transcript of a similar intensity to that of the polycistron (Figure 10D, c, f). Densitometric analyses revealed a 1 to 1 ratio of the polycistron (*aad*A-*orf*1-*orf*2-*cry*2Aa2) versus the *aad*A-*orf*1-*orf*2 tricistron (Figure 10E), due to processing in the intergenic region between *orf*2 and *cry*2Aa2 (Figure 10A, B). These results showed that the two transcripts produced by the processing in the intergenic region between *orf*2 and *cry*2Aa2 resulted in transcripts with a similar abundance to the complete polycistron containing all four genes and the 3'UTR. The fact that the tricistron containing the *aad*A, *orf*1 and *orf*2 genes did not contain a chloroplast 3'UTR but still was very stable, suggests that polycistrons are stable in the chloroplast even in the absence of 3'UTRs. Additionally, when the *aad*A probe was used, no

transcripts smaller than tricistrons (2,528 nt) were observed. This confirms the lack of further processing of polycistrons. Furthermore, computational analyses predicted that the 90 nt intergenic sequence between the *orf*2 and the *cry*2Aa2 genes could form a highly-stable secondary structure (Figure 10H), with a free energy of −16.44 kcal/mol, which masked the Shine-Dalgarno sequence (ribosome binding site) of the *cry*2Aa2 gene.

Western blot analyses revealed that the *Cry*2Aa2 protein (65 kDa; Figure 10F) and the *ORF*2 protein (45 kDa; Figure 10G) were hyperexpressed in the transgenic lines. The abundant expression of the *orf*2 confirmed that polycistrons were efficiently translated without the need for processing into monocistrons.
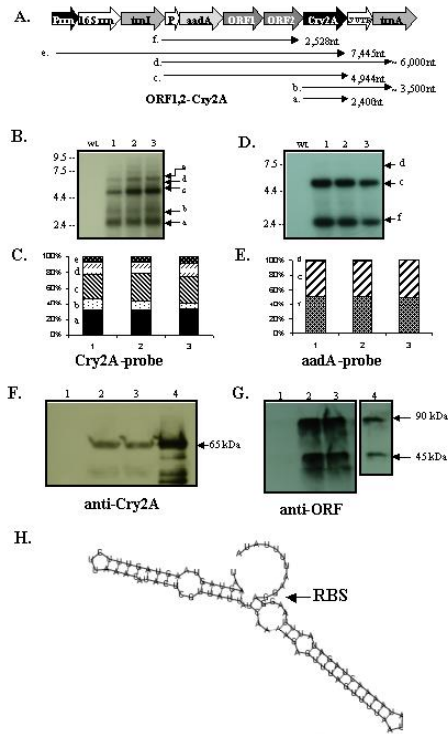
Figure 10: Transcriptional and translational analysis of the *Cry*2Aa2 operon

A. Schematic representation of the *orf*1-orf2-*cry*2Aa2 operon in transgenic lines, including the *aad*A gene and the upstream P*rrn* promoter; upstream native chloroplast 16S ribosomal RNA gene with its respective promoter and the *trn*I and *trn*A are shown. Arrows represent expected transcripts and their respective sizes. B. RNA hybridized with the *cry*2A probe, loaded as follows: wt: wild type control; lanes 1, 2 and 3: *cry*2Aa2 operon transgenic lines. Transcripts of the *cry*2Aa2 operon are indicated by lowercase letters and correspond to the transcripts depicted in A. C. Relative transcript abundance for each sample hybridized with the *cry*2A probe. D. Transcript analysis showing RNA hybridization with the *aad*A probe, loaded as follows: wt: wild type control, lanes 1-3 = *cry*2Aa2 operon transgenic lines. Transcripts of the *cry*2Aa2 operon are of sizes as described for the *cry*2Aa2 probe; f is *aad*A/*orf*1/orf2 tricistron, 2,528 nt. E. Transcript quantification for samples hybridized with the *aad*A probe. F. Western blot analysis using the *Cry*2Aa2 antibody. Lane 1: wild type control; lanes 2 and 3: *cry*2Aa2 operon transgenic lines; lane 4: positive control (*Cry*2Aa2 protein). The expected polypeptide of 65 kDa is shown in both transgenic plants and the positive control. G. Western blot analysis using the *ORF*2 antibody. Lane 1: wild type control; lanes 2 and 3: cry2Aa2 operon transgenic lines; lane 4: positive control (*ORF*2 protein). The expected polypeptide of 45 kDa is shown in both transgenic plants and the positive control. H. Predicted secondary mRNA structure of the intergenic sequence between the *orf*2 and *cry*2Aa2 genes. The arrow indicates the Shine-Dalgarno sequence or ribosome-binding site (RBS).

Transcription and Translation of the *hsa* Operon

Chloroplast transgenic lines transformed with 3 different multigene constructs, all containing the human serum albumin (*hsa*) gene, were used to study transcription, translation and posttranscriptional modifications. The *Prrn* promoter drives the operon downstream in all three constructs. The first transgenic line has an operon formed by the *aad*A followed by the *hsa* gene, the second transgenic line contained a four gene operon formed by the *aad*A, *orf*1, *orf*2 and *hsa* genes. The third transgenic line harbored an expression cassette that contained the *aad*A gene under the direct transcriptional regulation of the P*rrn* promoter, and the *hsa* gene under the transcriptional regulation of the *psb*A promoter and the translational enhancement of the 5' UTR *psb*A. This transgenic line was predicted to produce a monocistronic *hsa* transcript (Figure 11A).

Northern blot analyses of the transgenic lines expressing the *aad*A-*hsa* operon with the *hsa* and the *aad*A probes revealed that the most abundant transcript was a dicistron of a predicted 2,797 nt (*aad*A-*hsa*, Figure 11B, D, b), followed by two polycistronic transcripts, one transcribed from the native 16S *Prrn* promoter of an expected size of 5,298 nt (Figure 11B, D, h), and the other of 3,200 nt transcribed (Figure 11B, D, d) from the engineered *Prrn* promoter, terminating downstream of the 3'UTR of the gene cassette. No monocistrons were detected in these transgenic lines. Quantification of transcripts from the northern blots obtained with the *hsa* probe revealed that the polycistrons transcribed from the engineered P*rrn* promoter accounted for 65.49 ± 3.26% of the transcript detected in these lines (Figure 11C). The polycistrons terminating downstream of the 3'UTR in the *trn*A region and the one transcribed from the native16S P*rrn* were 24.93 ± 2.11% and 9.57 ± 1.29% of the total transcripts, respectively (Figure 11C). Values

for the northern analysis performed with the *aad*A probe were similar, with 61.83 ± 3.37% for the polycistron transcribed from the engineered P*rrn* promoter, 31.71 ± 3.14% and 6.46 ± 0.26% for the read-through transcript and the polycistron transcribed from the native promoter, respectively (Figure 11E). This analysis confirmed that there is abundant read-though transcription.

The transgenic lines containing the *aad*A-*orf*1-*orf*2-*hsa* operon showed a similar transcription pattern with respect to the *aad*A-*hsa* transgenic line when probed with the *hsa* or *aad*A probe. The most abundant transcript was the polycistron containing all four genes (predicted size of 4,397 nt), which was transcribed from the engineered *Prrn* promoter, representing 68.08 ± 1.72 % and 65.54 ± 1.49% of the total transcripts detected with the *hsa* or *aad*A probe, respectively (Figure 11B, D, f). Additionally, the predicted 6,898 nt polycistron originating at the *Prrn* native promoter was also detected (Figure 11D, k) and this represented 6.79 ± 0.44% of the polycistrons (Figure 11E). A ~5,200 nt transcript (Figure 11B, D, g) obtained from the engineered 16S P*rrn* and processed downstream of the 3'UTR was also detected. This transcript was about 27.67 ± 1.08% and 31.92 ± 1.72% of the polycistrons detected with the *aad*A or *hsa* probes (Figure 2C, E), respectively. Finally, transgenic lines engineered with the *aad*A-5'UTR-*hsa* construct produced transcripts about 200 nt longer than the transgenic lines transformed with the *aad*A-*hsa* construct (Figure 11B, D, c, i). This increase in transcript size is due to the presence of the *psb*A5'UTR and promoter. Additionally, this transgenic line produced an abundant *hsa* monocistron (2,069 nt) that accounted for 50% of the total transcript detected with the *hsa* probe (Figure 11B, C a); this transcript was not detected with the *aad*A probe (Figure 11D). The polycistrons transcribed from the engineered P*rrn* and

native promoter were 28.72 ± 3.04 % and 9.36 ± 1.66% of the transcripts produced (Figure 11A). Similar transcript abundance was detected in northern blot analyses in which the *aad*A probe was used. Additionally, read-through transcripts processed downstream of the transgene cassette, in the *trn*A native gene, were detected (Figure 11B, D letters e, j). The combined abundance of these transcripts was 15.5% (Figure 11A), whereas the overall polycistron abundance in this transgenic line was as much as the monocistronic transcript.

Western blot analyses of the different constructs showed abundant expression of the HSA protein (69 kDa) in the transgenic lines harboring the 5'UTR-*hsa* and the *orf*1,2-*hsa* constructs (Figure 11F). Transgenic lines expressing the monocistrons showed expression levels similar to the *orf*1-*orf*2-*hsa* transgenic line where only polycistrons were translated. The abundant translation of ORF2 protein (45 kDa) from the *aad*A-*orf*1-*orf*2-*hsa* transgenic lines (Figure 11G) transcribing only tricistrons and polycistrons support the view that polycistrons are highly stable in the chloroplast and can be efficiently translated without further processing. Hyperexpresion of the *hsa* gene in this transgenic line at levels similar to *psb*A-5'UTR-*hsa* transgenic lines again confirm similar translation efficiency of polycistrons and monocistrons (Figure 11F).

HSA accumulation under different periods of illumination was monitored by ELISA analyses on the *aad*A-*orf*1-*orf*2-*hsa* transgenic line to determine whether *hsa* expression under the *cry* 5'UTR is light dependent or developmentally regulated. The data obtained after young, mature and old leaves were exposed to periods of 0, 4, 8, and 16 hours of light, revealed no significant differences among age of leaf or among different periods of illumination. Therefore, HSA accumulation in this transgenic line regulated by a heterologous UTR is independent of light regulation and is free of cellular control (Figure 12).
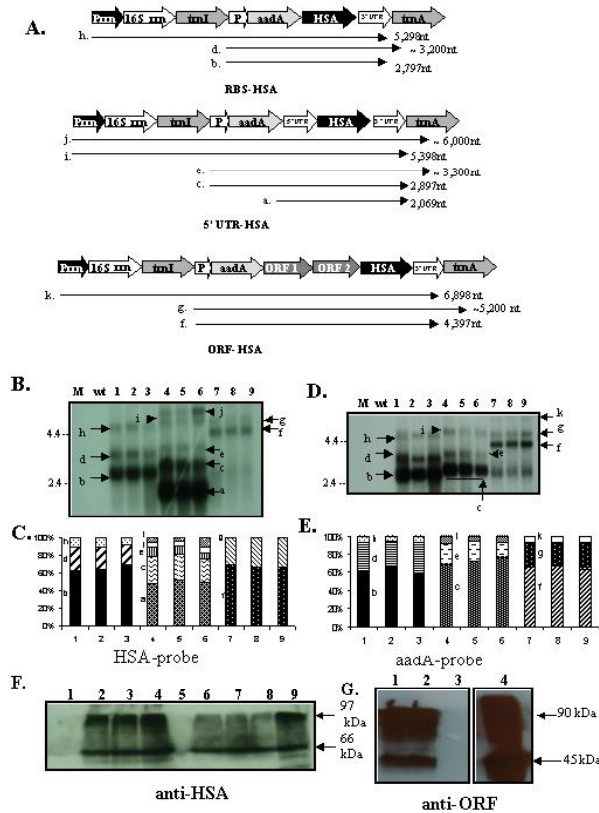
Figure 11: Transcriptional and translational analysis of the *HSA* operons

