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# Chloroplast Biotechnology in Higher Plants: Expressing Antimicrobial Genes in the Plastid Genome

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CHLOROPLAST BIOTECHNOLOGY IN HIGHER PLANTS:  EXPRESSING
ANTIMICROBIAL GENES IN THE PLASTID GENOME

A Thesis

Submitted to the Graduate Faculty of the
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in
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Tracey A. Ruhlman

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# Acknowledgment

Had anyone told me four or five years ago that I would someday be a research biologist I would have found it highly amusing. When I began my graduate career in June 2003 I had virtually no lab experience and only a layman's concept of the work that goes on in a molecular biology lab. I owe the deepest gratitude to my thesis advisors at the University of New Orleans, Drs. Candace Timpte and Mary Clancy, for somehow recognizing potential in me and giving me the opportunity to realize what has now become my passion. The experience that I have had at the USDA Southern Regional Research Center (SRRC) has established a direction for my life that I had never imagined.

I would like to thank Drs. Kanniah Rajasekaran and Jeffery Cary, my advisors at SRRC, for their guidance and friendship. I suspect that when I first came to the lab my training must have demanded a wealth of patience on their part. I look forward to a time in the future when I may be able to make a valuable contribution to their work as a collaborator and colleague. I would also like to thank Pam Harris who helped so much in my training, always warm and supportive.

To my husband, my son, and all of our family and friends, your encouragement continues to be the source of my determination to improve myself as an investigator and as an individual. No one stands alone in accomplishment, we are held up by those who offer us love and support; thank you for yours.

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# Abstract

While genetic improvement of susceptible crop species may enhance resistance to microbial pathogens and facilitate reduced pesticide load, the possibility for transmission of novel genes to wild relatives has hampered acceptance of GM crops in some markets. Chloroplast transformation presents an attractive alternative to nuclear transformation and offers the potential to ameliorate these environmental concerns. Most agronomically important species exhibit maternal inheritance of organellar genomes which eliminates the threat of transgene escape through pollen. Gene silencing is absent due to site directed, single copy insertion by homologous recombination. Foreign proteins can accumulate to high levels (up to 50% of total soluble protein) and are retained within the chloroplast envelope protecting them from degradation by host cytoplasmic proteases. A bacterial chloroperoxidase gene (*cpo-p*) was transformed into the tobacco chloroplast genome to test its efficacy against plant pathogens and the mycotoxin producing saprophyte *Aspergillus flavus*.

# Introduction

The current global human population of 6.4 billion is expected to reach 10 billion by the year 2050. The rate of agricultural yield at present is not sufficient to meet this demand and already malnutrition and starvation are taking a toll world wide. In the past increased productivity of primary producers, namely higher plants, has been accomplished through selective breeding programs but the successes in this area have reached a plateau. Bringing new acreage under cultivation is not a viable option as many lands in developing nations are marginal and serious environmental consequences prohibit agricultural development on remaining fertile preserves.

The architects of the 'Green Revolution' envisioned that increased global carrying capacity would result from the development of new crop cultivars, the use of irrigation systems, and the application of chemical fertilizers and pesticides. Food production increased over 1000% from 1960 to 1990 but not without consequences in terms of production cost, dependence on chemical inputs, top soil erosion and salinization due to irrigation practices and the development of pesticide-resistant species.

Nobel Peace Prize winner Norman Borlaug, considered the Father of the Green Revolution, suggests that in biotechnology lies the potential to ameliorate environmental concerns while meeting the rising demand for agricultural production (Borlaug, 2005). Indeed, through biotechnology many improvements have been made. Crop species have been genetically engineered to resist pests (Cary *et al.*, 2000; Rajasekaran *et al.*, 2000 Rajasekaran *et al.*, 2002), tolerate drought (Shou *et al.*, 2004) and herbicide treatment (Chin *et al.*, 2003), and to enhance nutritional value (Goto *et al.*, 1999; Ye *et al.*, 2000) through the incorporation of novel DNA in the nuclear genome. Specific technical

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challenges related to the random integration of foreign DNA sequences such as transgene silencing (Fagard *et al.*, 2000) are further compounded by negative public sentiment partially fueled by the fear of transgene escape via pollen or unintended effects on pollen feeding, non-target insects such as the monarch butterfly (Shelton, 2003). Without the acceptance of regulatory agencies and the general public, the advances made through biotechnology are of limited use, isolated in the laboratory and unable to facilitate much needed improvements in the field. The responsibility of finding new platforms for increased agricultural productivity without concomitant threat to the environment falls to science.

Higher plants possess two genomes in addition to that of the nucleus; the organellar genomes of mitochondria and chloroplasts. Genetic engineering of higher plant chloroplasts may offer the potential to mitigate certain distress in terms of agricultural productivity. Technological advances, most notably the invention of the particle accelerator (Boynton *et al.*, 1988), have provided the opportunity to explore the chloroplast genome as a platform for the expression of genes which may help address current and future demands for improved food production.

The genetic potential of the chloroplast lies in its negatively supercoiled, double stranded, circular DNA molecule referred to as the plastome. Within the angiosperms the plastome carries approximately 120 to 130 genes and ranges in size from 120 to 180 kilobases (kb) (Sugiura, 1992). Plastome molecules are clustered in nucleiods which are associated with plastid membranes and readily observed by fluorescent microscopy following DAPI staining (Mache *et al.*, 2001). Of the estimated 3000 or so proteins found in the higher plant chloroplast (Colas des Francs-Small *et al.*, 2004; Richly *et al.*,

2004), only a small fraction are encoded by the plastome (Shimada *et al.*, 1991). The bulk of the chloroplast proteome is nuclear encoded, translated on cytosolic ribosomes and subsequently translocated across the chloroplast envelopes (Zerges, 2000).

The plastome exists in a highly polyploid state with up to 100 identical copies present in each plastid of a mature leaf cell (Maier *et al.*, 2004). In a mature leaf, mesophyll cells carry up to 100 chloroplasts with the result that this genome alone can comprise up to 20 % of the total cellular DNA content (Bendich, 1987). The plastome persists in all plastid differentiation types: the proplastids of meristematic tissues, green chloroplasts, red or yellow chromoplasts, the colorless plastids amyloplasts and leucoplasts (starch containing), and elaioplasts (oil containing) (Maliga, 2004).

The plastome is strictly maternally inherited in most species of agricultural interest (Hagemann, 2004). In maternal inheritance systems, paternal transmission of plastids is impeded during either the first pollen mitosis *via* unequal plastid distribution, or during generative or sperm cell development *via* plastid degeneration (Birky Jr., 2001; Mogensen *et al.*, 2000; Zhu *et al.*, 1992). Therefore, the generative and sperm cells in mature pollen tend to be free of plastids.

Plastid gene organization and structural features are conserved among eukaryotic photosynthetic organisms (Mache and Lerbs-Mache, 2001; Sugiura, 1992). The circular molecule can be divided into three distinct domains: large single copy (LSC), small single copy (SSC) and the inverted repeat (IR) which is present in exact duplicate separated by the two single copy regions (Figure 1-1). Restriction fragment length polymorphism (RFLP) analysis indicates that the molecule exists in two orientations present in equimolar proportions within a single plant (Palmer, 1983). The circular

molecule undergoes interconversion to a dumbbell-shaped conformation that is believed

to be facilitated by the presence of the IR.  Concerted evolution within the IR (Kolodner

*et al.*, 1975;Kolodner *et al.*, 1976) suggests intramolecular recombination between the

repeats is a possible mechanism.
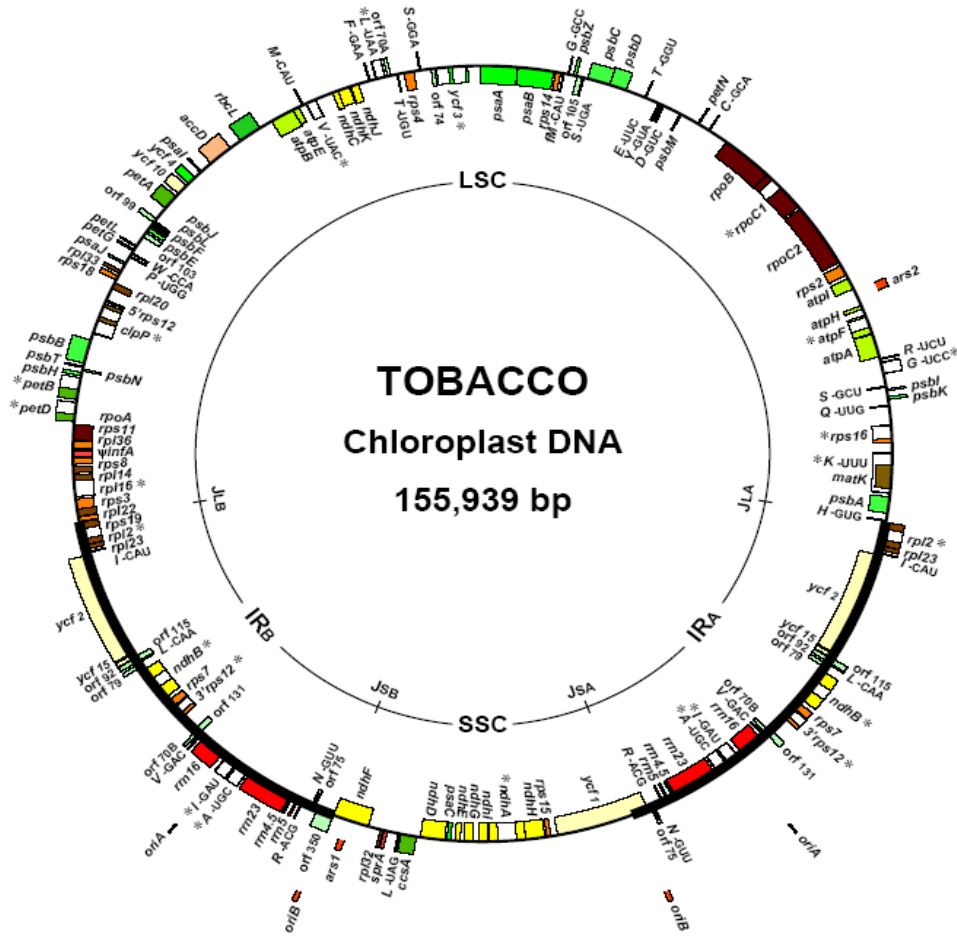
**Figure 1-1**:  **Map of a typical angiosperm chloroplast genome**, *Nicotiana tabacum* (from Wakasugi, 2001).

As sessile organisms plants are unable to escape potential DNA damage induced by ultraviolet (UV) radiation. A wide spectrum of DNA modifications such as base damage, strand breaks and DNA and protein crosslinking can arise from exposure to UVA and UVB light (Cadet *et al.*, 1992; Ravanat *et al.*, 2001). Additionally plastid DNA is subject to photooxidative damage due to the presence of reactive oxygen species generated through photosynthetic metabolism (Adam *et al.*, 2001). The need for a plastid localized DNA repair system is evident. In *Escherichia coli* the RecA protein is essential for homologous recombination and for a variety of SOS responses to DNA damage (Smith *et al.*, 1989; Witkin *et al.*, 1987). A related protein exists in stromal extracts of *Pisum sativum* (pea) and *Arabidopsis thaliana* as revealed by immunoblotting with *E. coli* RecA antisera (Cerutti *et al.*, 1992).