A. Schematic representation of the *hsa* operons (rbs*hsa*, 5'UTR*hsa*, *orf*1-*orf*2-*hsa*) in transgenic lines, including the *aad*A gene and upstream P*rrn* promoter; upstream native chloroplast 16S ribosomal RNA gene and promoter as well as *trn*I/*trn*A genes are shown. Arrows represent expected transcripts and their respective sizes. B. RNA hybridization with the *hsa* probe. M: molecular weight marker; wt: wild type; lanes 1-3: rbs-*hsa* transgenic lines; lanes 4-6: 5'UTR-*hsa* transgenic lines; lanes 7-9: *orf*1,2-*hsa* transgenic lines. The transcripts observed correspond to those indicated in A. by lowercase letters. C. Relative abundance of the transcripts obtained with the *hsa* probe. D. mRNA transcripts hybridized with the *aad*A probe and loaded in the same order as in B. Transcripts a-i corresponded to the same transcripts observed in B, "k" is 16*rrn*/*hsa* polycistron, 6,898 nt. E. Quantification of the transcripts obtained with the *aad*A probe. F. Western blot analysis using the *HSA* antibody. Lane 1: wild type control. Lanes 2-3: RBS-*hsa* transgenic lines; lanes 4-6: 5'UTR*hsa* transgenic lines. Lane 5 was left blank. Lanes 7 and 8: *orf*1,2-*hsa* transgenic lines. Lane 9: positive control (HSA protein). All samples presented 66 kDa and 97 kDa peptides, corresponding to the size of the *HSA* protein, and its dimeric form, respectively. G. Western blot analysis using the *ORF*2 antibody. Lanes 1-2: *orf*1,2-*hsa* transgenic lines; lane 3: wild type control; lane 4: positive control (ORF protein). 45 kDa ORF2 and 90 kDa dimer are shown.
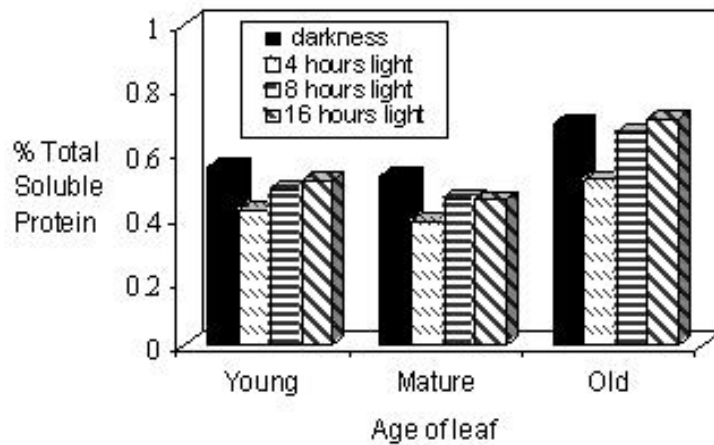
Figure 12: ELISA analysis of the *orf*1-*orf*2-*hsa* transgenic line.
Total soluble protein content of young, mature and old leaf extracts of the *orf*1-*orf*2-*hsa* transgenic lines determined by ELISA analyses. Transgenic plants were subjected to the following light conditions: 4, 8, and 16 hours of light, as well as total darkness.

Transcription and Translation of the *tps*1 Operon

The *tps*1 gene coding for trehalose phosphate synthase was engineered into a two-gene operon, formed by the *aad*A and the *tps*1 genes, and transcribed from the engineered *Prrn* promoter (Figure 13A). Northern blot analyses with either the *tps*1-specific or *aad*A-specific probes detected the expected 2,717 nt dicistron (*aad*A-*tps*1) as the most prominent transcript (Figure 13B, D, a). Densitometric analyses of the northern blots showed that the *aad*A-*tps*1 dicistron accounted for 43.27 ± 2.85% of the total transcripts detected by the *tps*1 probe, and 59.78 ± 5.45 % of the total transcript when the *aad*A probe was used (Figure 13C, E). A predicted 5,218 nt polycistron observed in the northern blots with either the *tps*1 and *aad*A probes (Figure 13B, D, c), is transcribed from the native 16S *Prrn* (Figure 13A). Additionally, the *tps*1 probe detected less abundant polycistrons of sizes ~3,500 nt (Figure 13B, D, b) and ~6,500 nt (Figure 13B, d), transcribed from the engineered *Prrn* promoter and the native 16S P*rrn* promoter, respectively, terminating downstream of the 3'UTR. The 3,500 nt polycistron was also detected by the *aad*A probe. Transcripts that ended in the *trn*A intron region (downstream of the engineered 3'UTR) were also detected in the *cry* transgenic lines (Fig 13B,D), indicating that this region may contain processing sequences. The transcripts processed at the *trn*A location account for about 37% of the total transcripts detected in the transgenic lines (Figure 13C, E). Transcripts longer than the 6,500 nt polycistrons may be read-through, terminating at undetermined locations and these were not quantified densitometrically. No monocistron was detected in the northern blots with the *tps*1 probe or the *aad*A probe, indicating

that there is no processing of the polycistron in these transgenic lines, whereas larger transcripts detected are likely to be read-through.

Western blot analyses performed to detect the trehalose phosphate synthase revealed efficient translation of polycistrons, as shown by the abundant accumulation of a 65 kDa polypeptide corresponding to this protein (Figure 13F). Because no monocistrons for *tps*1 or *aad*A were detected in the northern blot analyses, hyperexpression of TPS1 should thus be the result of efficient translation of polycistrons in transgenic chloroplasts.
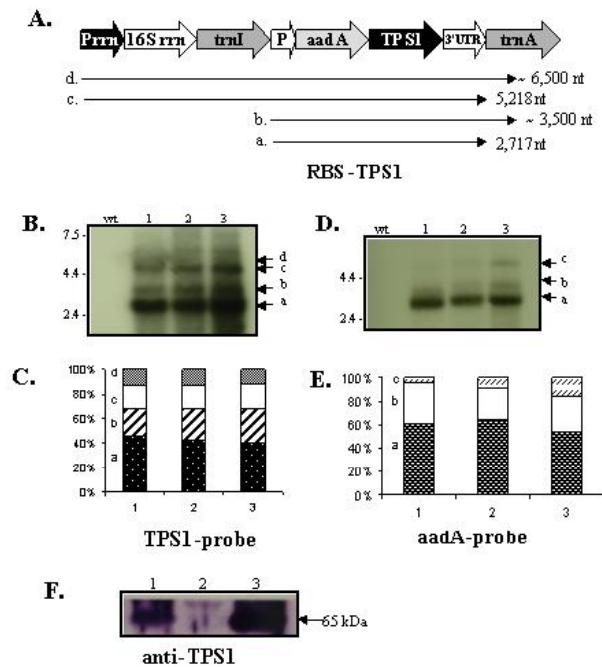
Figure 13: Transcriptional and translational analysis of the *tps*1 operon

A. Schematic representation of the *tps*1 operon in transgenic lines, including the *aad*A gene and upstream P*rrn* promoter; upstream native chloroplast 16S ribosomal RNA gene and promoter as well as *trn*I/*trn*A genes are shown. Arrows represent expected transcripts and their respective sizes. B. Northern blot analysis obtained by hybridization with the *tps*1 probe, loaded as follows: wt: wild type control; lanes 1, 2 and 3: *tps*1 transgenic lines. Transcripts of the *tps*1 operon corespond to those depicted in A, indicated with lowercase letters. C. Relative transcript abundance obtained with the *tps*1 probe. D. RNA transcripts hybridized with the *aad*A probe, loaded as follows: wt: wild type control; lanes 1-3: *tps*1 transgenic lines. Transcript bands obtained for the *tps*1 operon are of sizes as described for *tps*1 probe (B). D. Relative abundance of transcripts in each sample after hybridization with the *aad*A probe. E. Western blot analysis using the TPS1 antibody. Lane 1: positive control (TPS1 protein); lane 2: wild type control; lane 3: *tps*1 transgenic line. A polypeptide of 65 kDa was observed in the transgenic clone, corresponding to the expected size of the TPS1 protein, as observed in the positive control.

95

Transcription and Translation of the *ctb* Operon

RNA from chloroplast transgenic lines transformed with the *aad*A-*ctxb* or 5'UTR-*ctxb*-*gfp* fusion constructs were also analyzed by northern blots. The *aad*A-*ctxb* transgenic lines showed dicistrons and polycistrons, whereas the 5'UTR-*ctxb*-*gfp* transgenic lines showed monocistrons along with several polycistrons. Predicted dicistrons of 1,317 nt (Figure 14B, D, a) and 2,322 nt (Figure 14B, D, d) transcribed from the engineered *Prrn* promoter were detected with either the *ctxb* or *aad*A probe. Additionally, polycistrons transcribed from the native 16S *Prrn* were observed in both transgenic lines. In the *aad*A-*ctxb* transgenic line, the polycistron was of a predicted size of 3,818 nt (Figure 14B, D, f), while in the *aad*A-5'-UTR-*ctxb*-*gfp* transgenic line was 4,823 nt (Figure 14B,D, g); both polycistrons code for four genes (16 rRNA gene, *trn*I gene plus the two foreign genes). Polycistronic transcripts of higher molecular weight were read-through (Figure 14B, D, i, h), as well as transcripts of ~ 2,200 nt (Figure 14A,B letter c) and 3,500 nt (Figure 14A, B, e) obtained from the engineered *Prrn* promoter and processed downstream of the 3'UTR present in the gene construct. The *cxtb*-*gfp* monocistron of 1,389 nt (Figure 14B, b) was detected with the *ctxb* probe but not with the *aad*A probe; beside this transcript, no other monocistron was detected in these analyses. Its relative abundance was 42.10 ± 3.18% of the total transcripts in the 5'UTR-*ctx*B-*gfp* transgenic lines, while the total combined abundance of the polycistrons was about 56%, with the polycistron transcribed from the engineered P*rrn* accounting for 22.94 ± 1.31% of the total transcripts (Figure 14C). For the *aad*A-*ctx*B transgenic line, 100% of the transcripts were polycistrons, of which the most abundant transcript was the *aad*A-*ctx*B dicistron, with about 45% of the total transcripts,

followed by approximately 30% of the polycistron transcribed from the engineered P*rrn* and processed downstream at the *trn*A gene (Figure 14D, E).

The western blot analyses showed that transgenic lines expressing either CTB or CTB-GFP fusion produced large amounts of either protein (Figure 14F, G). CTB protein was detected as a higher molecular weight polypeptide (trimer of 35kDa) than the *E. coli* expressed CTB (Figure 14F). High protein levels were also detected for the *gfp-ctx*B fusion protein (Figure 14G) and was detected in the monomeric form (45 kDa). Interestingly, expression levels in both transgenic lines were similar, even though in the *aad*A-5'UTR-*ctxb-gfp* transgenic line the more abundant transcript was the monocistron. This again suggests that polycistrons are translated as effectively as monocistrons.
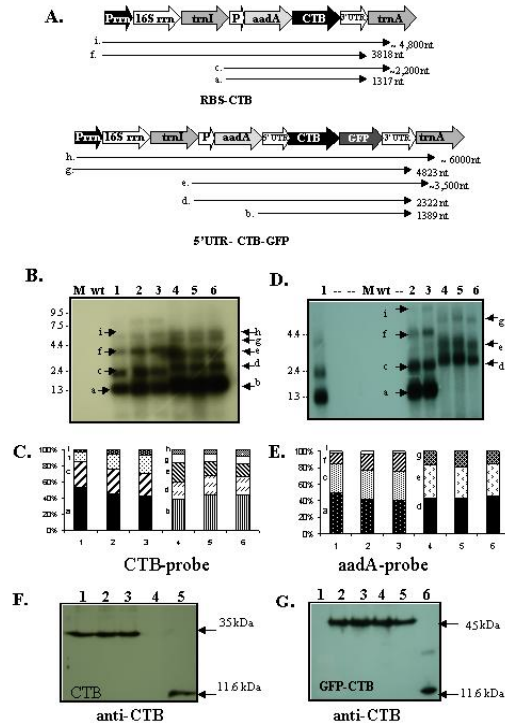
Figure 14: Transcriptional and translational analysis of the CTB operons

A. Schematic representation of the 5'UTR*ctb-gfp* and RBS-*ctb* operon in transgenic lines, including the *aad*A gene and the upstream P*rrn* promoter; upstream native chloroplast 16S ribosomal RNA gene with its respective promoter and the *trn*I and *trn*A are also shown. Arrows represent expected transcripts and their respective sizes. B. Northern blot analysis showing RNA hybridized with the CTB probe. Samples were loaded as follows: M: molecular weight marker; wt: wild type control; lanes 1-3: 5'UTR-*ctb-gfp* transgenic lines; lanes 4-6: *rbs-ctb* transgenic lines. The transcripts and respective sizes correspond to those indicated in A with lowercase letters. C. relative transcript abundance of the samples shown in B. D. RNA hybridization using the *aad*A probe, and loaded according to the following: M: molecular weight marker; wt: wild type control; lanes 1-3: RBS-*ctb* transgenic lines. Lanes 4-6: 5'UTR-*ctb-gfp* transgenic lines. Lanes marked with (--) are blank. The transcripts observed correspond to the same as in B. E. Relative transcript abundance for the samples shown in C. F. Western blot analysis of the RBS-CTB transgenic lines using anti-CTB antibody. Lanes 1-3: transgenic clones; lane 4: wild type control; lane 5: positive control (CTB protein). CTB from transgenic lines is in trimeric form. E. Western blot analysis of the 5'UTR-*ctb-gfp* transgenic lines using the CTB antibody. Lane 1: Wild type control. Lanes 2-5: transgenic lines. Lane 6: positive control (CTB protein).

Discussion

The chloroplast genome has been engineered with single genes to confer useful agronomic traits including herbicide resistance (Daniell *et al.,* 1998), insect resistance (McBride *et al.,* 1995; Kota *et al.,* 1999, De Cosa *et al.,* 2001), disease resistance (DeGray *et al.,* 2001), drought (Lee *et al.,* 2003), salt tolerance (Kumar *et al.,* 2004a), and phytoremediation (Ruiz *et al.,* 2003). Recent success in transforming the chloroplast genome of several major crops, including cotton (Kumar *et al*., 2004b) and soybean (Dufoumantelr *et al.,* 2004) has opened this field for commercial development. Because most of the desired traits require multigene engineering, it is important to understand transcription, posttranscriptional changes and translation of heterologous polycistrons within plastids.

Transcript analyses performed in this study repeatedly confirmed that different transgenic lines harboring multigenic operons generated polycistrons as the most abundant transcript form. Additionally, in most of the transgenic lines analyzed, these were not processed to monocistrons, in contrast to the general consensus for native chloroplast translation mechanisms (Barkan, 1988; Barkan *et al.,* 1994; Zerges, 2000; Meierhoff *et al.,* 2003). This shows that multigene operons engineered into the chloroplast genome do not require processing of polycistrons to monocistrons or dicistrons for efficient translation. It is also evident that heterologous bacterial sequences can form stable secondary structures, which are recognized by the chloroplast processing machinery. This is the case for the *cry*2Aa2 transgenic line, in which the native *Bacillus thuringiensis* operon containing an intergenic sequence between the *orf*2 and *cry*2Aa2 genes formed a stable secondary structure. Densitometry analyses of northern blots for the

99

*cry*2Aa2 transgenic lines revealed that the processing in the *orf*2-*cry*2Aa2 intergenic region occurred in about 50% of the polycistronic transcripts transcribed from the engineered P*rrn* promoter. De Cosa et al., (2001), reported that the Cry2Aa2 protein accumulated up to 46% of the total soluble protein in the transgenic leaves, the highest ever reported in transgenic plants. To achieve such levels of expression of Cry2Aa2, optimal translation should have been achieved. Additionally, the formation of Cry2Aa2 cuboidal crystals detected in the transgenic lines depended on hyperexpression of the chaperone encoded by the *orf*2 gene. This chaperone was found to be in a 1:1 ratio to the Cry2Aa2 protein (DeCosa *et al.,* 2001). *Orf*2 transcripts were found only in polycistronic forms, indicating that hyperexpression was the result of translation of polycistrons.

Previous reports have shown that intergenic sequences that form stable secondary structures, masking the ribosome-binding site may affect the translation of the downstream gene (Barkan et al., 1994; Hirose and Sugiura 1997; Del Campo et al., 2002). Additionally, processes of these secondary structures have been reported as part of a release mechanism in chloroplasts which endonucleolytically free the ribosome-binding site and allow translation of the downstream gene (Zerges, 2000). Enhancement of translation has been observed through the use of the 5'UTR *psb*A upstream of a foreign gene, in which nuclear encoded factors in response to light bind to the 5' UTR and release the multiple ribosome binding sites from the inhibitory effect of the stem loop, allowing efficient ribosome binding and increasing translation (Bruick and Mayfield, 1999; Eibl et al., 1999; Fernandez-San Millan et al., 2003). However, in the case of the *cry* 5'UTR, although translation enhancement occurs, it is not light-dependent, as the *aad*A-*orf*1-*orf*2-*hsa* transgenic lines showed no difference in HSA accumulation in response to

light or dark conditions, in contrast to those transformed with the *hsa* gene and native *psb*A 5'UTR. This shows that the heterologous *cry*2aA2 operon UTR region is independent of nuclear and chloroplast control, unlike the *psb*A regulatory sequences (Fernandez San Millan, *et al.,* 2003; Zerges, 2004).   Such heterologous UTRs have played a major role in transgene expressionin non-green tissues, such as carrot roots (Kumar *et al.,* 2004a), or in non-green cultured cells (Kumar *et al.,* 2004 a, b), to facilitate transformation of recalcitrant crops.

Data shown here supports the idea that engineered operons in the chloroplast, which do not carry any intergenic sequences capable of forming stable secondary structures can be translated very efficiently and are not processed into monocistrons. The processing observed in the *cry*2Aa2 transgenic lines may be due to endonucleolytic cleavage of the stem loop, but it does not indicate that this processing has to occur in order for translation to take place.   An interesting observation is that the *aad*A-*orf*1-*orf*2 tricistron produced by the processing event does not contain a 3' UTR region, yet this transcript is as abundant as the polycistrons, which contain the 3'UTR and translated efficiently. This shows that polycistrons may be stable in chloroplast even in the absence of 3'UTR.

In chloroplasts, all of the genes in the 16S *rrn* operon, including the *trn*A, *trn*I, as well as 23S, 4.5S and 5S *rrn* genes (which are downstream of the integrated transgenes), are transcribed from the native P*rrn* promoter. Therefore, disruption of these polycistrons by the insertion of the foreign operon due to effective termination at the 3' untranslated regions would mean that the *trn*A and other downstream genes will not be transcribed, affecting chloroplast protein synthesis. However, this was not the case; all the transgenic lines grew similar to the wild type plants, indicating that the read-through transcripts formed by the insertion of foreign operons were

sufficient for optimal ribosome synthesis in chloroplasts. Read-through transcripts processed at the *trn*A region accounted for about 26 to 39% of the total transcripts in all transgenic lines tested whereas, in HSA-expressing transgenic lines, this percentage was between 15% and 32%. Introns within the *trn*A gene undergoes splicing and other posttranscriptional modifications in order to produce the functional *trn*A (Barkan et al., 2004). Therefore, such processing may modify polycistronic transcripts that read through from the 3'UTR *psb*A engineered in these chloroplast vectors. Additionally, larger size polycistrons were also detected, although they were not quantified.

The transcript profile for the transgenic lines 5'UTR-*hsa* and 5'UTR-*ctx*b-*gfp*, which are the only two transgenic lines in this study that transcribed monocistrons, was very similar. The monocistronic transcripts accounted for about 42% to 50% of the total transcripts examined. The total polycistronic levels in these two transgenic lines, including read-through transcripts were between 50% and 57%. In all the transgenic lines that do not transcribe monocistrons, the most abundant transcript was transcribed from the engineered P*rrn* promoter, terminating at the 3'UTR; this transcript accounted for 43% to 59% of the total transcripts detected.

CTB protein was detected in the western blots as a higher molecular weight polypeptide (trimer of 35 kDa) than the *E. coli* expressed CTB. Functional CTB is pentameric (58 kDa) in structure, with monomers linked by disulfide bonds. *E. coli* cannot form disulfide bonds in the cytoplasm (Daniell et al., 2001); therefore this pentameric structure is produced. Even when - mercaptoethanol was used and the samples were boiled, no monomeric CTB (11.8 kDa) was obtained, and suggesting that expression in the chloroplast facilitates the assembly and increase the stability of foreign proteins.

The ability to engineer foreign genes without promoters or other regulatory sequences has several advantages. Repetitive use of promoters or regulatory sequences is known to cause transgene silencing. Also, repeated sequences may cause deletion of the transgene (Iamtham and Day, 2000). Observations reported here clearly demonstrate the uniqueness of transcription and processing of heterologous operons.

While endogenous polycistrons require processing for effective translation, this is not required for hyperexpression of foreign operons. Native polycistrons require chloroplast specific 3'UTR for stability, which is not required for heterologous polycistrons. Untranslated regions in native transcripts are regulated by nuclear factors, whereas heterologous transcripts are free of such regulations. Specific nuclear-encoded factors recognize sequences in native transcripts for the processing of primary mRNA (Barkan, 2004). This is not the case in foreign operons where heterologous sequences can be recognized and processed by the chloroplast posttranscriptional machinery. Finally, in both native and foreign operons there are abundant read through transcripts that allow the expression of genes downstream of 3'UTRs. Addressing questions of the translation of polycistrons and sequences required for transcript processing and stability is essential for metabolic engineering. Knowledge of such factors will allow us to engineer pathways that will not be under the complex post-transcriptional regulatory machinery of the chloroplast.

One of the primary advantages of using heterologous sequences for the enhancement of gene expression is the lack of cellular control over these sequences, allowing the enhancement of transgene expression in green and non-green tissues. Recently, the use of the g10 5'UTR made possible the transformation of non-green plastids of carrot (Kumar et al., 2004a). Additionally,

the use of a gene cassette containing the selectable marker genes under the regulation of heterologous UTRs, increased transformation efficiency and made cotton plastid transformation possible (Kumar et al., 2004b). In this study, we report the translational enhancement by heterologous 5'UTR, the translation of polycistronic transcripts without processing, and the stability of heterologous polycistrons lacking a 3'UTR. These results suggest that is possible to effectively express multiple genes via the chloroplast genome without significant intervention of chloroplast regulation. The findings of this study should facilitate multigene engineering via the plastid genome in both green and non-green plastids.

CHLOROPLAST GENETIC ENGINEERING FACILITATES MERCURY

PHYTOREMEDIATION: STUDIES AND CAPABILITIES

Introduction

Phytoremediation is the use of plants for *in-situ* restoration of contaminated environments. This technique would be a cost-effective and environmently friendly approach with big advantages for large-scale cleanups of contaminated sites (Meagher et al., 2000; Doucleff and Terry, 2002). Other techniques have been implemented in the past with little success because they were costly and threatened the safety of our environment. Current remediation methods to clean up heavy metal-contaminated soils include flushing, chemical reduction/oxidation and excavation, retrieval, and off-site disposal, all of which are expensive, environmental invasive, and labor intensive (Karenlampi et al., 2000).

Plants have a high capacity for adaptation to different environments and a natural resistance against different toxic pollutants. Some plants possess intrinsic bioremediation pathways that allow them to brake down and bioremediate various organic compounds, such as: PCBs, PAHs, chlorinated solvents, explosives (TNT), nutrients and surfactant agents (GWRTAC: http://gwrtac.org). In the environment, plants like *Brassica juncea* (Indian mustard), *Brassica oleracea* (cabbage) and sunflower can accumulate high concentrations of heavy metals and radionuclides (hyper-accumulator plants), and have been used for uptake of cesium-137, strontium-90, and uranium in places like Chernobyl, Ukraine and Ashtabula, Ohio (US Department of Energy: http://www.em.doe.gov). In order to determine that phytoremediation is

cost effective, the rate of plant uptake should be greater than one percent of the weight of the plant per harvest, and the time to complete the remediation treatment must be between two and ten years (US Department of Energy: http://www.em.doe.gov). Today, phytoremediation has been cost effective in soil remediation of low cesium and strontium concentration with a cost that ranges from 50 to 100 dollars per ton. Water phytoremediation, also called rhizofiltration, has been effective in uranium cleanup, the cost for removing close to 100% of the contaminant being between 2 and 6 dollars per 1,000 gallons of water. The expenses involved in phytoremediation are much lower compared with other non-plant techniques (US Department of Energy: http://www.em.doe.gov). Plants also have high public acceptance (especially via chloroplast engineering), they are cheap and non-invasive, and they help contain disrupted ecosystems. Plants have different mechanisms to absorb and detoxify pollutants from the environment, examples of these are: phytoextraction (the uptake and translocation of metals from the soil to the tissue by the root), phytodegradation (the brake down of contaminants), and phytovolatilization (the uptake and transpiration of the contaminant in a modified form by the plant) (http://www.epa.gov.htm). These characteristics, among many others, make plants advantageous for bioremediation. This is true though plants have limited applicability for treatment of polluted places with intermediate and high levels of toxic compounds or for metals or compounds to which the plant is not adapted. Genetic engineering can integrate new traits from organisms like bacteria (which have a higher capability to survive and change their environment) to confer new traits to plants in order to enhance their phytoremediation capabilities and the resistance of the plants to the toxic pollutants.