The nuclear localization of the higher plant r*ecA* gene has been supported. The cyanobacteria *Synechococus recA* gene hybridized to pea and *Arabidopsis* genomic DNA, whereas homology was not detected in purified chloroplast or mitochondria DNA. The isolation of a cDNA for the *recA* gene from *Arabidopsis thaliana* that included a predicted chloroplast transit peptide further indicated the nuclear residence of this gene whose product is subsequently targeted to the chloroplast (Cerutti *et al.*, 1992). DNA strand transfer activity has been detected in pea (Cerutti *et al.*, 1993), and *Chlamydomonas reinhardtii* transformants expressing a dominant negative mutant of the *E. coli* RecA protein showed reduced survival rates when exposed to mutagenic agents (Cerutti *et al.*, 1995). An active recombination system appears to be the mechanism of DNA repair in plastids.

At least two distinct RNA polymerases are responsible for transcription of chloroplast genes: the plastid encoded, multisubunit RNA polymerase (PEP) and the nuclear encoded, single polypeptide RNA polymerase (NEP) (Allison *et al.*, 1996). Many chloroplast genes of higher plants are co-transcribed from operons producing polycistronic primary transcripts. Several monocistrons are also transcribed including *psbA*, encoding the D1 core polypeptide of photosystem II, and most of the 30 tRNA genes (Bonen, 2004). Plastid genes and operons can be classified according to the promoter sequences upstream of the transcription initiation site: those that have sequences recognized by PEP only, by NEP only, or those that contain recognition sequences for both PEP and NEP (Hajdukiewicz *et al.*, 1997).

Study of these promoter regions indicate that NEP is active in the early stages of development, establishing the chloroplast genetic system, whereas PEP is the predominant activity in mature chloroplasts and responsible for the transcription of the photosynthesis genes (Mullet, 1993; Sugita *et al.*, 1996). The core subunits of eubacterial-like PEP are encoded by the plastid *rpoA, rpoB, rpoC1* and *rpoC2* genes (transcribed by NEP) (Baumgartner *et al.*, 1993). Disruption of these genes leads to a pigment deficient phenotype, eventual loss of stacked thylakoid membranes and the disappearance of normally abundant transcripts for photosynthesis related proteins (Allison *et al.*, 1996).

While the plastid ribosomal RNA operon (*rrn*) is constitutively transcribed, the process is regulated in response to environmental and developmental cues (Baumgartner *et al.*, 1993). Transcription of the operon is accomplished by at least two different RNA polymerases from one of four promoter elements (Iratni *et al.*, 1997; Suzuki *et al.*, 2004;

Vera *et al.*, 1995). In tobacco, transcription by the PEP from a sigma 70 ($\sigma^{70}$) type promoter (P1) with conserved -10 and -35 elements is thought to predominate. $\sigma^{70}$ like factors encoded in the nucleus and imported from the cytoplasm are thought to be required by PEP for transcription initiation, allowing for highly concerted regulation of chloroplast gene expression (Kanamaru *et al.*, 1999; Kanamaru *et al.*, 2004). Deletion series analysis of P*rrn*P1 has identified a hexamer, GTGGGA, directly upstream of the -35 box as essential to P*rrn*P1 promoter activity. This element is referred to as RUA (rRNA upstream activator) (Suzuki *et al.*, 2003). Mutation of the -10 element had only a moderate effect on transcription of the operon, whereas other chloroplast genes transcribed by PEP, such as *psbA* and *rbcL*, require this sequence element for promoter strength (Eibl *et al.*, 1999; Kim *et al.*, 1999). This suggests that RUA may have replaced a sigma-type interaction with the -10 element and itself interacts with PEP directly or via some other protein factor which binds to the RUA. Abolition of PEP activity by mutation of the *rpoB* operon, did not lead to a dramatic reduction in transcript levels for *rrn* indicating effective transcription of the ribosomal operon can also be accomplished by the NEP (Allison *et al.*, 1996) from a novel promoter element (P2) located at -64 relative to the transcription start site and downstream of P1 (Vera and Sugar, 1995). Ultimately, transcription of all chloroplast genes is under control of the nucleus at several levels.

Transcript availability is not the rate limiting factor for protein accumulation in mature leaf chloroplasts. Photosynthetic proteins, the most abundant proteins in chloroplasts, are closely regulated post-transcriptionally and changes in steady-state levels are attributed to the stability of messenger RNA (mRNA) rather than the transcription of these molecules from their respective genes (Barnes *et al.*, 2004; Zerges,

2000).  Transcript stability, maturation and subsequent translation into protein products is influenced by several features of the mRNA.  Chloroplast mRNAs include 5' and 3' untranslated regions (UTRs), both of which confer on the molecule distinctive elements necessary for the eventual production of chloroplast proteins from mono- or polycistronic transcription units (Monde *et al.*, 2000b).

Mature chloroplast mRNAs do not have 3' poly A tails *per se* although similar structures can be detected in low abundance and appear to be involved in marking transcripts for degradation (Monde *et al.*, 2000a; Sugita and Sugar, 1996).  Most transcription units studied in chloroplast systems, including monocistronic *psbA,* carry a short inverted repeat (IR) region within the 3' end that is predicted to form a stem loop.  The 3' IR present in *psbA* is not an effective terminator of transcription (Deng *et al.*, 1987) and polycistronic transcripts have been shown to have embedded IRs (Marchfelder *et al.*, 2004; Westhoff *et al.*, 1988).  Rather these IRs appear to act as RNA processing signals for endonucleolytic cleavage of pre-mRNAs and further serve to stabilize the transcript by blocking the activity of 3'➜5' exonucleases resulting in homogeneity of 3' ends (Adams *et al.*, 1990; Chen *et al.*, 1991).  As demonstrated in *Chlamydomonas*, *atpB* mRNAs with discrete 3'end were associated with polysomes, while those with heterogeneous ends remained in non-polysomal fraction (Rott *et al.*, 1998) indicating a 3'end effect on translation.  In higher plant chloroplasts though, a substantial portion of some mRNAs, such as *psbA*, are not found to be associated with polysomes (Hirose *et al.*, 1996) but are immunoprecipitated with plastid RNA binding proteins.  It has been suggested that these proteins stabilize non-polysomal pre-RNAs prior to assembly into

ribosomal complexes (rRNA), or until subsequent replacement by ribosomes is accomplished (Sugita and Sugiura, 1996).

Experiments employing gene fusion constructs for reporters such as β-glucuronidase (GUS) and green fluorescent protein (GFP) have demonstrated that polycistronic mRNAs can be efficiently translated in plastid systems. The plasmids utilized in these transformation experiments included a single promoter for transcription of an antibiotic marker fused to the promoterless GFP or GUS sequence with a Shine Delgarno (SD)-like ribosome binding site introduced between the two sequences. Accumulation of dicistronic mRNAs as well as their efficient translation leading to high levels of foreign protein has been established (Jeong *et al.*, 2004; Staub *et al.*, 1995).

Maturation of plastid pre-mRNA, rRNA and tRNA into functional molecules requires the coordination of *cis* and *trans* acting factors and may occur co- or post-transcriptionally. C to U as well as U to C editing is found in plastid mRNA and U to C conversion is observed in plastid tRNAs (Mulligan, 2004). Additionally, the higher plant chloroplast genome contains ~40 introns which must be spliced according to environmental and developmental cues before the respective RNAs are functional in the organelle (Barkan, 2004). Stepwise assembly of the translation competent ribosomal complex is fully accomplished at the first codon (AUG) of the coding region (Zerges, 2000; Zerges, 2004) and, in terms of translational efficiency, initiation may be the most strictly regulated step. Recruitment of a host of factors, both nuclear and plastid encoded, is required for protein synthesis on 70S plastid ribosomes (Bruick *et al.*, 1999; Manuell *et al.*, 2004). SD-like sequences (most commonly GGAGG or GGAG), ribosome binding sites (RBS), stem loop structures and various "box" sequence elements are found to exist

within plastid mRNA  5' UTRs and are considered the cis-acting factors involved in translation initiation.

In tobacco chloroplasts, 30 of 79 protein coding genes examined lack a SD-like sequence within 20 nucleotides of the start codon and the remaining 49 posses these motifs but not at a conserved position (Sugiura *et al.*, 1998).  Bacterial SD sequences interact with the anti-SD region at the free 3' end of 16S rRNA to facilitate translation of the protein products and elements both up and downstream of the translation start site are believed to play a role in the efficiency of this process.  Increasing the complementarity of the 15 nucleotide downstream box (DB) to the anti-DB region of the 16S penultimate stem lead to enhanced protein accumulation in *E. coli* (Etchegaray *et al.*, 1999).  A similar experiment in a tobacco plastid system did not demonstrate improved protein expression.  Transformation with a chimeric construct including the strong P*rrn*P1 promoter, phage T7 gene10 5'UTR and *E. coli* DB resulted in neomycin phosphotransferase II (NTPII) accumulation to 16% of total soluble protein (TSP), while eliminating the DB boosted NTPII in mature leaves to ~23% of TSP.  Increasing complementarity between sequence downstream of the start codon and 3' 16S rRNA through the inclusion of plastid native down stream sequences rather than *E. coli* DB in the transformation cassette dramatically reduced NTPII accumulation in part due to increased turnover in mRNA (Kuroda *et al.*, 2001).  This result suggests that the 5' region involved in transcript stability and complementary interactions with the ribosome lies within the UTR for plastid genes.

In depth analyses have detailed the structure of the 85 nucleotide *psbA* 5' UTR which contains representatives of the translational motifs discussed above occurring
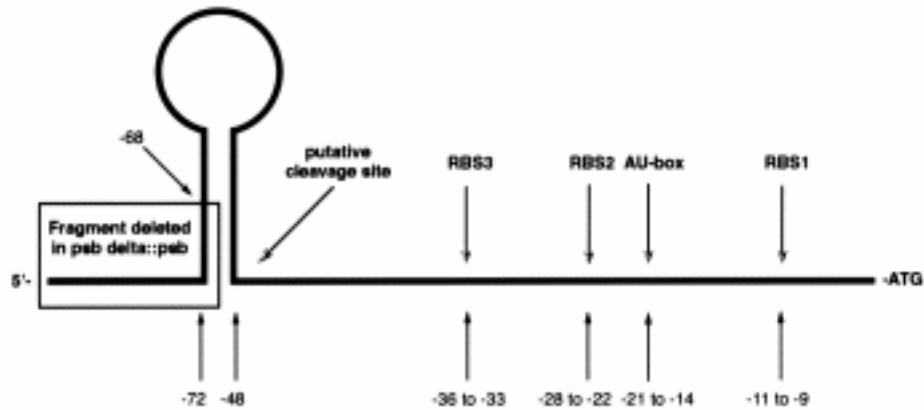
**Figure 1-2:  Schematic representation of the *psbA* 5' UTR.**  Boxed area indicates 17 nucleotide deletion (from Eibl, 1999).

in this order:  5'stem loop, RBS3, RBS2, AU box, RBS1 3' (Figure 1-2).  RBS2 and

RBS1 appear to act cooperatively, as deletion of both sites has a more severe effect than

single deletions for either (Hirose and Sugiura, 1996).  A stem loop structure is predicted

between the two which allows the intervening AU box to loop out resulting in a model
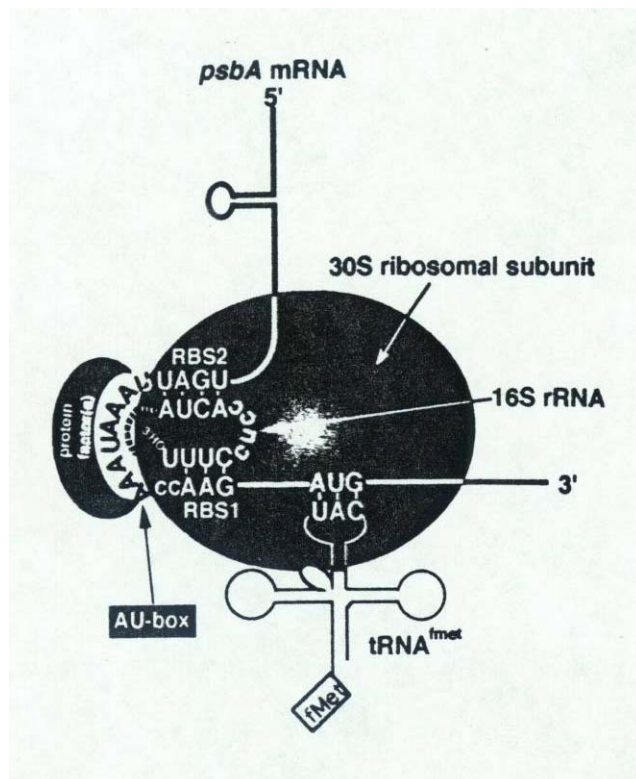


**Figure 1-3:  Model of possible orientation of the *psbA* 5'UTR within the 30s ribosomal subunit.** Predicted base pairing between RBS1 and RBS2 may facilitate binding of a protein factor to the AU Box (from Hirose, 1996)

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for interaction with a protein factor within the translation complex (Figure 1-3) (Hirose and Sugiura, 1996; Zerges, 2000) Detection of initiation complexes corresponding to ribosomes with initiator tRNA on RBS3 lead to the proposition that the small subunit first binds transiently here then scans in the 5'➔3' direction to the legitimate AUG downstream. This scanning does not appear to be an absolutely required step as mutation or deletion of RBS3 reduced translation by less than 15% of wild-type levels. (Hirose and Sugiura, 1996; Kim *et al.*, 1994). The stem loop structure found at -48 through -72 upstream of AUG possesses a predicted endonuclease cleavage site at the 3' end (Alexander *et al.*, 1998). Mutant lines for the stem loop structure demonstrated a twofold lower transcript abundance following a 17 nucleotide deletion at the 5' end of the UTR (Figure 1-2). An eight-fold depression in protein accumulation was observed resulting in an overall 4-fold depression of translation efficiency. A predicted cleavage site at the 3' end of the stem loop accounts for the differently sized transcripts found among native *psbA* mRNAs. The smaller sized transcript is present at much higher steady state levels. The longer species is not found to be associated with polysomes suggesting that it is not translatable (Bruick *et al.*, 1998).

As many endogenous chloroplast proteins function in the photosynthetic unit it is only logical to predict their expression will by regulated by light. Translation of *psbA* is stimulated by light to a greater extent than other mRNAs (Eibl *et al.*, 1999; Staub *et al.*, 1993; Staub *et al.*, 1994) reflecting the requirement for replacement of the PSII core subunit following photooxidative damage. Upregulation of D1 expression in response to light has been mainly investigated in etiolated seedling versus those grown in 16 h light/8 h dark growth chambers. Transplastomic lines for chimeric *psbA* 5' region (including

promoter) and *uidA* (GUS) demonstrated 135- to 200-fold higher expression in light grown plants over those deprived of light based on fluorogenic assay and illumination of dark grown seedlings enhanced expression by 32-43 fold within 36 hours. In these constructs the 3' UTR was varied, with *rcbL* 3' UTR resulting in the greatest enhancement. When constructs were employed with constant *psbA* 3' regions and variable 5' UTRs results were less dramatic, with light enhancement of GUS expression in the 2 to 4 fold range. Analysis of transcript abundance demonstrated only modest increases among transgenic lines exposed to light versus dark grown (Staub and Maliga, 1994). This indicates that enhancement of translation resides in the 5' leader for *psbA*.
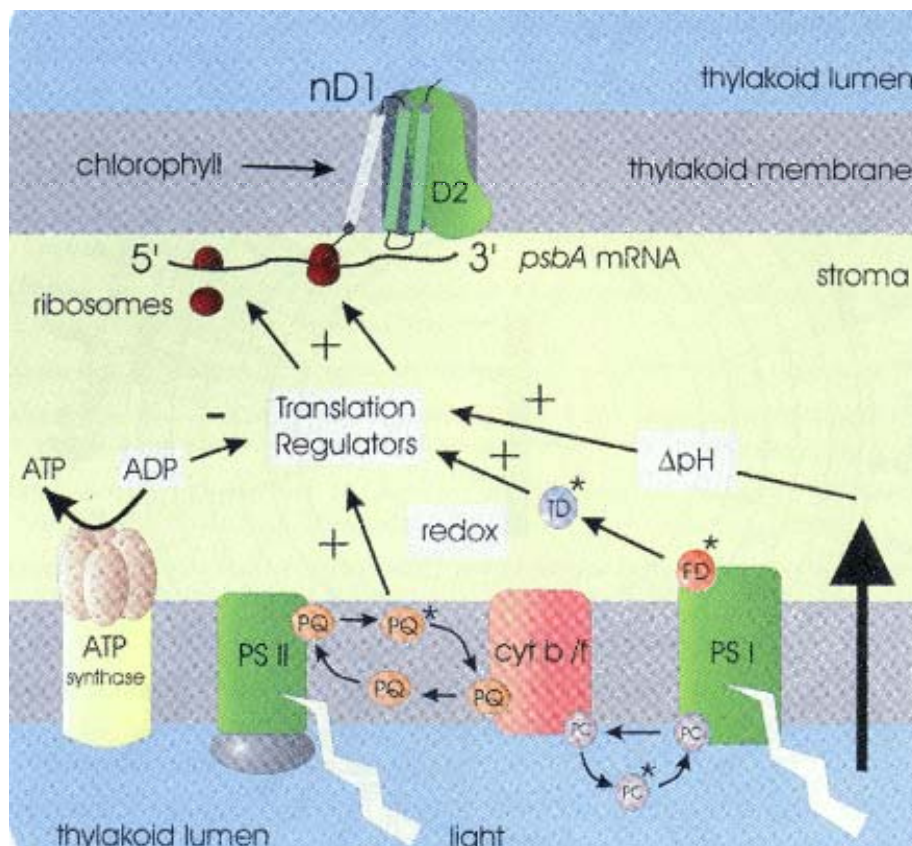


**Figure 1-4: Translation of *psbA* mRNA is mediated by light through photosynthesis.** ADP concentration, the redox state of electron carriers plastoquinone (PQ) and thioredoxin (TD) and the electrochemical proton gradient (large arrow) regulate D1 synthesis. Abbreviations: nascent D1, nD1; ferredoxin, FD; photosystem II, PSII; cytochrome b6/f complex, cyt b6/f; photosystem I, PSI; plastocyanin, PC. Asterisks indicate reduced state (from Zerges, 2004).

Interestingly, while transcription of nuclear and chloroplast genes is regulated by light via photoreceptors and signal transduction pathways, translation of chloroplast mRNAs appears to be regulated by light through the effects of photosynthesis.  Binding proteins involved in translation activation have been identified by RNA affinity purification from cells grown in the presence or absence of light.  In *Chlamydomonas* light regulation of *psbA* translation is mediated by a set of four proteins that bind as a complex to the 5' UTR.  One member of this complex, RB47, is a homolog of polyadenylate-binding protein (PABP).  This nucleus-encoded factor binds exclusively to
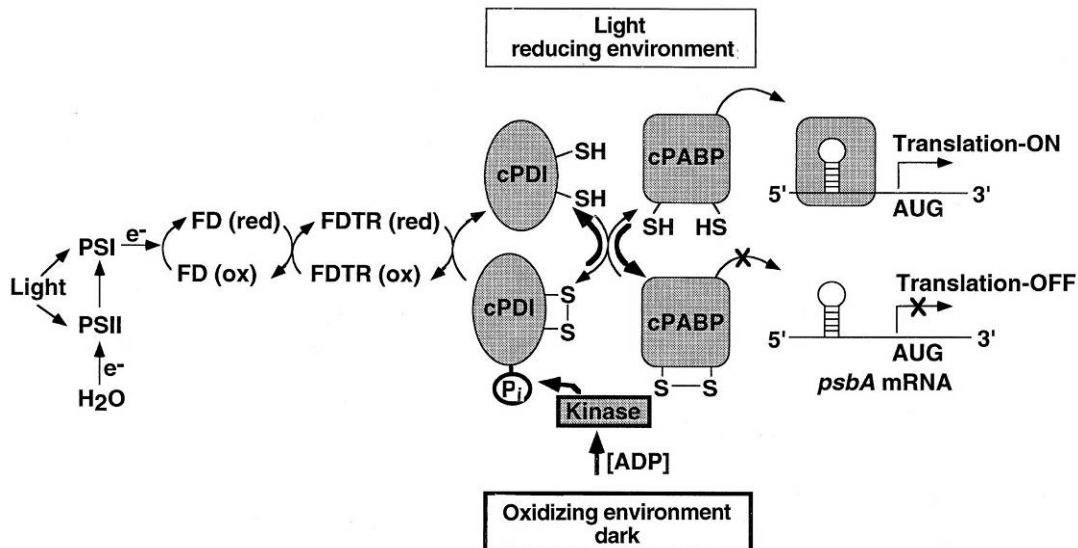


**Figure 1-5:  A model of light-regulated translation of *psbA* mRNA in *C .reinhardtii.***
Thin arrows and thick arrows indicate the light pathway and the dark pathway, respectively.  Abbreviations:  photosystem I and I , PSI and PSII; ferredoxin, FD; ferredoxin-thioredoxin reductase, FDTR; chloroplast protein disulfide isomerase (RB60), cPDI; chloroplast polyadenylate-binding protein (RB47), cPABP; oxidized, ox; reduced, red; inorganic phosphate group, $P_i$ (from Kim, 1997)

the 5' UTR of *psbA* mRNA in competition experiments (Danon *et al.*, 1991; Yohn *et al.*, 1998a; Yohn *et al.*, 1998b) lending support to the concept that many chloroplast mRNAs have their own specific activation factors.  Another member of this complex, RB60 (60

kDa), is phosphorylated by a serine/threonine protein kinase found to be associated with the complex. Transfer of the β-phosphate of ADP to RB60 abrogates the binding activity of the complex (Kim *et al.*, 2002). Sensitivity to the redox state of electron carriers involved in the light reactions is thought to activate RB60, a homolog of protein disulfide isomerase (PDI). The activity of RB60 within this RNA binding complex provides a direct link between exposure to higher light intensities and the rate of D1 synthesis in response to photodamage (Barnes *et al.*, 2003; Kim *et al.*, 1997; Trebitsh *et al.*, 2000).