Urgent Need for Phytoremediation of Mercury and Organomercurials

One of the most toxic pollutants threatening our health and ecosystem is mercury. Mercury is a toxic heavy metal that is commonly released into the environment as a byproduct of different chemical reactions of modern industries and can also be introduced from environmental sources (Patra and Sharma, 2000). Power plants in the US emit about 48 tons of mercury annually and it would cost 40,000 to 70,000 dollars to remove each pound of mercury with currently available technologies (New York Times Feb 24, 2004). The present world production of mercury is about 9,000 tons/year (http://www.chem.ualberta.cal.htm). Mercury (Hg) pollution of soil and water is a world-wide problem (Dean et al., 1972; Kramer and Chardonnens, 2001). The extent to which Hg is harmful depends on the form of mercury present in the ecosystem. Inorganic forms of Hg are less harmful than organic forms partly because they bind strongly to the organic components of soil. Because of this, Hg does not tend to contaminate the ground water except when Hg leaches from a municipal landfill (USEPA, 1984). On the other hand, organomercurial compounds may be 200 times more toxic than inorganic Hg (Patra and Sharma, 2000) and methyl-Hg is especially toxic (Meagher and Rugh, 1997).

Mercury is usually released in its metal or ionic form, accumulating in the sediments of lakes and oceans, becming rapidly methylated by bacteria (Ex. *Desulfovibrio desulfuricans*), producing 200 fold more toxic methylmercury or organomercurial (Compeau et al.1985; Gilmour et al. 1992) which is eventually released into water (Harada et al. 1995). Methylmercury is trapped into small fish when the water passes through their gills, or they feed on phytoplanktons that carry high concentrations of the pollutant. Predatory fish, as bass in fresh water and tuna in

salt water, live for long periods of time feeding on smaller fish. During their life span, they can accumulate high levels of methylmercury that can reach 1.0 ppm in water with normal levels of mercury, and up to 30 ppm in areas with high mercury pollution (http://ehpnet.niehs.nih.gov). The biomagnification of methyl mercury represent a risk for people and animals that feed from these fishes (Minamata Disease Research Group, 1968; Harada et al.1995; Patra and Sharma, 2000).

Organomercurials are more toxic due to their increased hydrophobicity, which allows them to cross lipid membranes. Organomercurials are neurotoxins easily absorbed into blood and known to harm fetuses and young children; over 90% of the intake of methylmercury is absorbed into blood compared with only 2% of inorganic mercury (http://www.chem.ualberta.cal.htm). Both organomercurials and mercury have the tendency to accumulate in the tissue (biomagnification), especially in the membrane bound organelles. In plants, organic mercury crosses the lipid membrane of organelles poisoning essential oxidative and photosynthetic electron transport chains more easily than metallic mercury (Rugh et al. 1996). These properties, in addition to the wealth of knowledge on genes that catalyze the degradative pathways for mercury and organomercurials make this an ideal target for phytoremediation.

Bacterial Mer operon

The genes for mercury resistance are widespread among the prokaryote kingdom and are known as *mer* genes. These genes are found in operons of bacterial plasmids, transposons and in bacterial chromosomes (Foster, 1983, 1987; Summers et al. 1978, 1986; Radford et al., 1981).

Different genes constitute these operons, some examples are: the transport and uptake genes (*mer* T, P, C and F) and the regulatory genes (*mer* R and D). But the two most important genes are the *merA* that codes for the mercuric ion reductase, and the *merB* that codes for the organomercurial lyase (Foster, 1983, 1987; Summers et al. 1978, 1986). *MerA* is a 1.7 kb gene that codes for a 69 kDa soluble enzyme (Jackson and Summers, 1981). *MerA* enzyme is a NADPH-dependent flavin adenine dinucleotide-containing disulfide oxidoreductase. The mercuric ion reductase enzyme contains an active-site thiolate that acts as an electron donor (Stanisich et al., 1977), and it has been shown *in vitro* to requiere NADPH for activity (Fox and Walsh, 1982, 1983; Schottel, 1978; Summer and Sugarman, 1974). The flavoprotein encoded by *merA* uses NADPH as a co-factor to catalyze the enzymatic reduction of Hg^{2+} to a volatile, non-reactive and less toxic form of mercury (Hg^0) (Begley et al. 1986). *MerB* is a 638 bp gene that codes for the soluble 24 kDa organomercurial lyase. The organomercurial lyase undergoes the protonolysis of organomercurials by cleavage of the carbon-mercury bond (Resing et al., 1992), releasing a reduced organic moiety and the mercury ion; which is then detoxified by the mercuric ion reductase (Jackson et al. 1982; Barrineau, 1983). A polycistron containing both genes should allow effective degradation of mercury and organomercurials. The main site of action of organomercurial is within chloroplasts in plants, and the ability of chloroplasts to express bacterial operons, and several other environmental benefits of chloroplast genetic engineering make this an ideal pathway for engineering via the chloroplast genome.

Site of Action of Organomercurials and Mercury in Chloroplast

Organomercurials and mercury accumulate in the tissue, especially in the membrane bound organelles (Rugh et al.1996). In photosynthetic organisms, the main site of action of mercury damage seams to be the chloroplast thylakoid membrane and photosynthesis. Organomercurial compounds strongly inhibit electron transport, oxygen evolution (Bernier et al., 1993), Hill reaction, photophosphorylation, and quench chlorophyll fluorescence in photosystem II (Kupper et al., 1996). Mercury affects the oxygen-evolving complex (OEC) that is found in the photosystem II bounded to the thylakoid membrane (Bernier et al. 1993). Among the constituents of this supramolecular complex are three extrinsic polypeptides (EP) of 16, 23, 33 kDa respectively; and two inorganic cofactors, chloride and calcium (Bernier and Carpentier, 1995). It has been shown that 30 minutes of incubation of PSII sub-membrane fragment in the presence of 10 μM $HgCl_2$ causes 40% depletion of the EP33 (Bernier and Carpentier, 1995). EP33 is gradually released with increasing concentrations of mercury up to 10 μM (Bernier and Carpentier, 1995) but decreased activity seems to take place at much lower mercury concentrations than the required for the release of the EP33 polypeptide (Bernier and Carpentier, 1995). Mercury treatment of PSII leads to a strong inhibition of oxygen evolution by removal of EP33 (Bernier and Carpentier, 1995). Mercury reduces chlorophyll fluorencence due to additional inhibitory sites at the donor side of PSII, including damage to the light-harvesting complexes and structural changes in the antenna pigments that affect the primary photochemistry (Rashid et al., 1990; Bernier et al., 1993; Rashid et al., 1994; Bernier and Carpentier, 1995; Sabat et al., 1996). Mercury can replace the Mg ion from chlorophyll, leading to the reduction of

chlorophyll content (Prasad and Prasad, 1987). Sen and Mondal (1987) and Sinha et al., (1996) reported a 26% reduction of chlorophyll content in *Silvia natans* and 35% in *Bacopa monnieri* at 5 μg/mL HgCl$_2$, although this two species have inherent mercury resistance. Additionally, mercury is an inhibitor of photosynthetic electron transport at the level of plastocyanin (Trebst, 1980).

Phytoremediation Studies via Nuclear Transformation

All of the attempts to genetically engineer plants with improved phytoremediation have previously been based on transformation of nuclear genome. Several studies have successfully integrated bacterial genes into nuclear genome to produce plants that were specifically engineered for phytoremediation of metal-polluted environment (Heaton et al., 1998; Rugh et al., 1998; Nies, 1999) and TNT (Salt et al. 1998). Among several reports on phytoremediation, one of the most interesting is the use of nuclear modified plants for bioremediation of mercury and organomercurials. In these report a nuclear modified form of the *merA* (mercury ion reductase) and *merB* (organomercurial lyase) genes were used to transform plants, conferring resistance to mercury and organomercurials respectively (Bizily et al. 1999, 2000; Rugh et al. 1996). One drawback of nuclear genetic engineering is that it requires several backcrosses to create the complete pathway that detoxifies mercury and organomercurials (Bizily et al. 2000). This results in variations in expression levels among different transgenic lines and tolerance to different concentrations of organomercurials, up to 10 μM (Bizily et al. 2000). Another concern over the use of nuclear transformed plants *in-situ* is the escape of the foreign genes via pollen (Daniell,

1999; Bogorad, 2000). Most importantly, the enzymes were not targeted to chloroplasts where mercury accumulates and is most toxic.

Mercury Translocation from Root to the Above Ground Tissues

The integration of foreign genes into plant genomes has been shown to alter the mobilization of metal ions, including uptake into the root, sequestration and detoxification (Kärenlampi et al., 2000; Singh et al., 2003). However, a major limitation of all of these studies is the inability to transport organic or inorganic mercury to above grown tissues, which constitute more than 90% of the plant biomass. There is considerable body of evidence to show that translocation of Hg[II] from root to shoot is negligible or non-existent (Hannerz, 1968; Boney, 1971; De et al., 1985; Suszcynsky and Shann, 1995). Heaton et al (2003) compared the accumulation of Hg[II] by wild type and nuclear transgenic rice plants expressing the *merA* gene, while growing in hydroponic media or in soil with $HgCl_2$; mercury accumulated in high concentrations in roots when grown in hydroponic media, and was poorly translocated to shoots. This same study showed that translocation of Hg[II] into shoots decreased even more when the plants were grown in soil. According to Godbold and Huttermann, (1986), mercury induced root damage may have serious consequences for nutrient and water supply to above ground plant parts, and should be taken into account when assessing the effect of mercury on the physiology of leaves. However, there are differences in how the inorganic and organic forms of mercury carry out cell injury. While inorganic mercury ($HgCl_2$ in particular) affects the plasma membrane, methyl mercury may primarily affect organelle metabolism in the cytoplasm, which

112

subsequently affects membrane integrity. Furthermore, there are no reports showing that organic mercury is transported from roots to shoots.

In sharp contrast to lack of transport of Hg[II] from root to shoot, several investigators have reported that the main site of damage of organomercurial compounds is the chloroplast (Kupper et al., 1996, Patra and Sharma, 2000). Organomercurial compounds have been shown to strongly inhibit chloroplast functions, including electron transport, oxygen evolution (Bernier et al. 1993), Hill reaction, photophosphorylation, chlorophyll fluorescence (Kupper et al., 1996) and chlorophyll content (Prasad and Prasad, 1987; Sen and Mondal, 1987). Such discrepancy may be due to the fact that photosynthesis studies were carried out in isolated chloroplasts in which transport of Hg[II] from root to shoot was irrelevant. In order to address these conflicts, the *merA* and *merB* genes were engineered via the chloroplast genome and both enzymes were compartmentalized within the chloroplasts (Ruiz et al., 2003). In contrast to nuclear transformation and expression of both enzymes in the cytosol, compartmentalization within chloroplast dramatically increased the resistance of transgenic plants to the toxic effects of the organomercurial, phenyl mercuric acetate (Ruiz et al., 2003). If the chloroplast is the main site of mercury damage as has been proposed, then detoxification in transgenic plants expressing the *merA* and *merB* genes should occur in the chloroplast. However, transport of Hg[II] from root to shoot is negligible to non existent (Sen and Mondal, 1987; Suszcynsky and Shann, 1995).

Rationale and Approach

We reported for the first time the use of chloroplast genetic engineering to enhance the capabilities of plants for phytoremediation (Ruiz et al., 2003). More importantly, we report the construction of and optimized operon for the phytoremediation of organomercurials via chloroplast genetic engineering. This work will increase the applicability of the system for polluted environments with extremely high levels of organomercurial contamination. To accomplish the aforementioned, the native bacterial genes *mer*A and *mer*B were placed under the translation enhancement of chloroplast specific 5'UTR*psb*A and g10 5'UTR. Additionally, 3'UTRs are also engineered in the operon to increase transctipt stability and abundance. Finally, we report the molecular characterization of chloroplast transgenic plants expressing the optimized *mer*A and *mer*B operon. The effective integration and expression of this operon in chloroplast will also increase our knowledge of multigene engineering in chloroplast, validating the applicability of the chloroplast system for the expression of complex metabolic pathways.