Elucidation of transcription and translation mechanisms in chloroplasts has been largely facilitated by the ability to manipulate the plastid genome employing a reverse genetics approach. A greater understanding is developed with each experiment through the confirmation of expected results and the thrill of unexpected discovery. The application of this knowledge permits the opportunity to explore the plastome as a platform for the expression of recombinant proteins.

By far the most common tool for introducing foreign DNA into chloroplasts is the particle accelerator, or "gene gun" (Boynton *et al.*, 1988). Chloroplast transformation was first accomplished in *Chlamydomonas* (Blowers *et al.* 1989; Boynton *et al.*, 1988)
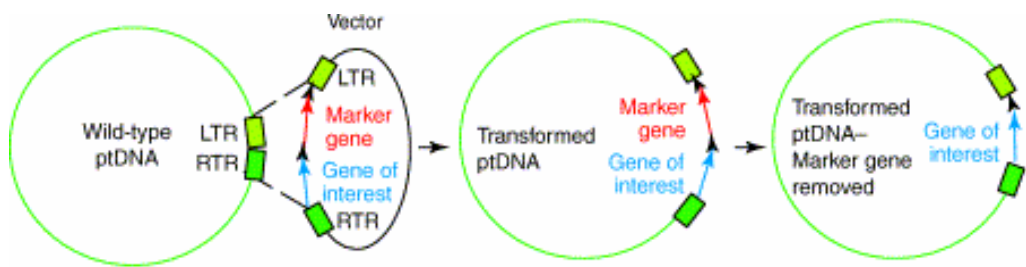


*TRENDS in Biotechnology*

**Figure 1-6: Diagram of foreign DNA insertion into the chloroplast genome.** Site specific integration by homologous recombination is directed by left (LTR) and right (RTR) targeting regions encoded on transformation vector (from Maliga, 2003).

and soon after requirements were established for application in higher plants (Svab *et al.*, 1990a). First, plasmid vectors designed for chloroplast systems are precipitated onto microprojectile particles. Tungsten particles on the order of 1μm in size were used in the early experiments and are still common in the transformation of *C. reinhardtii* chloroplasts (Cheng *et al.*, 2005; Sommer et al,. 2002), whereas gold particles are now more prevalent for higher plants. The smooth surface, homogeneity of size and size choice possible with milled gold particles may be in part responsible for higher transformation efficiency now routine in higher plant model systems. Particles are driven by pressurized helium (1100-1300 psi) into an evacuated chamber containing tissue or cells for transformation penetrating cells and the double membrane of chloroplast (Ye *et al.*, 1990). Once a plasmid coated particle is, by chance, introduced into a chloroplast integration is possible via homologous recombination.

One of the great advantages of plastid transformation systems is the ability to direct single copy insertion of transgenes. The location of insertion is controlled by flanking the transgene with plastid endogenous sequence 1 to 2 kb in length (Maliga, 2003) on the transformation vector (Figure 1-6). Intergenic regions are attractive for expression of recombinant proteins (Kumar *et al.*, 2004a; Zoubenko *et al.*, 1994) while specific mutant lines can be established by replacement of wild-type genes with nonfunctional versions carrying reporters or resistance markers (Allison *et al.*, 1996; Takahashi *et al.*, 1991).

The achievement of homoplasmy is paramount to the establishment of stable transplastomic lines is the. Due to the highly polyploid nature of the plastid genome the initial transformation event, that of recombination between the vector and a single

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plastome copy, must be iterated throughout all plastome molecules until no wild-type copies remain.  The mixed genotype is unstable and this condition does not persist, resolving into one state (wild-type) or the other (transformed) (Maliga *et al.*, 1993).  The transformation of all copies is facilitated by culturing on non-lethal selective media. Plastids carrying the resistance marker, and in turn the cells that harbor these plastids, are preferentially maintained as plastome molecules are divided up between daughter chloroplasts and subsequently as plastids are partitioned between daughter cells at mitosis (Maliga *et al.*, 1993; Moller, 2005).

The earliest reports of stable transformation of higher plant plastids replaced the native 16S rRNA gene with a copy that had received a base mutation proximal to the tRNA binding domain (Svab *et al.*, 1990a).  This mutation confers resistance to the antibiotic spectinomycin (Svab *et al.*, 1991) which is known to prevent initiation of protein synthesis on plastid ribosomes (De Stasio *et al.*, 1989).  Spectinomycin selection remains predominant although newer constructs utilize the bacterial *aadA* gene encoding aminoglycoside-3'-adenyltransferase which also confers resistance to streptomycin.  First explored as a dominant selectable marker in nuclear transformation experiments (Svab *et al.*, 1990b), then for *Chlamydomonas* plastid transformation (Goldschmidt-Clermont, 1991), *aadA* proved 100 times more effective than the 16S RNA mutation in the recovery of tobacco plastid transformants (Svab *et al.*, 1993)  A current focus in the field is the development of systems for the removal of antibiotic resistance markers (Corneille *et al.*, 2001; Corneille *et al.*, 2003; Day *et al.*, 2005; Iamtham *et al.*, 2000) or efficient selection programs that do not employ antibiotics (Daniell *et al.*, 2001b). Beyond those for antibiotic resistance, several genes have now been expressed via the plastid genome.

Improved agronomic performance (De Cosa *et al.*, 2001; DeGray *et al.*, 2001),

production of human therapeutic proteins (Fernandez-San Millan *et al.*, 2003; Staub *et al.*, 2000) , vaccine antigens (Daniell *et al.*, 2001a; Tregoning *et al.*, 2003; Watson *et al.*, 2004), and polymer compounds of commercial importance (Guda *et al.*, 2000; Lossl *et al.*, 2003; Viitanen *et al.*, 2004) are being explored (see Tables 1-1 and 1-2).

| Trait | Transgene | Promoter | 5'/3' UTRs | Integration site | Refs |
|---|---|---|---|---|---|
| Insect resistance | Cry1A(c) | Prrn | rbcL/Trps16 | trnV/rps12/7 | McBride *et al.*, 1995 |
| Herbicide resistance | AroA | Prrn | ggagg/T*psbA* | rbcL/accD | Daniell *et al.*, 1998 |
| Insect resistance | Cry2Aa2 | Prrn | ggagg (native)/T*psbA* | rbcL/accD | Kota *et al.*, 1999 |
| Herbicide resistance | bar | Prrn | rbcL/*psbA* | rbcL/accD | Iamtham and Day, 2000 |
| Insect resistance | Cry2Aa2 operon | Prrn | native 5'UTRs/T*psbA* | trnI/trnA | De Cosa *et al.*, 2001 |
| Disease resistance | MSI-99 | Prrn | ggagg/T*psbA* | trnI/trnA | DeGray *et al.*, 2001 |
| Drought tolerance | tps | Prrn | ggagg/T*psbA* | trnI/trnA | Lee *et al.*, 2003 |
| Phytoremediation | merA/merB | Prrn | ggagg,/T*psbA* | trnI/trnA | Ruiz *et al.*, 2003 |
| Salt tolerance | badh | Prrn | ggagg/rps16 | trnI/trnA | Kumar *et al.*, 2004b |
| Cytoplasmic male sterility | phaA | Prrn | P*psbA*/T*psbA* | trnI/trnA | Ruiz and Daniell, 2005 (in press) |

**Table 1-1: Some examples of chloroplast transformation experiments for genes of agronomic interest.** A "P" indicates the inclusion of the promoter sequence in 5' regulatory element.

| Proteins | Transgene | Promoter | 5'/3' UTRs | % of TSP | Integration site | Ref |
|---|---|---|---|---|---|---|
| Elastin-derived polymer | EG121 | Prrn | T7gene10/ T$psbA$ | ND | trnI/trnA | Guda *et al.*, 2000 |
| Human somatotropin | hST | Prrn$_a$ P$psbA_b$ | T7 gene10$_a$, $psbA_b$/Trps16 | 7.0%$_a$, 1.0%$_b$ | trnV/rps12/7 | Staub *et al.*, 2000 |
| Cholera toxin | CtxB | Prrn | Ggagg/T$psbA$ | 4% | trnI/trnA | Daniell *et al.*, 2001a |
| Human serum albumin | hsa | Prrn$_a$, P$psbA_b$ | ggagg$_a$, $psbA_b$/ T$psbA$ | 0.02%$_a$, 11.1%$_b$ | trnI/trnA | Fernandez-San Millan *et al.*, 2003 |
| Interferon g | IFN-g | P$psbA$ | P$psbA$/T$psbA$ | 6% | rbcL/accD | Leelavathi *et al.*, 2003 |
| Interferon-$\alpha$5 | INF$\alpha$5 | Prrn; | P$psbA$/T$psbA$ | ND | trnI/trnA; | Torres, 2002 |
| Monoclonal antibodies | HSBV- | atpA/rbcL | AtpA/rbcL | ND | $psbA$/5S/23S | Mayfield *et al.*, 2003 |
| Anthrax protective antigen | Pag | Prrn | P$psbA$/T$psbA$ | 18.1% | trnI/trnA | Watson *et al.*, 2004 |
| Plague vaccine | CaF1~LcrV | Prrn | P$psbA$/T$psbA$ | 4.6% | trnI/trnA | Singleton, 2003 |
| Tetanus toxin | Tet C | Prrn | T7gene10$_a$ atpB$_b$/TrbcL | 25%$_a$, 10%$_b$ | Trnv/rps12/7 | Tregoning *et al.*, 2004 |

**Table 1-2: Some examples of chloroplast transformation experiments for the production of pharmaceuticals and polymers.**
Where more than one construct is used, the corresponding results are identified with the same subscript within a given row. A "P" indicates the inclusion of the promoter sequence in 5' regulatory element.

The identification and cloning of antimicrobial compounds from cellular organisms in combination with transgenic technologies offers the possibility to enhance disease resistance in agricultural crops. When higher plant cells are invaded by microbes such as fungi or bacteria they respond with a respiratory burst generating, among other things, hydrogen peroxide (Wojtaszek, 1997). A similar disease response occurring in animals goes one step further; the enzyme mediated generation of peracetic acid and hypohalites from hydrogen peroxide (Jacks *et al.*, 2000).

$$H_2O_2 + X- ==> H_2O + -OX$$

$$AcOH + H_2O_2 ==> AcOOH + H_2O \qquad\qquad Equation\ 1$$
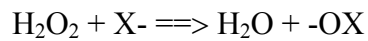
Peracetic acid and hypohalites are much stronger antimicrobial agents than hydrogen peroxide but plants lack a haloperoxidase enzyme (van Pee, 1996).