Additionally, we test the hypothesis that mercury detoxification occurs in leaves (chloroplasts) and that sufficient quantities of mercury are transported from roots to shoots for this to occur. Such transport showed dramatically enhancment of phytoremediation capabilities, which is currently limited to roots, which account for less than one tenth of the total plant biomass. Finally, we determined whether the insertion of *merA* and *merB* genes into the tobacco chloroplast genome is of value in the phytoremediation of mercury supplied in inorganic forms and in organic forms. Furthermore, since the combined action of *merA* and *merB* is to convert mercury into the volatilizable elemental form, we determined whether detoxification in the

transgenic plants is accompanied by the conversion of mercury into the volatile form. The specific objectives of this study therefore are to determine the extent to which chloroplast transformation with *merA* and *merB* genes results in the uptake and translocation of Hg compounds from roots to shoots when plants are supplied with PMA or $HgCl_2$. Additionally, to study the volatilization rates of elemental mercury in transgenic plant and determine their applicability for phytoremediation when mercury is supplied in organic as well as inorganic forms. Because mercuy is such a toxic environmental pollutant there is urgent need for the cleanup of the contaminated areas. Here we show that by genetically engineer the chloroplast with a multigene operon for the degradative pathay of organomercurials we could bioremediate extremely high levels of organic-mercury. Additionally, the results shobioof mercury we coulchloroplast transgenic plants as an effective phytoremediation system for extremely polluted environments. This study also supports the idea that the chloroplast can be transformed with complex operons for the development of novel metabolic pathways in plants.

<center>**Materials and Methods**</center>

Construction of tobacco Optimized Phytoremediation Vectors (Second Generation)

The bacterial native genes, *mer*A (1.69 kb) and *mer*B (638 bp) that encode the mercuric

ion reductase and the organomercurial lyase, respectively, were amplified by PCR from *E. coli*

strains harboring plasmids NR1 (containing the full length *mer*A) and R831b (containing full the

length *mer*B). Isolation and amplification of the *mer*A and *mer*B gene from the native plasmids

was performed by polymerase chain reaction (PCR) with the utilization of specific 5' and 3'

flanking DNA primers. All primers were designed using the QUICKPRI program of the

DNASTAR software. *Nde*I and *Not*I restriction sites were engineered at the 5' and 3' of the

*mer*B primers, respectively, to allow directional cloning into the suitable vectors. The 5' and 3'

primers had 15 bases of homology with the 5' and 3' ends of the *mer*B gene, respectively. Either

primer contain 10 bases, which are upstream of the *Nde*I restriction site in the 5'primer or

downstream of the *Not*I restriction site in the 3' primer to facilitate subsequent restriction

digestions, these regions base-pare with the regions flanking the *mer*B gene in the native plasmid

DNA. The 5' primer of *mer*A was designed with a *Sma*I restriction site followed by an *Nde*I

restriction site that flanked the *pha*A coding region. The 3' primer of *mer*A was engineered with

a *Bam*HI restriction site flanking the *mer*A coding region. Either primer contain 10 bases, which

are upstream of the *Sma*I restriction site in the 5'primer or downstream of the *Not*I restriction site

in the 3' primer to facilitate subsequent restriction digestions, these regions base-pare with the

regions flanking the *mer*A gene in the native plasmid DNA. The PCR reaction and amplification

<center>116</center>

protocol for the *mer*A and *mer*B genes were performed as explained in the material and methods section in chapter 1, with the exception that the gene elongation time for the PCR reaction was adjusted as follows; 2 minutes for *mer*A and 1 minute for *mer*B.

The PCR amplified *mer*B gene was cloned into the vector pCR2.1-5'UTR*psb*A, which contained the functional *psb*A gene promoter and 5' regulatory sequence, by directional cloning after *Nde*I and *Not*I restriction digestion of the PCR product and vector. The digestion reactions for the PCR amplified *mer*B and the pCR2.1-5'UTR*psb*A were performed as follow: 5 μL of vector DNA (0.25 μg) or 5 μL of *pha*A (1.0 μg), 1.0 μL of *Nde*I, 1.0 μL *Not*I, 2μl of 10X reaction buffer compatible with *Nde*I/*Not*I, and 11 μL of sterile Milli Q water were added for a final volume of 20 μL. The restriction digestions were incubated at 37°C for 2 hours. The reactions were run in a 0.8% agarose gel and the digested vetor and *mer*B gene were eluted together and used for ligation and subsequent *E. coli* transformation. Finally, transgenic *E. coli* clones containing the pCR2.1-5'UTR-*mer*B vector were verified for the integration of the *mer*B gene by restriction digestion with *Nde*I and *Not*I to cut out the *mer*B gene from the pcR2.1-5'UTR-*mer*B vector and positive identify the integration of the *mer*B gene. All the cloning and reaction setting were performed as explained in the materials and methods section in chapter 1.

The vector pCR2.1-5'UTR-*mer*B and the chloroplast transformation vector pLD-ctv were restriction digested with *Nde*I and *Not*I, the samples were resolved in a 0.8% agarose gel an the DNA fragment corresponding to the digested pLD-ctv vector and to the 5'UTR-*mer*B construct were eluted from the gel. Followed, these samples were used for ligation and subsequent *E. coli* transformation and confirmation of integration of the 5'UTR-*mer*B insert into the pLD-ctv vector. These procedures were performed essentially as described ealier.

The PCR amplified *mer*A gene and the pBSK vector were restriction digested with *Sma*I and *Bam*I restriction enzymes, and their digested products were ligated and cloned into *E. coli* to produce the vector pBSK-*mer*A. This vector was used for the successive directional cloning of the 5'UTR g10 into the *Sma*I and *Nde*I restriction sites flanking the 5' end of the *mer*A coding region to produce vector pBSK-g10-*mer*A. The g10 5'UTR was obtained from the pBSK-g10 vector by double digestion with *Sma*I and *Nde*I. The 3'*rps*T was inserted downstream of the *mer*A gene by digestion of both pBSK-g10-*mer*A and pCR2.1-*rps*T with *Bam*HI and *Not*I restriction enzyme. The restriction-digested product of the reaction were ligated and cloned into *E. coli* to produce vector pBSK-g10-*mer*A-*rps*T. The construct g10-*mer*A-rpsT was cloned into the pLDR-5'UTR-*mer*B vector by digesting both vector with *Eco*RV and ligating the g10-*mer*A-*rps*T fragment into the pLDR-5'UTR-*mer*B to produce the final chloroplast transformation vector pLDR-g10-*mer*A-*rps*T-5'UTR-*mer*B.

*Mer*A and *mer*B Gene Sequencing

The pCR2.1-5'UTR-*mer*B and the pBSK-g10-*mer*A-rpsT vector were use for to sequence the complete merA and merB constructs before any successive cloning step. Vector specific M13 forward and M13 reverse primers, which flank the multiple cloning site of the pCR2.1 vector, along with an internal primer specific for each of the *mer*A gene were used to sequence the entire 5'UTR *psb*A-*pha*A gene, as well as the g10-*mer*A-*rps*T. Three independent reactions for each of the gene constructs, using the suitable primers were performed as explained in the material and methods section in chapter 1. The DNA sequencing was performed in a Beckman

Coulter sequencer at the UCF sequencing facility. Finnally, electrophoregram was compared with gene bank sequences by using the BLAST 2 Sequences program from the NCBI website.

Bombardment and Selection of Transgenic Plants

The steps involved in the gene delivery by particle bombardment and the selection process of the transgenic *Nicotiana tabacum* var Petit Havana lines were performed essentially as describe in the Material and Methods section in Chapter 1 (Daniell, 1997; Daniell et al., 2004; Kumar and Daniell; 2004. Tobacco leaves were bombarded using Bio-Rad PDS-1000/He biolistic device (Bio-Rad, Hercules, CA). After bombardment, leaves were placed on Regeneration Medium of Plants (RMOP) containing 500 µg/ml spectinomycin for two rounds of selection on plates and subsequently moved to jars on Murashige and Skoog medium (MS) containing 500 µg/mL spectinomycin. Finally, homoplasmic plants were transferred to high nutrient soil and grown in a growth chamber at a temperature of 26°C in a 16 hours ligh photoperiod.

Confirmation of Chloroplast Integration by PCR

Plant DNA was isolated using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The PCR primer pairs 3P-3M and 5P-2M were used to confirm the integration of the gene cassette into the chloroplast and the presence of the genes of interest respectively essentially as

described in the Materials and Methods section in Chapter 1 (Guda et al., 2000; Daniell et al, 2004; Kumar and Daniell; 2004). PCR analysis was performed using the Perkin Elmer Gene Amp PCR System 2400 (Perkin Elmer, Chicago, IL).

Southern Blot Analysis

The total plant DNA was obtained from transgenic plants as well as from untransformed tobacco plants using the Dneasy Plant Mini Kit (Qiagen, Valencia, CA). Southern blot analyses were performed essentially as described previously (Daniell et al., 2004; Kumar and Daniell, 2004) Briefly; 2 µg of plant DNA was digested with *Bgl*II and separated on a 0.8% agarose gel at 50 V for 2 hours. The gel was soaked in 0.25 N HCl for 15 minutes and then rinsed 2 times with water. The gel was then soaked in transfer buffer (0.4 N NaOH, 1 M NaCl) for 20 minutes and transferred overnight to a nitrocellulose membrane by capillarity. The next day the membrane was rinsed twice with 2X SSC (0.3 M NaCl, 0.03 M Sodium citrate), dried on a Whatman paper, and then cross-linked in the GS GeneLinker (Bio-Rad, Hercules, CA) at setting C3 (150 njouls). The flanking sequence probe was obtained by *Bgl*II/*Bam*HI digestion of the plasmid pUC-ctv that contains the chloroplast flanking sequences (*trn*I and *trn*A genes). The *mer*AB probe was obtained by *Eco*RI digestion of plasmid pCR2.1-*mer*AB (Ruiz et al., 2003).   Probes were radiolabeled with ^{32}P by using Ready Mix and Quant^{tm} G-50 Micro columns for purification (Amersham, Arlington Heights, IL). Prehybridization and hybridization were performed using the Quick-Hyb solution (Stratagene, La Jolla, Ca). The membrane was washed twice for 15 min

at room temperature in 2 X SSC with 0.1% (w/v) SDS, followed by two additional washes at 60°C (to increase the stringency) for 15 min with 0.1 X SSC with 0.1% (w/v) SDS. Radiolabeled blots were exposed to x-ray films and then developed in the Mini-Medical Series x-ray film processor (AFP Imaging, Elmsford, NY). Detailed methods for each of the steps described above can be find in the Material and Method section in Chapter 1.

Northern Blot Analysis

The RNeasy Mini Kit and protocol was used to isolate total RNA from plant tissues (Qiagen, Valencia, CA). Northern blot analysis were performed essentially as described previously (Ruiz et al., 2003). The *mer*A, *mer*B and *mer*AB probes were used to probe different RNA blots. The *mer*A probe was made by cutting out the *mer*A gene from the pCR2.1-merA vector with *Eco*RI. The *mer*B probe was made by cutting out the *mer*B gene from the pCR2.1-*mer*B vector with *Eco*RI. The *mer*AB probe was produced by digesting the pCR2.1-*mer*AB vector with *Eco*RI. Restriction fragments were exiced and eluted from the gels. Total RNA (2.5 μg) per plant sample was resolved in a 1.2% agarose/formaldehyde gel. The probe labeling reaction, pre-hybridization/hybridization steps, membrane washing step and autoradiography were performed as explained in the Southern blot section in the materials and methods.

Results

Chloroplast Transformation Vector

The bacterial genes, *merA* (1.7 kb) coding for mercuric ion reductase and the mer gene, which code for organomercurial lyase wer amplified by PCR and subsequently cloned into the chloroplast transformation vector (pLD-ctv) to finally produce the pLDR-g10-*mer*A-*rps*T-5'UTR-*mer*B vector (Fig 15). Detailed vector construction including PCR amplification and intermediary vectors can be found in the Material and Methods section. The vector contains homologous recombination sequences (flanking sequences), which allow site-specific integration by homologous recombination into the inverted repeat region of the chloroplast genome in between the *trn*I (tRNA Ile) and *trn*A (tRNA Ala) genes. This specific targeting mechanism allows high efficiency integration of the transgene construct containing the *aad*A (aminoglicoside 3'-adenylyltransferase) gene, which confers spectinomycin resistance and the *pha*A genes. The *aad*A, *mer*A and *mer*B genes are transcribed constitutively from chloroplast 16S ribosomal RNA gene promoter (*Prrn*), which is found upstream of the *aad*A gene and should produce tricistrons (*aad*A-*mer*A-*mer*B). Additionally, the *psb*A gene promoter, which is a constitutive promoter, and the *psb*A 5' regulatory sequence (5' untranslated region; 5' UTR), which is known to enhance translation of foreign genes, was used upstream of the *mer*B gene and should produce *mer*B monocistrons. The organomercurial lyase encoded by *mer*B is the limiting factor in the bioremediation pathway of mercury, because of that we enhace the translation of this gene by using the 5'UTR from the psbA gene. At the 3' end of the gene

construct is the 3' *psb*A untranslated region (3'UTR), which is known to be involved in mRNA abundance and stability in chloroplast. The gene 10 5' UTR derived from bacteriophage is known to enhance translation in chloroplasts considerably when fused to endogenous promoters (Staub et al., 2000). In addition, it is free from developmental or light regulation, because it is derived from a foreign source. In this expression cassette, *mer*A should be transcribed as a dicistron in the chloroplasts, because the upstream gene, *aad*A, is devoid of a 3' UTR and the gene 10 5' UTR is promoterless, been the gene cassette P*rrn* promoter the only promoter upstream of the aadA and merA genes. Additionally, the g10-merA cassette was coupled to the 3'UTR of *rps* gene. This region has shown to increase transcript stability and abundance.

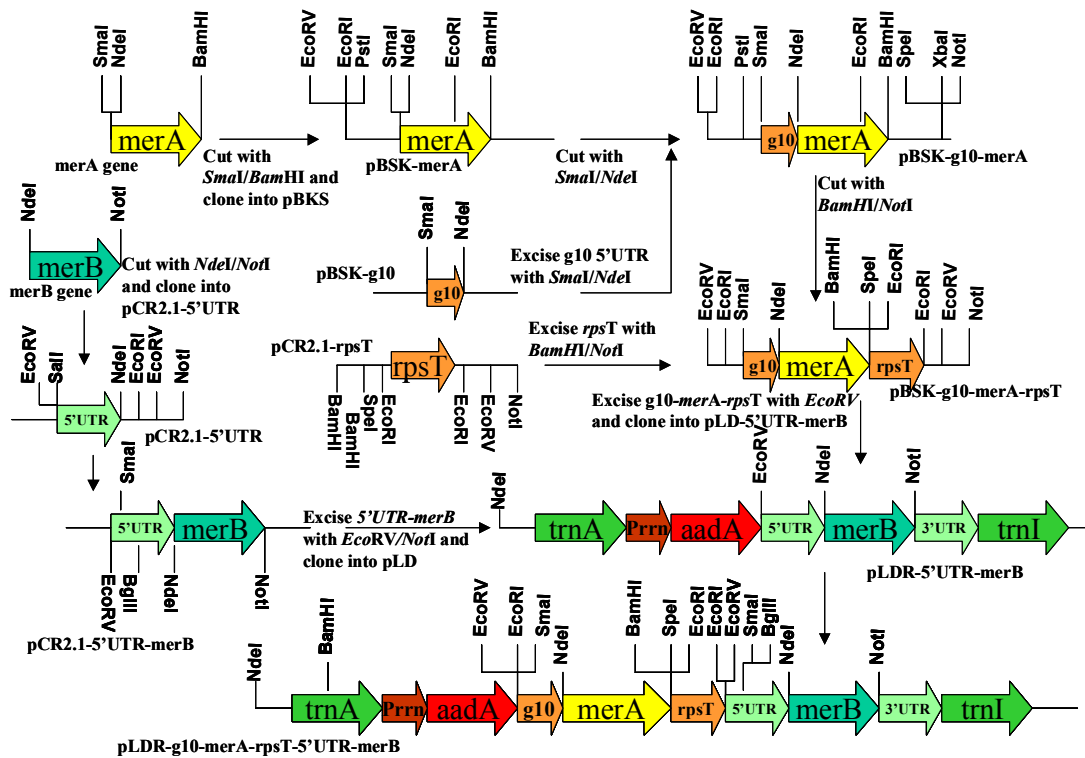**Figure 15: pLDR-g10-*mer*A-*rps*T-5'UTR-*mer*B**
The map shows the specific steps and restriction enzymes used in the vector construction. PCR products, mer A and merB were cloned into suitable vectors inframe to 5' untranslated regions and 3'UTRs. The final step shows the integration of the merA and merB optimized operon into the pLD-ctv vector to form the functional pLDR-g10-merA-rpsT-5'UTR-merB vector.

Transformation and Selection of Chloroplast Transgenic Plants

Transgenic lines transformed with the pLDR-g10-*mer*A-rpsT-5'UR-*mer*B vector as explained in the Materials and Methods section were passed through a selection process. Three days after bombardment the bobarded leaf tissue was placed on regeneration media with 500 μg /mL spectinomycin antibiotic. After 3 to 4 weeks in selection the first putative transgenic shoots were observed (Fig 16A). The transgenic shoot is characterized by being green over a background of bleach leaf material (Fig 16A). From the first selection the transgenic lines were move to second selection, were pieces of leaf material from the transgenic plant recovered from first selection were placed in media with 500 μg/mL spectinomycing for selection (Fig 16B). During this selection many more shoots identical (clones) to the parental primary transgenic shoot are generated (Fig 16B). Because the initial leaf tissue use in secondary selection is transgenic the callus does not bleach as much as in first selection, allowing faster regeneration of the transgenic clones. This step increases homoplasmy by selecting the transgenic chloroplast and genomes. Finally, once fully regenerated plants are recovered from second selection, these are transfer to third selection. Third selection is performed in MS medium amended with 500 μg/mL spectinomycin to induce rooting and the developments of a fully normal plant (Fig 16C). Finally, the transgenic plants were move to soil and allowed to grow to collect seeds. Transgenic seeds were recovered from the different transgenic lines and were germinating in MS medium with 500 μg/mL spectinomycing to demonstrate maternal inheritance of the genetic engineered traits.

Figure 16: Selection of Chloroplast Transgenic Plants
A, Transgenic plant shoot in first selection (RMOP medium with 500 µg/mL spectinomycing).
B, Second selection in RMOP medium with spectinomycin show multiple transgenic shoot. C, Third selection in MSO medium with spectinomycin.

Molecular Characterization of Chloroplast Transgenic Plants

PCR Analysis

Chloroplast transgenic plants were obtained as described previously in the material and methods section in chapter I. More than 20 positive independent transgenic lines were obtained with the contruct. In this report, we show the results from transgenic lines transformed with the pLDR-G10-*mer*A-*rsp*T-5'UTR-*mer*B. The variability in expression levels among independent chloroplast transgenic lines were minimal as reported previously (Daniell et al., 2001A) and the results shown here correlate well with the results of other transgenic lines with the same chloroplast vectors.

The primer pair 3P and 3M was used to test the integration of the transgene cassette into the chloroplast genome at very early stages during the selection process. The 3P primer lands in the native chloroplast genome and the 3M primer lands in the *aad*A gene that is present within the gene cassette (Fig 17A). If integration has occurred, a 1.65 kb PCR product should be obtained (Fig. 17A, E). The untransformed control and the mutants (caused by the spontaneous mutation of the 16S rRNA gene which confers resistance to spectinomycin) did not show any product, confirming that these plants are negative for integration of transgenes (Fig. 17B, E). The integration of transgenes (*aad*A, *mer*A and *mer*B), was further tested by using the 5P/2M primers and PCR analysis. The 5P and 2M primers annealed to the internal region of the *aad*A and *trn*A

127

genes, respectively (Fig. 17A). The product size of positive transgenic clones was 3.89 kb, while

the mutants and untransformed control did not show any PCR product (Fig. 17 C, D, F).
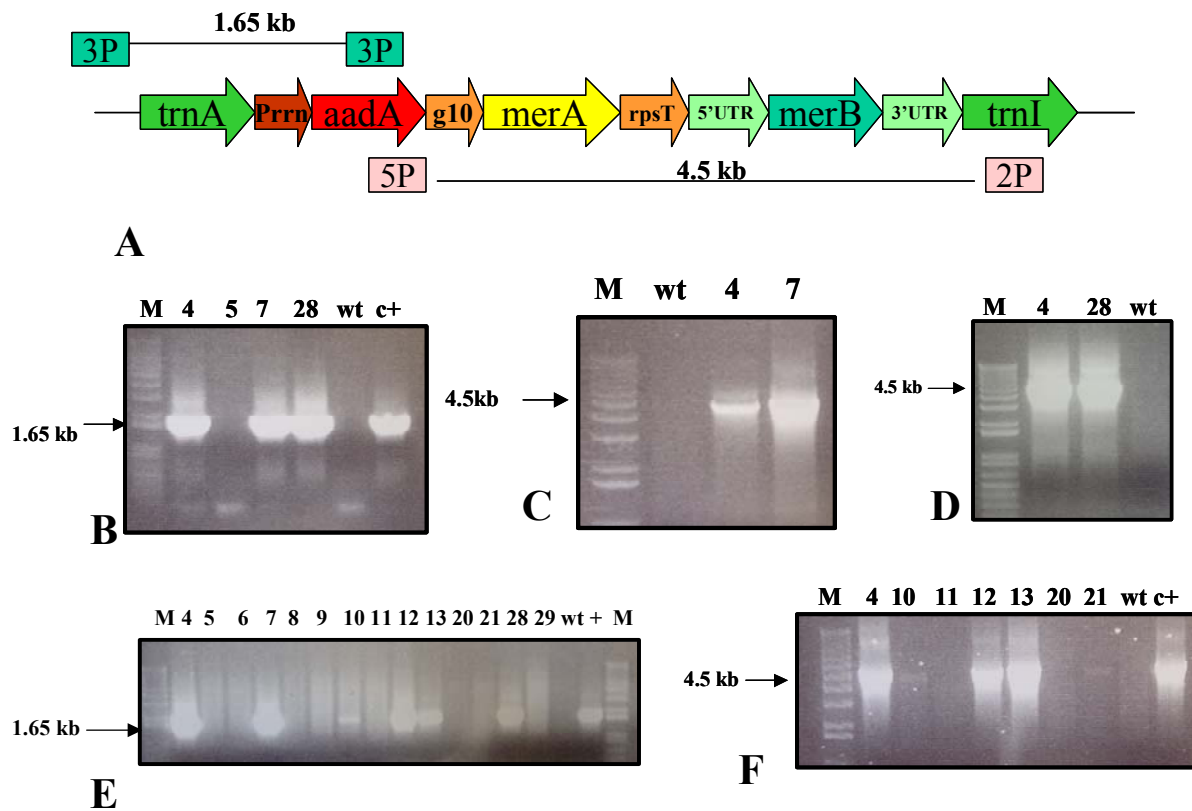
Figure 17: PCR Analysis of Control and Putative Transformant

a) Schematic representation of the landing sites for the primer pairs 3P-3M and 2P-2M. Additionally, amplification product sizes are indicated. **b and e)** Multiple putative transgenic lines show a 1.65 kb amplification product when the 3P-3M primer pair was used, indicating chloroplast integration. **c, d, f)** Transgenic lines test positive (4.5 kb PCR product) for the presence of aadA, merA and merB genes in chloroplast; 5P-2M primer pair was used.