A cDNA clone showing 38% sequence identity to a nonheme bromoperoxidase from *Streptomyces aureofaciens* was isolated from *Pseudomonas pyrrocinia.* Called chloroperoxidase (CPO-P) (Wiesner *et al.*, 1988), the gene for this enzyme has been integrated into the nuclear genome of *Nicotiana tabacum* by *Agrobacterium* mediated transformation to investigate its potential to enhance disease resistance in higher plants (Rajasekaran *et al.*, 2000). The CPO-P lines demonstrated significantly reduced anthracnose severity *in planta*. Incubation with crude leaf extracts from the CPO-P transformants was up to 100% effective in preventing colony establishment by pregerminated conidia of several pathogenic fungi as well as the mycotoxin producing saprophyte *Aspergillus flavus.*

*Aspergillus flavus* is of particular interest as it poses a significant threat in terms of food and feed safety. *A. flavus* infestation occurs on seeds of cotton, corn, peanut and

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tree nuts and market samples of sorghum, groundnut, cassava and soybean from

developing nations routinely test positive for aflatoxin (*Aspergillus flavus* **toxin**)

contamination (Williams *et al.*, 2004).  Aflatoxin contamination is a perennial concern

between the latitudes of $40^o$ north and south of the equator, in drought years, crops

outside of this range are threatened.  Aflatoxins are a closely related group of compounds

of with small differences in chemical composition.  The most prevalent, and most potent,

aflatoxin $B_1$ (AFB$_1$) is tolerated at very low levels by humans and livestock, on the ppb

scale, and contaminated stores are destroyed resulting in dramatic economic losses



Aflatoxin B$_1$

Aflatoxin G$_1$

Aflatoxin B$_2$
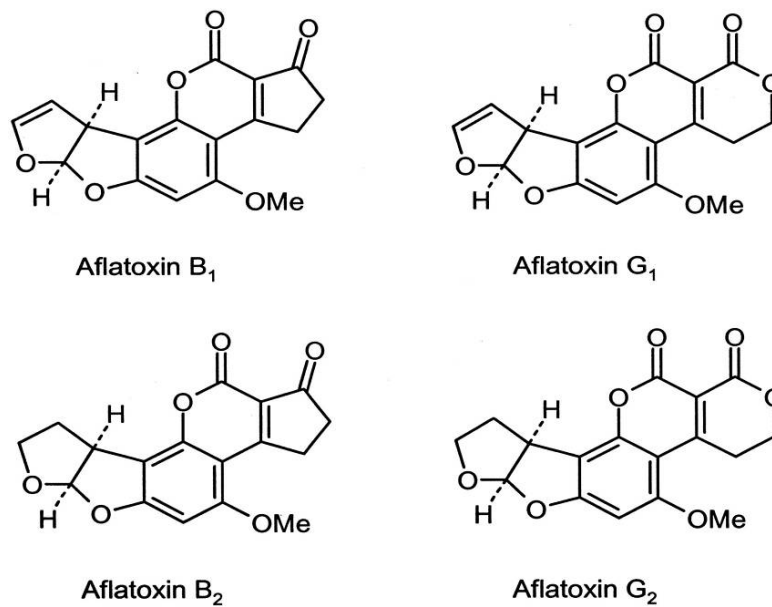
Aflatoxin G$_2$

**Figure 1-7:  Structure of aflatoxins.**  Aflatoxin B$_1$ is the most potent natural carcinogen known (from Williams 2004)

(Bennett *et al.*, 2003).  If the toxin goes undetected and enters food system as livestock

fodder, it is incorporated into the hosts' tissues and can be transmitted though milk.  In

developing nations where regulatory mechanisms are lacking early exposure results in

stunting and underweight in children.  Immunologic suppression and nutritional

consequences such as loss of food conversion efficiency is observed in livestock animals subjected to chronic aflatoxin exposure.  The International Cancer Research Institute identifies $AFB_1$ as a Class 1 carcinogen primarily perceived as an agent promoting liver cancers.  There appears to be a synergistic relationship between aflatoxin and hepatitis B virus (HBV) and hepatitis C virus (HCV) in the occurrence of hepatomas.  In areas where aflatoxin and HBV occur together hepatomas represent 64% of all reported cancers (Williams *et al.*, 2004).

The focus of this project was to exploit the potential of the chloroplast expression system in order to achieve high levels of CPO protein accumulation in tobacco for the purpose of enhancing plant resistance to phytopathogens and mycotoxin producing saprophytes such as *A. flavus*.

# Materials and Methods

## *Materials*

Plant tissue culture media components (agar #A023, MS packets #MSP009) were purchased from Caisson Laboratories (Rexburg, ID). RNA ladder, restriction enzymes and buffers were purchased from New England Biolabs (Beverly, MA). TOPO® cloning kits, DNA ladders and protein molecular weight markers were purchased from Invitrogen (Carlsbad, CA). Oligonucleotide primers were purchased from Sigma-Genosys (The Woodlands, TX). Expendable supplies (macrocarriers, microcarriers, stopping screens, gold particles) for use with PDS1000/He Biolistic® particle delivery system were purchased from BioRad (Hercules, CA). All chemicals and reagents were of analytical grade and purchased from Sigma (St. Louis, MO) unless otherwise noted. Statistical analyses to test significance of results were performed using GraphPad Prism® software, version 4 (San Diego, CA)

## *Plasmid Construction*

Chloroplast transformation vector (pLD-CtV) was obtained from Dr. Henry Daniell (University of Central Florida, Orlando, FL). This vector carries a multiple cloning site (MCS) and all necessary elements for cloning, selection and tobacco chloroplast transformation by homologous recombination (Figure 2-1). Sequences include endogenous *trnI* and *trnA,* which encode the plastid transfer RNA isoleucine and alanine respectively, to facilitate integration by homologous recombination in the intergenic spacer region of the 16S ribosomal operon (*rrn*) of tobacco chloroplasts. In addition to the *Escherichia coli* origin of replication, pLD-CtV carries a copy of the plastid origin of replication, *oriA* within the sequence of *trnI* (Kunnimalaiyaan *et al.*,

1997).  Transcription of integrated transgenes by the plastid encoded RNA polymerase

(PEP) is enhanced by inclusion of an engineered *rrn* promoter region which includes P1

but not P2.  The vector carries two selectable markers:  *amp^r* (β-lactamase) for selection

in *E. coli* and *aadA* (aminoglycoside-3'-adenyltransferase) for selection of transplastomic

tobacco cells.  The tobacco native full length *psbA* 3' UTR is included to impart

transcript stability.
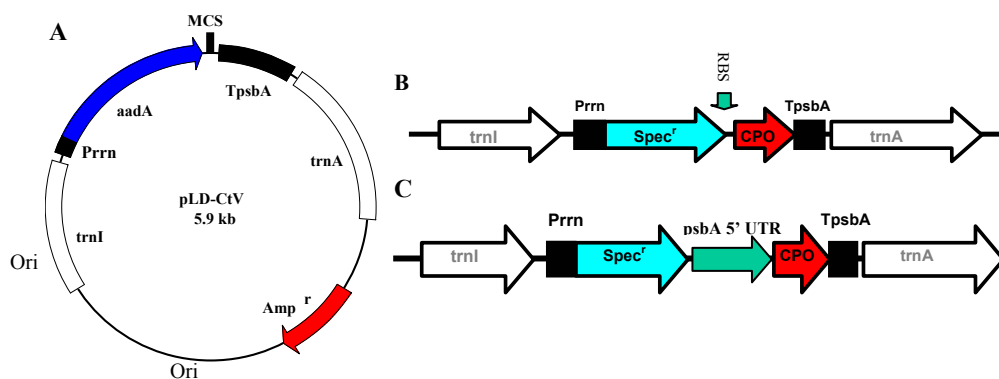


**Figure 2-1: Schematic representation of (A) chloroplast transformation vector**, (B)
original chloroperoxidase (CPO) construct and (C) construct with tobacco native *psbA* 5'
UTR.  Transformation cassette employed for *uidA* has same sequence as (B) except that
this gene is present in place of *cpo*.

For chloroplast transformation *cpo* was amplified by polymerase chain reaction

(PCR, general parameters indicated below) from pUC19-*cpo* template DNA employing

primers to introduce restriction sites compatible with those available in the multiple

cloning site of pLD-CtV (5' GAATTC**GGAGG**GATTTATGGCATACGT

CAC TACGAAGGAT 3', 5'CGGCCGCTTAAGCTTGTTGCACGAACGCC

AGCAGGT 3') as well as a 5' Shine Delgarno type ribosome binding site (RBS),

GGAGG, to facilitate translation (Figure1b).  This *cpo* PCR product was ligated into

TOPO® pCR®2.1 (Invitrogen #460801) vector and transformed into TOPO®OneShot®

TOP10 (Invitrogen #44-0301) cells according to the manufacturer's protocol. Putative

transformants were inoculated into 5 mL Luria-Bertani (LB) medium with 50 μg mL$^{-1}$

kanamycin and grown overnight. Plasmids were isolated by QIAprep spin miniprep kit

(QIAGEN #27106) according to the manufacturer's protocol. Sequence was determined

by capillary electrophoresis prior to excision from TOPO® vector and ligation into

polylinker of pLD-CtV.

The *psbA* 5' UTR was PCR amplified from tobacco genomic DNA incorporating

5' *Eco*RI and 3' *Nco*I restriction sites (5'GAATTCGTCATGTTATACTGTTGAATAA

AAGC 3', 5' CCATGGTAAAATCTTGGTTTATTTAATCATCAGG 3') and subcloned

into TOPO® pCR®2.1 for sequencing. The *psbA* 5' UTR was excised from pCR®2.1 as a

*Bam*HI, *Eco*RV fragment and subcloned in to *Bam*HI-*Eco*RV digested pBluescript®

(pBS, Stratagene #212207) vector. The *cpo* coding region was PCR amplified from

pUC-19-*cpo* template incorporating 5' *Nco*I and 3' *Not*I sites (5'CCATGGCATACGTC

ACTACGAAGGATAACG 3', 5' GCGGCCGCTTAAGCTTGCACGAACGCCAGCA

GGT 3') and ligated into TOPO® pCR®2.1 for sequencing. *Nco*I-*Not*I digest released

cpo from pCR®2.1 vector for ligation into *Nco*I-*Not*I digested pBS-*psbA* 5' UTR. *Eco*RI-

*Not*I digest released *psbA* 5' UTR:*cpo* fusion from pBS for ligation with *Eco*RI-*Not*I

digested pLD-CtV. pLD-CtV.*psbA* 5' UTR:*cpo* was sequenced from the 5' end of *aadA*

through the 3' end of *cpo*. The *E. coli uidA* (GUS) gene was also transferred to pLD-CtV

incorporating a GGAGG RBS allowing for fluorometric analysis of transplastomic

expression.

TOPO®OneShot® DH5α™ (Invitrogen #18263-012) cells were transformed with

pLD-CtV vector, carrying gene of interest, according to the manufacturer's protocol.

Plate grown colonies were used to inoculate 10 mL cultures (LB with ampicillin 100 µg mL$^{-1}$). These were grown overnight and used in turn to inoculate 1 L LB with ampicillin 100 µg mL$^{-1}$ cultures which were grown overnight. Plasmids were harvested by Plasmid Maxi kit (QIAGEN #12163) and plasmid DNA was resuspended in TE buffer. Concentration was determined with spectraphotometrically and adjusted to ~1 µg µL$^{-1}$.