Southern Blot Analysis

DNA from full-grown transgenic plants was extracted and used for the Southern blot analysis (Fig. 18). The 0.81 kb flanking sequence probe that hybridizes with the *trn*I and *trn*A genes (Fig. 18A) allowed detection of the site-specific integration of the gene cassette into the chloroplast genome. The transformed chloroplast genome digested with *Bgl*II restriction enzyme produced two fragments, one of 4.7 kb and a shorter one of 1.5 kb (Fig. 18A, 3B, 3C). The untransformed chloroplast genomes digested with BglII yield a 4.47 kb fragment (Fig. 18A, B, C). The flanking sequence probe also showed that homoplasmy of the chloroplast genomes was achieved through the selection process. Southern blots confirmed stable integration of foreign genes into all of the chloroplast genomes because the 4.47 kb untransformed fragment was not detected in the transgenic lines, this confirmed homoplasmy. Southern blots screened with the *mer*AB probe (2.3 kb in size) showed integration of specific genes, *mer*A and *mer*B by two fragments of 7.3 kb and 1.5 kb (Fig. 18A, 3D). The control untransformed tobacco plants and mutants did not show this fragment (Fig. 18D). If the *mer*AB probe would have detected any unexpected size fragments, it might be a non-specific integration into other plant genomes (nuclear or mitochondria) as discussed elsewhere (Daniell and Parkinson, 2003); but this was not observed. The transgenic plants were fully characterized via PCR and Southern blot analysis, which showed site-specific integration of the genes into the chloroplast genome and achievement of homoplasmy even at very early stages of selection (T0).
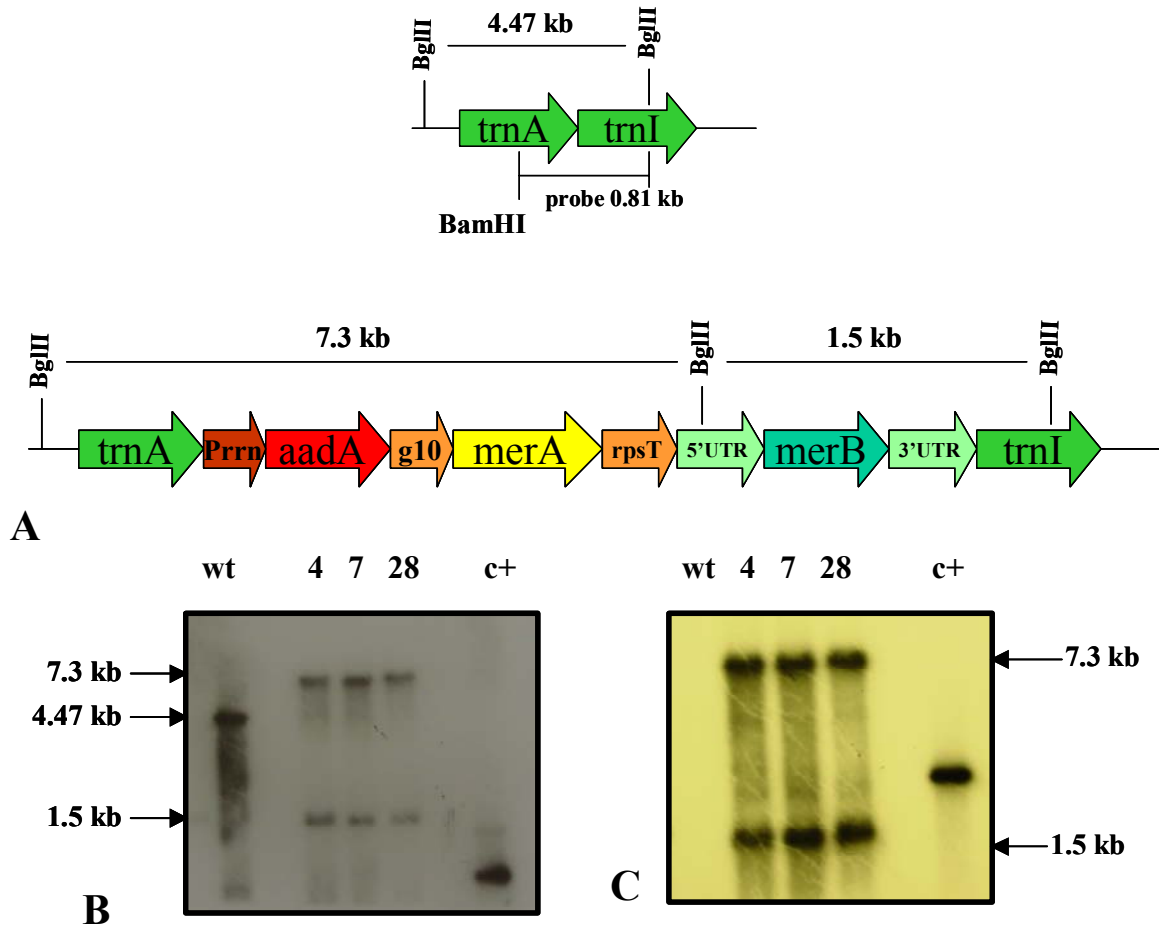
Figure 18: Southern Blot Analysis

A, Schematic representation of the untransformed chloroplast genome, size of the wild type DNA fragment produced by BglII restriction digestion as well as the flanking probe used for Southern blot analysis are shown. B, Schematic representation of the transformed chloroplast genome, BglII restriction sites used for Southern blot analysis and the specific DNA fragments sizes are shown. C and D, Southern blot analysis of transgenic lines (4, 7, 28) with the flanking probe and the merAB probe, respectively. 7.3 kb and 1.5 kb fragments observed in transgenic lines but not in wild type. The wild type (wt) fragment of 4.47 kb detected with the flanking probe is shown.

Northern Blot Analysis

Total RNA from plants transformed with the pLDR-g10-*mer*A-*rps*T-5'UTR-*mer*B was extracted and used to perform the northern blot analysis with three different probes (the *mer*A, *mer*B, *mer*AB). The *mer*A probe clearly showed a tricistron containing the *aad*A, *mer*B and *mer*A genes of size 4,214 nt, which is transcribed from the engineered P*rrn* promoter. Addtionally, a polycistron of size 6,715 nt transcribed from the native 16S ribosomal RNA promoter (P*rrn*) was also detected, this polycistron contained the 3 foreign gene plus the upstrams native 16S *rrn* and *trn*I genes (Fig. 19A). The *mer*B probe detected the tricistron (4,214 nt), the polycistron transcribed from the native 16S promoter and a monocistron (838 nt) corresponding to the *mer*B transcript (Fig. 19B). This monicistron is more abundant due to the action of its upstream *psb*A promoter, which is a strong promoter. The *mer*AB probe helped to visualize different transcripts in a single blot, the *aad*A-*mer*A-*mer*B tricistronic transcript (4,214 nt), the *mer*B monocistron (838 nt) and the polycistron transcribed from the native 16S promoter (Fig. 19C). The northern blot analyses showed that the monocistron was the most abundant transcript detected although the dicistron and polycistron transcribed from the engineered and native 16S P*rrn* promoter, respectively, were also very abundant. Other transcript sizes detected can be explained as readthrough transcripst. Interestingly, read though polycistrons transcribed from the *psb*A promoter (engineered), the engineered P*rrn* promoter and the native P*rrn* promoter and which termininate in the intronic region of the *trn*A gene downstream of the gene construct were also bundant, demonstrating that 3' untranslated region in chloroplast are not strong termination.
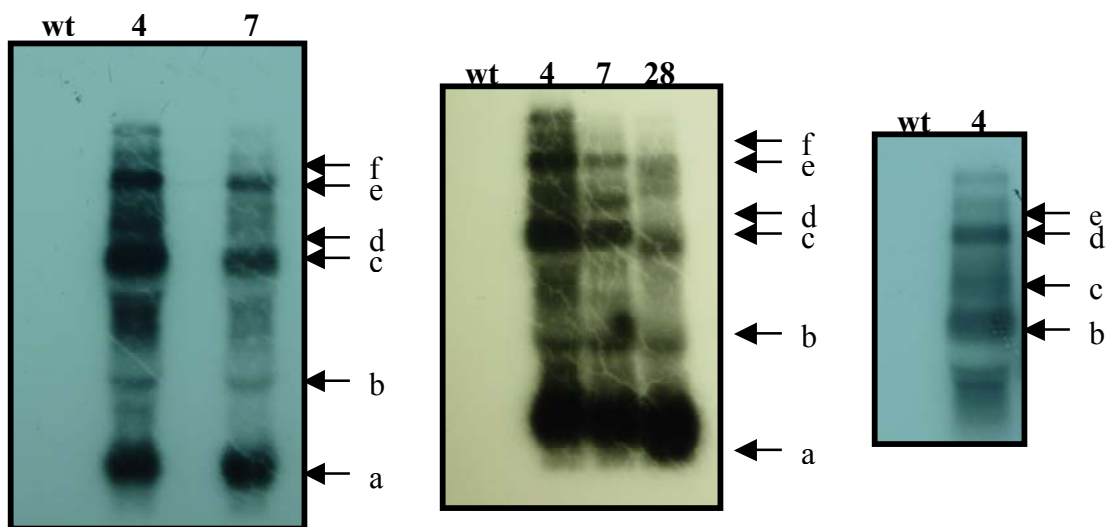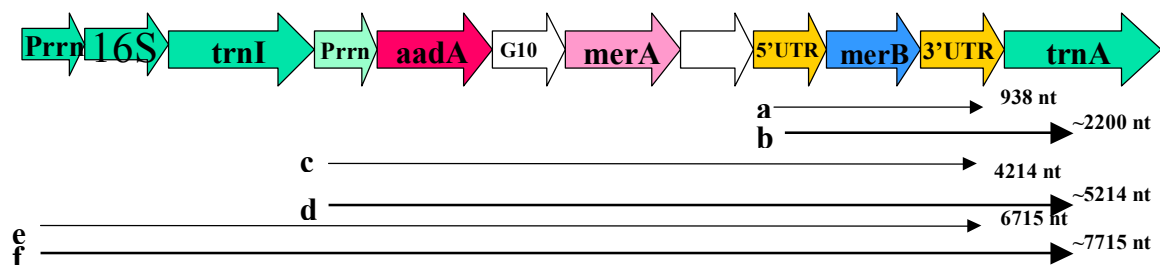
Figure 19: Northern Blot Analysis

A, Schematic representation of the different RNA species detected in the transgenic lines and the spected sizes. B, *mer*B probe was used. C, *mer*AB probe was used D, *mer*A probe was used. wt: wild type; 4, 7 and 28 are chloroplast transgenic lines.

Protein Expression in Chloroplast Transgenic Plants

To confirm hyperexpression of the Mer enzymes in the chloroplast transgenic lines, untransformed and transformed plants were subjected to SDS-PAGE and subsequent stained with Coomasie dye. In our previous report (Ruiz et al., 2003), chloroplast transgenic lines expressing the MerA and MerB enzymes were able to resist high concentration of phenylmercuric acetate, although this was true expression level of the two enzymes was not detectable in Coomasie stained gels. A good way to characterize enhancement in expression produced by the use of the 5' regulatory sequences, used in this study would be the detection in a Coomasie stained gel.

The appearance of a distinct band at 69 kDa in the Coomassie-stained sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel (Fig 20A lanes 1-4) but not in the unstransformed sample (lane 5) suggests that the chloroplast transgenic plants were expressing high levels of mercuric ion reductase (Fig 20A). Failiure to the detect MerB enzyme may be because the *mer*B gene is under the regulation of psbA 5'UTR, which is light regulated. Because this plants were not tested under continious light exposure, maximal expression of *mer*B was not achieved.
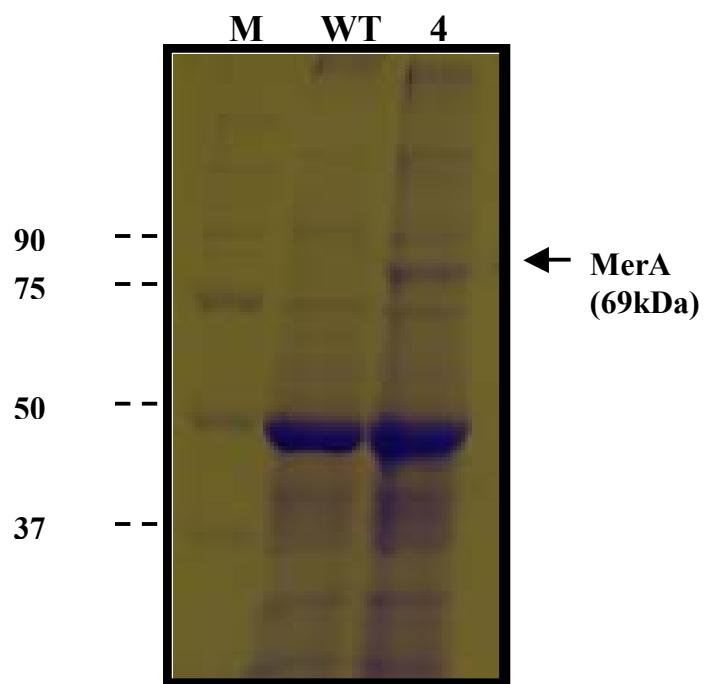
Figure 20: Coomassie Stained Gel

Abundant expression of mercuric ion reductase in the chloroplast of transgenic lines. 20 µg of total plant protein were loded per lane. M, molecular marker; WT, unstransformed tobacco plant protein; 4, chloroplast transgenic line 4.