## *Transformation and Regeneration of Transgenic Plants*

Seeds of *Nicotiana tabacum* var. Petit Havana or SR1 were surface sterilized with 1 % (w/v) silver nitrate and germinated on MS (Murashige *et al.*, 1962)basal salts (30 g L$^{-1}$ sucrose, 5 g L$^{-1}$ phytoagar). Sterile leaves approximately 6 cm x 3 cm were placed abaxial side up on regeneration media of plants (RMOP) for bombardment (Figure 2-2). Gold particles (0.6 µm) were coated with pLD-CtV-*cpo*, pLD-CtV.*psbA* 5' UTR:*cpo* or pLD-CtV-GUS plasmid DNA following the established method of Daniell *et al.* (2005). Rupture disks rated for 1100 psi were employed in bombardments with the PDS-1000/He Biolistic® device. Bombarded samples were held in the dark for 48 hours then cut into ~5 mm$^2$ pieces and placed abaxial side down on RMOP augmented with 500 mg L$^{-1}$ spectinomycin dihydrochloride for selective regeneration of transformed cells under 16 h light/8 h dark in growth chambers (Percival, Boone, IA) at 28 $^o$C.



**Figure 2-2:** Representation of transformation and selection process (from Bock, 2004).

Putative transplastomic regenerants bombarded with *psbA* 5'UTR:*cpo* (pCPO) construct were screened to confirm presence of transgenes. Positive transformants were subjected to one or two further regeneration cycles on the same selective media, explant size ~2 mm$^2$, followed by additional selection by rooting in MS media with 500 mg L$^{-1}$ spectinomycin. Primary regenerants (pCPO) were further screened for resistance to streptomycin sulfate by placing explants on RMOP with 500 mg L$^{-1}$ streptomycin.

## *Isolation of Plant DNA*

Unless otherwise indicated, all isolations of genomic DNA were performed on young, fully expanded green leaves taken from the third to sixth position below the apex. Midribs were removed and samples were ground in liquid N$_2$ with chilled, sterile mortar and pestle. Each sample, 75 mg, was transferred to a 2 mL microfuge tube and extraction was carried out using a QIAGEN DNeasy® Plant mini kit (#69104) according to the manufacturer's protocol. Each column was eluted twice with 200 μL EB, except where sample was to be used for sequencing in which case elutions were performed with nuclease free water. Eluates were combined in a 1.5 mL microfuge tube. To precipitate DNA, 10% 3M sodium acetate pH 5.2 and 1.5 volumes cold 100% EtOH were added before placing samples in either -20 °C overnight or -80 °C for two hours. Following precipitation, samples were centrifuged for 20 minutes at 18, 380g at 4 °C. Pellets were washed with 70% EtOH and centrifuged for 5 additional minutes. DNA pellets were dried under vacuum and resuspended in TE buffer (Amresco #E112) or nuclease free water.

## *Polymerase Chain Reaction (PCR)*

PCR reactions were carried out in the BioRad iCycler thermal cycler (#170-8720). Unless otherwise noted TaKaRa ExTaq™ kit (#RR006A) which includes polymerase buffer and dNTP mix, was employed according to manufacturer's protocol. The standard reaction volume of 25 μL included 1 μg of each oligonucleotide primer. Annealing temperatures were generally 5 °C below the oligo $T_M$, and 30 seconds was allowed for annealing time. Duration of extension was determined based on size of predicted product, according to a rate of nucleotide addition of approximately 1000 bases per minute at 72 °C. After an initial denaturing step of 2 min cycles included denaturation for 30 seconds at 94 °C and were repeated 34 times. PCR products were separated on 1% agarose gels in TAE buffer.

To confirm insertion of *aadA:cpo* gene fusion in putative transformants, primers specific for the 3' region of *aadA* and the 5' region of *cpo* were employed (5' CAGCC CGTCATACTTGAAGCTAGGCA 3', 5' CCATGACATGGATCATTACGCAGCG 3').

## *Generation of Probes for Hybridization Analyses*

Probe for Southern analysis was generated by PCR amplification of *trnI- trnA* junction within the tobacco *rrn* operon (5'ATAATTGCGTCGTTGTGCCTGGGCTG TGAC 3', 5' CTTTCAGTGGCACGTTTCGGTCCTCTT 3'). Gene specific probes (*cpo, aadA* and *uidA*) were cleaved from chloroplast transformation vector (pLD-CtV) by *Eco*RI-*Not*I. PCR product was ligated into TOPO® pCR2.1® cloning vector and transformed into OneShot® Top10 chemically competent cells according to manufacturer's protocol. Cells were plated on LB agar (25 g L$^{-1}$) with 50 μg mL$^{-1}$ kanamycin monosulfate, 40 mg L$^{-1}$ X-gal (5-bromo-4-chloro-3-indolyl-β-D-

galactopyranoside) (Promega #V3941), 0.8 µM IPTG (isopropyl-beta-D-thiogalactopyranoside) (Gold Biotechnology #I2481C5) and allowed to grow overnight at 37 $^{\circ}$C. White colonies were selected and used to inoculate 5 mL cultures (LB, 50 mg L$^{-1}$ kanamycin) for overnight growth. Plasmid DNA was isolated by alkaline lysis miniprep, digested to release insert and separated on 1% agarose II, low melt gels (Amresco #0851). Following ethidium bromide staining and washing, bands were excised using a sterile blade with the aide of a UV lamp. Gel fragments were stored at -20 $^{\circ}$C prior to α-$^{32}$P labeling. Probes were synthesized by Rediprime II$^{\circledR}$ random prime labeling system (Amersham Biosciences #RPN1633) and unincorporated radioisotope was removed using a QIAquick Nucleotide Removal Kit (QIAGEN #28304).

## *Dot Blot Analysis*

To positively identify transplastomic regenerants, ~2 µg genomic DNA from putative chloroplast transformants was boiled for 5 min in TE buffer. Tubes were transferred to wet ice for 1 minute before spotting on NYTRAN$^{\circledR}$SPC membranes (Schleicher and Schuell #10416289). Wild-type DNA and plasmid vector were included on each blot as controls. Membranes were UV crosslinked and stored at 4 $^{\circ}$C. Positive transformants were identified by hybridization with $^{32}$P labeled probe for gene of interest. Hybridization of probes to nucleic acid blots was carried out in ULTRAHyb buffer (Ambion #8670) at 42 $^{\circ}$C overnight. Membranes were washed once for 15 minutes in 2X SSPE (Amresco #0806) with 0.1 %SDS at 42 $^{\circ}$C. Three additional washes were carried out in 0.1X SSPE with 0.1 % SDS at 42 $^{\circ}$C, 50 $^{\circ}$C and 60 $^{\circ}$C. Damp membranes were placed on Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY) for exposure with intensification at -80 $^{\circ}$C.

### *Southern Blot Analysis*

Approximately 5 µg of total genomic DNA isolated from wild-type and transplastomic lines was digested to completion. GUS lines were digested with *Bgl*II. As *cpo* carries an internal *Bgl*II site sequential digest with *BamH*I and *Sac*I was performed. Initial digest with *BamH*I was carried out in 50 µL total volume with 2.5 µL of enzyme. Following completion of first digest, samples were subjected to phenol:chloroform:isoamyl alcohol (25:24:1) extraction, back extracted with TE buffer, followed by an additional extraction with chloroform:isoamyl alcohol (21:1). DNA was precipitated by addition of 0.1 volume 3M sodium acetate pH 5.2 and two volumes of 100% cold ethanol for 2 hours at -80 °C. Samples were centrifuged, washed with 70% EtOH and dried in speedvac before proceeding to second digest with 2.5 µL *Sac*1, 30 µL total volume. Digest reaction mixtures were prepared according to standard protocols (New England Biolabs). Digests were separated by electrophoresis on 1% agarose gels in TAE buffer (Amresco #0912), stained 6 minutes in ethidium bromide solution, washed twice in deionized water, ten minutes each, then imaged by UV camera. In preparation for transfer gels were placed in 0.25 M HCl for 25 minutes, moved to denaturing solution (1 M NaCl, 0.5 M NaOH) for 25 minutes, and subsequently placed in neutralization buffer (0.5 M Tris pH 7.4, 1.5 M NaCl) for 25 minutes. DNA was transferred to NYTRAN®SPC membranes by vacuum apparatus in 10X SSPE (Amresco #0806). Membranes were UV crosslinked prior to storage at 4 °C. Hybridization of $^{32}$P labeled probe and subsequent washings were carried out as described above.

## *RNA Extraction and Northern Analysis*

All aqueous solutions for RNA analysis were prepared in sterile, diethylpyrocarbonate (DEPC) treated water. RNA isolations were performed by RNeasy Plant Mini Kit (Qiagen #74904) according to manufacturer's protocol. Two 50 μL elutions in RNase-free water were performed for each sample. RNase-free water was allowed to stand on column in ice 10 minutes before spinning. First elution was centrifuged at 2042 x g for 5 minutes, second elution for 4 minutes, then 1 additional minute at 8168 x g. Two elutates were combined, 0.1 volumes 3M sodium acetate pH 5.2 and two volumes cold 100 % EtOH were added and samples were precipitated at -20 $^{\circ}$C overnight. Following precipitation, samples were centrifuged for 20 minutes at 18, 300 x g at 4 $^{\circ}$C. Pellets were washed with 70% EtOH, centrifuged for 5 additional minutes, dried under vacuum and resuspended in RNase free water. 5 μL of each sample was added to RNA sample buffer (10 μL formamide, 3.5 μL 37% formaldehyde, 2 μL 10x MOPS, 1 μL EtBr (1 mg mL$^{-1}$)). Samples were centrifuged briefly to collect volume in bottom of tube and incubated at 65 $^{\circ}$C for 10 minutes. Samples were separated by electrophoresis on 1.2 % agarose gels (5 mL10X MOPS and 9mL 37 % formaldehyde, 36 mL H$_2$O), in 1X MOPS buffer. Gels were destained 30 minutes in water before vacuum transfer onto NYTRAN$^{\circledR}$SPC membranes in 10X SSPE. Upon completion of transfer, membranes were UV crosslinked and held at 4 $^{\circ}$C. Presence of *cpo* mRNA was visualized by hybridization with $^{32}$P labeled full length *cpo*. Hybridization and wash conditions were as described above. Quantitation of northern blots was accomplished by STORM$^{\circledR}$ phosphorimaging system (Amersham Biosciences) and ImageQuant®5.0

software (GE Healthcare).  The one sample t-test was employed to analyze significance of quantitative results (Sokal and Rohlf, 1981).

## *Tobacco Protein Isolation and Western Blotting*

Total soluble protein (TSP) was extracted from leaves harvested from 6 week old plants, 4-6 nodes below the apex.  Tissue was ground in liquid nitrogen to a fine powder with a chilled mortar and pestle.  Ground sample (200 mg) was transferred to a 2 mL microfuge tube and 400 μL extraction buffer (see appendix) was added, then incubated on ice for 5 minutes.  Samples were centrifuged at 18, 380 x g for 20 min at  4 $^{o}$C. Supernatant was removed to a new 1.5 mL tube and centrifuged again for an additional 10 min to remove residual debris.  Supernatant was transferred to a new 1.5 mL tube.  Concentration of TSP was determined by the BioRad protein assay (#500-0006).  Samples, including purified CPO enzyme of known concentration, were combined with 1X Laemmli sample buffer (see appendix 1) to obtain equal TSP concentration of all samples.  These were boiled in a water bath for 5 minutes. Bromophenol blue (BPB, 1:100) was added to each sample (0.5 μL).  Samples were loaded into wells of precast 12% Tris-HCl polyacrylamide resolving gels (BioRad# 161-1102) and separated by electrophoresis in 1X SDS running buffer (Ameresco #0147) at 75 volts for 2 hours.  Gels were electroblotted onto PROTRAN$^{®}$ (Schleicher and Schuell #1040245) nitrocellulose membranes and stained with SYPRO™ ruby protein blot stain (BioRad # 1703127), according to the manufacturer's protocol, to visualize and quantitate TSP on blot.  Image was captured by Intelligent Dark Box$^{®}$ by Fuji (FujiFilm Tokyo, Japan) and analyzed for equal loading by Science Lab 2001 ImageGauge$^{®}$ V4.1 (FujiFilm Tokyo, Japan) software.  Blots were blocked overnight in 5% solution of

non-fat skim milk in TBS-T (0.15 M NaCl, 0.02 M Tris pH 7.4, 0.5 mL L$^{-1}$ Tween 20). Blocked membranes were rinsed with deionized water prior to incubation for 2 hours at 24 $^{\circ}$C with CPO polyclonal antibody (raised in rabbit) (Antibodies Incorporated, Davis, Ca) diluted 1:1000 in TBS-T on a rotary shaker. Membranes were washed 5 times, 5 min each, in cold TBS-T. Horseradish peroxidase conjugated goat anti-rabbit serum (Amersham Biosciences #RPN2124) was diluted 1:20, 000 and added to blots for 1 hour of incubation with shaking. Blots were washed 5 times, 5 minutes each, in cold TBS-T. ECL Plus western blot detection system (Amersham Biosciences #RPN2132) was used according to manufacturer's protocol for imaging of blots. Images were collected with the Intelligent Dark Box$^{\circledR}$ by Fuji and analyzed for concentration of CPO by Science Lab 2001 ImageGauge$^{\circledR}$V4.0 (FujiFilm). One way ANOVA was employed to analyze significance of quantitative results, mean separations were performed using the method of Tukey (Sokal and Rohlf, 1981).

## CPO Enzyme Activity Assay

All leaf tissue samples were ground in liquid nitrogen with mortar and pestle and allowed to thaw on ice. When material began to thaw 1 M sodium acetate buffer pH 5.2 augmented with 50 mg mL$^{-1}$ polyvinylpyrolidone (insoluble) and protease inhibitor cocktail (Sigma #9599) was added at a ratio of two parts buffer to one part sample. Continued grinding of sample with pestle was performed to produce homogenate. Samples were transferred to 2.0 mL microfuge tubes and centrifuged at 18, 380 x g for 10 min at 4 $^{\circ}$C. Supernatant was carefully removed to a clean 1.5 mL tube. Analysis for total soluble protein content was performed by BioRad protein assay (#500-0006). To assess enzyme activity, one volume (2.9 mL) of assay cocktail (see appendix) was placed

in a glass cuvette (calibrated to $\lambda$=290 nm), 100 µL sample was added and mixed by inversion 3 times.  Reaction mixture was assayed for activity by monitoring the change in absorbance ($A_{290}$) over time.  An observed drop in absorbance at this wavelength indicated the bromination of the substrate monochlorodimedon (MCD) by active CPO-P enzyme present in sample preparation.  Spectrophotometer (Jasco V530 UV/Vis) was blanked with a calibrated cuvette containing 1 M sodium acetate buffer alone and pure CPO-P was employed as positive control.

## *Pollination Experiments*

To confirm maternal inheritance of transplastomic traits controlled crosses were performed on mature plants in the greenhouse.  Just prior to anthesis, flowers were emasculated and pollen was transferred from wild-type plants to receptive stigma surface of transplastomic plants.  The reciprocal crosses were performed in the same manner.  Once pollinated, flowers were enclosed by envelopes that remained sealed until capsules had set.  Mature capsules were collected from crosses and sterile seeds, 250 from each cross, were plated on MS media with 500 mg $L^{-1}$ spectinomycin to assess transmission and expression of aminoglycoside-3'-adenyltransferase (*aadA)* in the progeny.

## *Assay for β-glucuronidase (GUS) Expression*

### *a) Fluorometric assay*

Samples were ground to a fine powder in liquid nitrogen using chilled mortar and pestle.  Ground samples (<1 g) were transferred to a tared 2 mL microfuge tube and sample weights recorded.  All samples were held on ice.  GUS fluorometric extraction buffer, 1 mL, (see appendix 1) was added for each gram of tissue and all samples were given a 3 second burst with a Vibra-Cell™ sonicator (Sonics and Materials Inc., Danbury,

CT) to facilitate mixing. Samples were centrifuged 10 minutes at 11, 700 x g in an Eppendorf benchtop microfuge with swinging bucket rotor. Each sample, 100 μL, was added to 900 μL GUS assay buffer (see Appendix) in a 1.5 mL tube. Samples were then placed in 37 $^o$C incubator for 1 to 3 hours. A 1:2 dilution series of 1 μM 4-MU stock solution was prepared in 0.2 M sodium carbonate ($Na_2CO_3$) as a concentration standard for relative fluorescence. Each standard, 200 μL, was loaded into wells of microtiter plate along with 200 μL of each sample, 3 replicate wells per sample. GUS assay buffer only was used as negative control measure. Measurement of fluorometric output was made using the HTS 7000 bioassay reader (Perkins Elmer, Boston, MA) with 360 nm excitation filter and 455 nm emission filter. One way ANOVA was employed to analyze significance of quantitative results, mean separations were performed using the method of Tukey (Sokal and Rohlf, 1981).

### b) Histochemical staining

Leaf disks were prepared with a 7 mm diameter hole punch and submerged in GUS histochemical stain (see Appendix). Samples were placed in an incubator 37 $^o$C for 12 hours. Stain was removed and leaf disks were bleached in 95 % EtOH. Ethanol was left on disks for 24 hours before replacement with fresh 95 % EtOH, this was repeated for a total of 3 ethanol cycles prior to photographing.

## Antimicrobial assays

### a) In planta assay for Pseudomonas syringae resistance

Glycerol stocks of *Pseudomonas syringae* pv *tabaci* (ATCC 17914) (Pst), stored at -84$^o$, were allowed to thaw on ice. *Pseudomonas* was streaked on two *Pseudomonas* Agar F (PAF) (Difco #244820) plates and incubated at 28$^o$ C for at least 18 hours. Cells

were transferred from culture plates to 100 mL liquid Nutrient Broth (NB) (Difco #234000) with glucose (2.5 g $L^{-1}$) in 500 mL Erlenmyer flask for culture overnight on a rotary shaker at $28^{o}$ C, 225 rpm.  Suspensions were transferred to two 50 mL tubes and harvested by centrifugation at 1570 x g for 20 minutes in a Beckman TJ-6 centrifuge (Beckman Coulter Inc., Fullerton, CA) fitted with a swinging bucket rotor.   Pst pellets were combined and resuspended in 100 mL bacterial phosphate ($PO_4$) buffer (0.01 M, pH 7.0) in a 1 L flask.  Cell suspension was adjusted to an optical density of 0.32 ($\lambda =$ 530 nm) by addition of $PO_4$ buffer to obtain approximately 1 x $10^8$ cells/mL.  Inoculum concentration was verified by dilution plating on PAF (1:10 serial dilution).  All leaves present at the time of treatment were marked, as plants will produce new leaves during sampling time.  Marking ensured sampling of treated leaves.  Plants were sprayed until runoff using a spray bottle.  Treated plants were covered with clear plastic bags placed in a growth chamber at 28 $^{o}$C and 64 % relative humidity.  Bags were removed after 48 hours.  Treated leaves were sampled every 48 hours by taking 3 leaf disks (~7mm diameter) with a sterile punch.  Fresh weight of sample was recorded (~14.5 mg for tobacco).  Samples were collected over 10 days.  Leaf discs were placed in $PO_4$ buffer and stored at 4 $^{o}$C until all samples had been collected.  For processing, samples were ground in 300 µL 10 mM $MgCl_2$ with the aid of a homogenizer (Glas-Col, Terre Haute, IN)) and homogenate was then transferred to 5 mL $PO_4$ buffer.  This suspension was further diluted by the addition of $PO_4$ buffer (1:100).  Remaining undiluted sample (in 5 mL $PO_4$ buffer) was filtered through 4 layers of cheesecloth.  Filtered, undiluted samples were plated in duplicate along with 3 platings of 1:100 dilution with the aid of a spiral plater (Spiral Systems Inc, Bethesda, MD).  Colony forming units were enumerated after

incubation at 28° C for 24 to 48 hours according to published procedures (DeGray *et al.*,

2001). Significance of results was tested by Spearman's and Pearson's correlation

analysis.

### b) In vitro bioassay for antifungal activity

Antifungal activity of crude leaf extracts of CPO chloroplast transformants was

assessed *in vitro* following the method of De Lucca *et al.*,(1997), as modified by

Rajasekaran *et al.*, (2000). Briefly, conidial suspensions of *Aspergillus flavus,*

*Verticillium dahliae* and *Fusarium vericillioides* were prepared from cultures grown on

potato dextrose agar (PDA, Difco #213400) slants for 7 days at 30 °C (*A. flavus,*

*F. verticillioides*) or 24 °C (*V. dahliae*).

Conidial suspensions prepared in 1% (w/v) potato dextrose broth (PDB) pH 6.0

(Difco #254920) were adjusted to $1x10^5$ conidia $mL^{-1}$ and allowed to germinate prior to

assays. Leaves were ground to a fine powder with liquid $N_2$. Two 2 mL tubes, ~750 mg

ground tissue per tube, were generally prepared for each chloroplast transformant sample.

A nuclear CPO transformant and a non-transformed wild-type control were also included

in the assay. One tube for each sample received a 40 μL aliquot of protease inhibitor

cocktail (Sigma #9599). Homogenates were centrifuged at 11, 700 x g for 10 minutes at

room temperature, and supernatants were tested for antifungal activity. Additional

control samples were prepared with 1% PDB in place of plant extract. 25 μL of conidial

suspension of was added to 225 μL of sample supernatant, vortexed, and incubated for 1

hour at 30 °C or 24 °C. A minimum of three 50 μL aliquots from each sample were then

spread onto PDA plates and incubated at 30 °C or 24 °C for 24-48 hours and fungal

colonies enumerated. Significance of results was determined by one-way ANOVA

(GraphPad Prism®V4, San Diego, CA), and mean separations were performed using the method of Tukey (Sokal and Rohlf, 1981).