Discussion

Several reports have shown that it is possible to enhance 300 to 500-fold the levels of transgene expression in chloroplasts if appropiate regulatory sequences (5'UTRs) are used instead of the ribosme binding site (RBS) alone (Staub et al., 2000; Fernandez-San Millan et al., 2003). Enhancing the levels of enzyme expression is definetly essential for the phytoremediation of polluted environments with very high levels of mercury. Additionally, organomercurials can effectively move across cellular membranes, achiving extremely high levels of mercury inside the cell or organelles. In this study, we developed a chloroplast transgenic line that harbours constructs with the *mer*A and *mer*B genes under the translational enhancement of the gene 10 5'UTR and the psbA 5'UTR, respectively. Additionally, 3'UTR for each of the two genes were engineered to provide increased stability of the transgene transcripts. The *mer* genes were directly amplified from the bacterial native plasmid with out codon optimization, because the chloroplast can effectively transcribe and translate genes from prokaryotic origin. This facilitates genetic manipulations (Kota et al., 1999; DeCosa et al., 2001; Ruiz et al., 2003). The chloroplast nature additionally facilitates the integration of the multigene construct through homologous recombination, allowing specific targeting of multigenes in a single transformation event without position effects.

Homoplasmic chloroplast transgenic lines harboring the construct aadA-g10 5'UTR-merA-rpsT-5'UTRpsbA-merB-3'UTRpsbA were achieved after 3 rounds of selection in medium with 500 μg/mL spectinomycin. Spectinomycin is a suitable selectable agent because it targets the chloroplast 70S ribosome. Several chlroplasts transgenic lines obtained after the first round

of selection tested positive by PCR for site-specific integration and for the complete presence of the multigene construct. Further characterization of the transgenic lines by Southern blot analysis after the third round of selection revealed that homoplasmy was achieved during T_0 generation. Absence of any detectable native untransformed chloroplast genomes not only confirmed homoplasmy, but also facilitated the detection of transgene copy numbers in each cell. It is known that mature leaf cells in tobacco contain about 10,000 copies of chloroplast genomes per cell (Bendich, 1987). By virtue of achieving homoplasmy, it is inferred that there are 10,000 copies of transgenes per cell. Achieving homoplasmy will also increase the gene dosage of the transgene and will assure that the foreign traits will not be dilited out over time. Additionally, the presence of the *mer*A and *mer*B genes in the targeted region (trnI-trnA spacer region) was also confirmed by Southern blot analysis. Any unspected DNA fragment detected in the Southern blot analysis will indicate that integration of the transgene construct occurred in the nuclear or mitochondrial genome.

Northern blot analysis of total plant RNA revealed that the transcriptional unit (operon) containing the *mer*A and *mer*B was efficiently transcribed. Because transcription inintiation may occurr from either of three promoters, three transcripts of major abundance were detected. The *mer*B monocistron transcribed from the psbA promoter was the most abundant transcript, followed by the aadA-merA-merB tricistron which is transcribed from the construct P*rrn* promoters. Finally, abundant transcription was also detected for the polycistron transcribed from the native P*rrn*. All of these promoters are constitutive promoters, which proves abundant transgenes transcripts. Interestingly, the northern blot analyses performed with the merA, merB and merAB probes revealed transcripts that read-through over the 3'UTR psbA, which is in the

gene construct, terminating drownstream at the trnA gene. This data coincides with the results shown in Chapter 2, where polycistronic transcripts were processed at a region located in the *trn*A gene. These results confirmed that 3'UTRs are not strong terminators in chloroplasts. This study demonstrates that the chloroplast can be engineered polycistronnically for metabolic engineering allowing the expression of multiple genes and the use of multiple regulatory sequences for the efficient transcription and translation of foreign operons without the disruption of the proper transcriptional function of the chloroplast.

In addition to the construction and molecular characterization of chloroplast transgenic lines harboring optimized vectors for organomercurial and mercury phytoremediation, we have also shown for the first time that chloroplast transgenic plants expressing the *mer*A and *mer*B genes can resist, accumulate, translocate, and bioremediate high concentrations of phenylmercuric acetate and mercury chloride. The transgenic lines that only contain Shine-Dalgarno sequences for the translation of the merA and merB genes were able to germinate and grow in concentrations of PMA of up to 200 μM. The resistance to organomercurials was increased to 400 μM if mature plants were used instead of seedlings.

This study shows that use of the chloroplast genome to express foreign genes offers several advantages to enhance mercury phytoremediation over nuclear transformation. High volatilization rates, as well as the Hg hyper accumulation in the root followed by the efficient translocation from roots to shoots in the chloroplasts transgenic plants provide support to the efficient conversion of organic-Hg and Hg[II] to the rapidly volatilized Hg[0] in the green tissues. Detoxifying organomercurial to elemental Hg, by expressing the MerA and MerB enzymes in the chloroplasts, protects cell normal functions and produce a continuous gradient for

137

Hg. In addition, this could allow Hg to move from one cell to the next either by crossing the cell membrane or via plasmodesmata, until reach the higher regions of the plant. In wild type plants, on the other hand, the greater toxicity of mercury is due to a higher toxicity to root metabolism, rather than a greater root uptake, disrupting normal cell functions and impeding the movement of Hg to other cells.

Most importantly, chloroplast genetic engineering presents an advantageous system for phytoremediation due to the uniparental inheritance of plastids, which prevents pollen transmission of foreign genes, making chloroplast genetic engineering a safer option for *in situ* phytoremediation. This study also provides a useful example of multigene engineering via the chloroplast genome for the production of a metabolic pathway.

GENERAL DISCUSSION

The scientific community has witnessed the evolution of plant genetic engineering from the expression of single genes of defined characteristics, to the development of polygenic metabolic pathways of complex functions. The expression of multiple genes is a difficult task when the nuclear genome is the target for the genetic manipulation. Alternatively, chloroplast transformation can result in a more efficient way to introduce and effectively express a multi gene pathway in plants. Because the chloroplast can transcribe polycistrons and multiple genes can be targeted to specific regions inside the chloroplast in a single transformation event, the chloroplast is a valuable tool for metabolic engineering.

Although the inherent advantages of the chloroplast system have been proven for the expression of agronomic traits and therapeutic proteins encoded by single genes, only few reports have shown the use of chloroplast genetic engineering for multigene metabolic engineering. The targeting of the *cry*2a2 operons into chloroplast encoding a total of four genes demonstrated that the chloroplast expresses very large multigene constructs effectively (De Cosa et al., 2001). More recently, the value of chloroplast metabolic engineering was established when a three gene operon expressing the enzymes for the degradative pathway of mercury conferred resistance and phytoremediation capabilities to chloroplast transgenic plants exposed to high levels of mercury (Ruiz et al., 2003). Finally, an operon containing the three genes for the biosynthesis of polyhydroxybutyrate was integrated into the chloroplast, producing the first report of PHB expression via chloroplast transformation (Lossl et al., 2003). However, this report was shadowed by limited expression levels and pleiotropic effects produced by the

expression of PHB in chloroplast. Aforementioned reports indicate that a better understanding of chloroplast biology at the levels of transcription, translation, post-transcription, and metabolism is needed for the efficient expression of complex pathways.

The expression of the biopolymer polyhydroxybutyrate represents a real alternative to the use of synthetic non-biodegradable polymers if it could be in cost-efective, yet abundant quantities. All attempts to express PHB in plants either by nuclear or chloroplast transformation have failed. To understand this pathway its detrimental effects to plants, we have turned our attention to the expression of β-ketothiolase, which is the first enzyme of the PHB biosynthetic pathway. This enzyme has been pointed to as the main cause for the detrimental effects observed in plants expressing PHB. So far, no reports of plants expressing β-ketothiolase have been found. Understanding the role of β-ketothiolase will help in designing of an effective metabolic pathway for the expression of PHB in chloroplasts and will further increase our knowledge of chloroplast biology and metabolism. We hyperexpressed β-ketothiolase via the chloroplast genome, and, in constrast to the previos reports for PHB expression, our chloroplast transgenic plants did not show any sign of pleiotropic effects, growing as well as wild type tobacco plants. Bohmert et al., (2002) reported that the integration of the *pha*A gene into the nuclear genome dramatically decreased transformation efficiency, inhibiting the recovery of fully regenerated transgenic plants. In contrast, we reported the characterization of multiple independent lines expressing the *pha*A gene.

The chloroplast transgenic lines expressing the phaA gene turned out to be male sterile. Molecular and histological characterization of the transgenic plants revealed that the expression of β-ketothiolase in the anthers led to a severe phenotype characterized by aberrant anther tissue

patterns, and collapsed pollen grains. Interestingly, this phenotype may represent a valuable tool for the production of male sterile lines. The expression of the *pha*A gene produced 100% male sterility; this is an advantage over current male sterility systems, which do not achieve complete male sterility. The expression of the *pha*A gene to produce male sterility has several advantages, among them is the idea that male sterility can be engineered by incorporating the *pha*A gene as a foreign gene in a multigene construct. Expression of the *pha*A gene can be targetted to anthers by the use of tissue specific or inducible promoters, and by the use of regulatory sequences. Tissue specific expression of β-ketothiolase in the anthers can lower concerns related to the constitutive expression of β-ketorhiolase. Additionally, this system has the possibility of being used as a uniform mechanism to produce male sterile lines in different plant species.

The study of β-ketohiolase via chloroplast genetic engineering has provided the first report of a genetically engineered cytoplasmic male sterily system in plants. Additionally, this report has increased our knowledge of the PHB metabolic pathways, revealing that β-ketothiolase is not the causative of the pleiotropic effect, at least when expressed by itself. Additional studies with constructs containing different combinations of the three genes involved in PHB expression are needed in order to pin point the key steps involved in toxicity by PHB.

We have consicely shown that chloroplasts are able to efficiently translate foreign polycistrons without processing into monocistrons. Also, we observed that the chloroplast can recognize and process stable secondary structures produced from heterologous sequences inserted during cloning or from the foreign organism. Intergenic sequences that form stable secondary structures and mask the ribosome-binding site may affect the translation of the downstream gene and are removed by processing of the transcripts, enabling translation (Barkan

141

et al., 1994; Hirose and Sugiura 1997; Del Campo et al., 2002). The factors involved in processing of the secondary structures in the native chloroplast transcripts have been shown to be target-specific, targeting specifc sequences inside the steem loops. Although, this is the case for native sequences, the processing event observed in our transgenic plants occurred within a heterologous sequence, indicating that there is some degree of flexibility in the sequences recognized inside the secondary structures. Alternatively, the processing machinery may target specific secondary structures beside specific sequences. Finally, we have shown that polycistronic mRNA lacking 3'UTR is stable and can be translated. These results are important for multigene engineering because they facilitate the expression of multigenes by unerstanding the role of different sequences withing a foreign operon.

Finally, we have shown that heterologous pathway can be engineered via the chloroplast genome to confer complex new traits that improve our environment. The expression of the *mer*A and *mer*B genes via the chloroplast genome promoted the detoxification of high concentrations of organomercurials, and confered resistance to high levels of the toxic chemical (Ruiz et al., 2003). We reported the hyperexpression of the *mer*A and *mer*B genes using chloroplast and heterologous 5'UTR. This is an important report that shows the increased capabilities, applicability and the potencial of multigene genetic engineering via the chloroplast genome. This provides an excellent example of how metabolic engineering can improve our quality of life. This pathway provides a good example for beneficial effects of plants genetic engineering. The knowledge adquired from the results of this research will certainly offer insight for the effective expression of multigene metabolic pathways via chloroplast genetic engineering.

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153

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