# Results

## *Transformation Efficiency and Regeneration*

The *Pseudomonas* chloroperoxidase (CPO) gene was ligated into the chloroplast transformation vector (pLD-CtV). This vector contains all the necessary elements for cloning, selection and tobacco chloroplast transformation by homologous recombination (Figure 2-1). Sequences include endogenous *trnI* and *trnA* to facilitate integration by homologous recombination in the intergenic spacer region of the 16S ribosomal operon (*rrn*) of tobacco chloroplasts as well as the *aadA* gene for selection of putative plastid transformants. In order to address the efficiency of CPO expression in the chloroplast genome, fully expanded leaves from aseptically grown 6 to 8 week old tobacco plants were bombarded with 0.6 μm Au particles coated with chloroplast transformation vector pLD-CtV carrying genes of interest (Figure 2-1). Spectinomycin resistant shoots arose from bombarded leaf explants via *de novo* organogenesis after 4 to 6 weeks in culture (Figure 3-1A). Transformed shoots were analyzed by dot blot hybridization, and
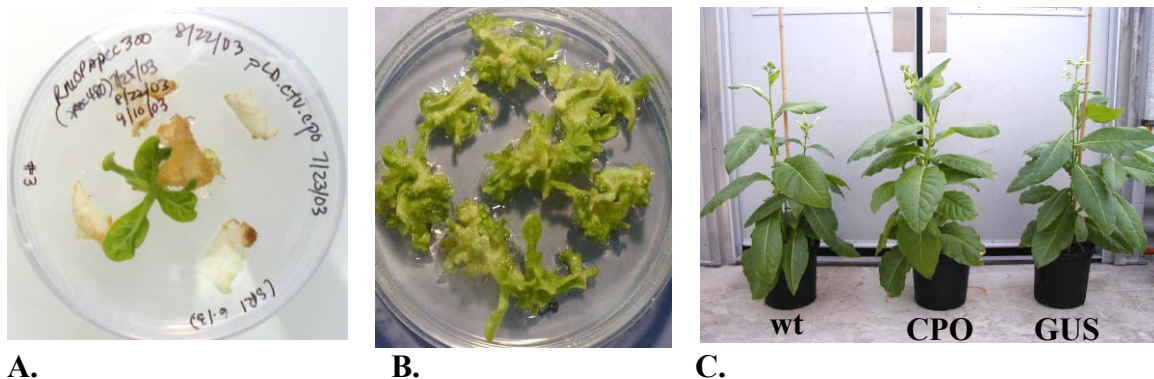


**Figure 3-1: Regeneration and growth of chloroplast transformants.** A) Appearance of primary regenerant, B) adventitious shoot formation on explants of transplastomic leaves, C) mature transplastomic plants display no visible phenotypic difference from wild-type.

positives were termed primary transformants. Leaf explants prepared from primary

transformants produced multiple resistant shoots in 2 to 3 weeks as did explants in

subsequent regenerations (Figure 3-1B). Initial regenerants in Petit Havana background

screened on streptomycin selective media indicated that the incidence of apparently

spectinomycin resistant yet streptomycin susceptible (escapes) increased after 7 to 8

weeks on primary regeneration medium. These escape events may be due to spontaneous

mutation in the 16S gene which confers resistance to spectinomycin but not streptomycin.

The number of escapes in *psbA* 5'UTR:*cpo* and empty vector experiments (Table 2-1)

| Vector | Variety | # of leaf samples | Spec resistant regenerants | Southern dot blot positive | Bleached on streptomycin |
|---|---|---|---|---|---|
| pLD.CtV.*cpo* | SR1 | 6 | 6 | 5 | NA |
| pLD.CtV gUS | SR1 | 9 | 4 | 2 | NA |
| pLD.CtV.*psbA:cpo* | Petit Havana | 22 | 35 | 10 | 6 (17%) |
| pLD.CtV.*cpo* | Petit Havana | 19 | 15 | 3 | 11 (73%) |
| pLD.CtV…EV | Petit Havana | 21 | 50 | 2 | 9 (18%) |

**Table 2-1**: **Regeneration results for 8 independent bombardment experiments**.
Expression of spectinomycin/streptomycin resistance and positive dot blot analysis
indicated incorporation of transgenic sequences for *aadA* and *cpo*.

was similar to published results from other labs (see Table 1-1 for references).

Bombardments with pLD-CtV-*cpo* (CPO) in Petit Havana resulted in much lower

transformation efficiency than bombardments with empty vector (EV) or *cpo* with *psbA*

5'UTR (pCPO) (Table 2-1). Primary regenerants from CPO experiments in Petit Havana

typically appeared after 8 to 12 weeks in culture. Plants transferred to the greenhouse

demonstrated no visible phenotypic difference from wild-type plants, flowered and set

abundant, viable seed (Figure 3-1C).

## *Foreign Genes are Present in Transplastomic Lines*

Primary transformants were analyzed by PCR to verify presence of transgenes. PCR analysis of primary transformants for pLD-CtV-*cpo* in SR1 background was performed by amplifying with one primer to the 3' region of *aadA* and the other to the 5' region of *cpo*. The expected product of 370 base pairs (bp) was amplified (Figure 3-2). This result confirmed the presence of the *aadA:cpo* gene fusion in the tobacco genome but does not address the site of integration.
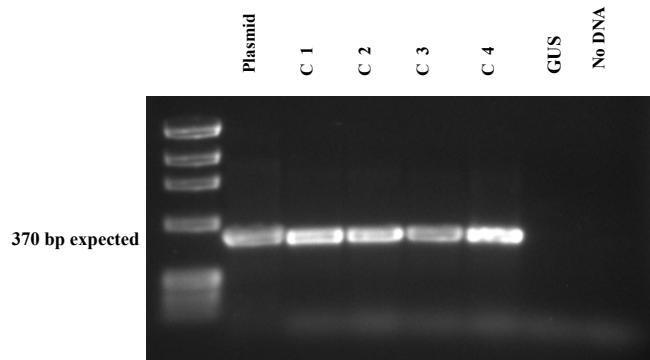


**Figure 3-2: PCR analysis** of 1$^{\circ}$ regenerants of leaves bombarded with pLD-CtV-cpo.

## *Localization of Inserts and Assessment of Homoplasmy*

In order to confirm that the foreign DNA was integrated into the chloroplast genome, Southern blots were probed with a 0.61 kilobase (kb) fragment spanning the *trnI-trnA* junction in the *rrn* operon of the tobacco plastid genome (Figure 3-3B). Insertion in this region is indicated by the presence of a hybridization band corresponding
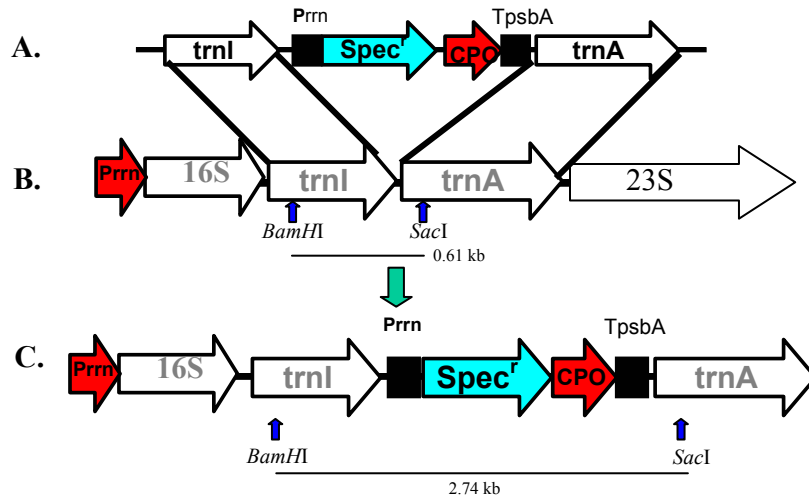
**Figure 3-3: Diagram of A) chloroplast transformation vector, pLD-CtV-cpo indicating site of homologous recombination into B) native plastid *rrn* operon**. Flanking sequences direct single copy insertion between trnI and trnA resulting in C) transformed plastid genome. Blue arrows indicate pertinent restriction sites for Southern analysis of CPO and pCPO lines. Fine black lines indicate size and origin of probe and expected band size in transformed lines.

to the size of foreign sequence plus the length of the probe whereas wild-type samples

will give bands of the probe length only. The presence of both the wild-type band in

addition to the larger band indicates that plants possess both transformed and

untransformed copies of the plastid genome, a condition referred to as heteroplasmy.

Where the wild-type band is absent and only the larger band is observed plants are

considered homoplasmic and contain only transformed copies of the plastome. Total

plant DNA was subjected to sequential digestion with *Sac*I and *BamH*I. Control DNA

isolated from untransformed wild-type plants yielded the expected band size of 0.61 kb

where as transformed plant lines for CPO and pCPO yielded bands of the expected sizes,

2.7 kb and 2.8 kb respectively indicating presence of exogenous DNA in the region

spanned by the probe. Of the two primary transformants for CPO analyzed one

individual, C 3, was homoplasmic while the other, C 6, harbored a mixed population of

wild-type and transformed plastome copies (Figure 3-4A) as indicated by the detection of

both the 0.61 and the 2.7 kb bands. $R_1$ progeny (first filial generation from primary
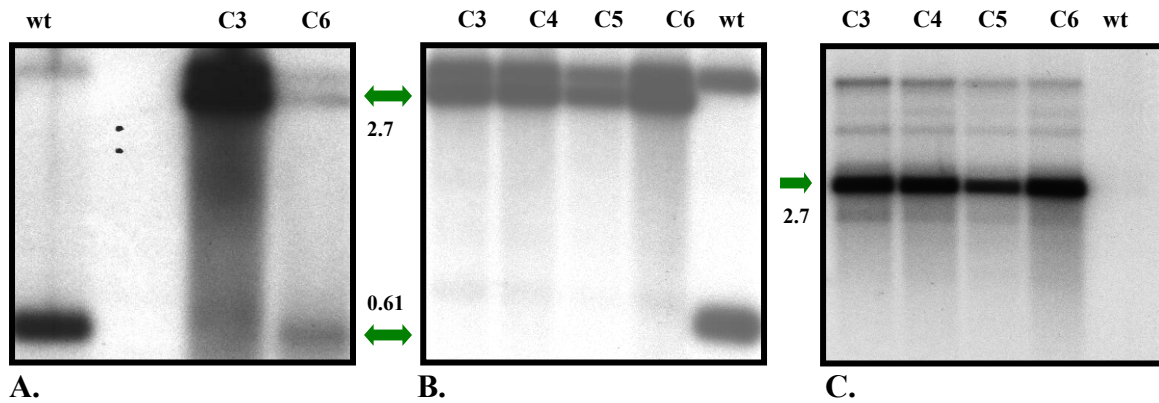


**Figure 3-4: Confirmation of chloroplast insertion.** Total DNA was hybridized to determine site of integration and homoplasmy. C3-C6 are chloroplast transformants, wt is non-transformed SR1 tobacco A) primary transformants probed with 0.61 *trnI-trnA* fragment. B) $R_1$ progeny after additional regenerations hybridized with same probe. C) Same blot as B stripped and probed with full length *cpo*.

transformants) of the CPO lines, which had been subjected to two additional regeneration

cycles, were all homoplasmic (Figure 3-4B) as indicated by the absence of the 0.61 kb

wild-type band. The $R_1$ blot was stripped and probed with full length *cpo* resulting in a

uniform band at 2.7 kb that is absent in the wild-type lane (Figure 3-4C), confirming that

the expected integration event had occurred.

Transformants for pCPO were analyzed by Southern analysis after two

regeneration cycles followed by rooting in spectinomycin media (Figure 3-5A). Of the

five lines analyzed, three were found to be homoplasmic (pC1, pC3, pC4), one was

heteroplasmic containing a mixed population of transformed and untransformed copies of

the plastome (pC5), and one line (pC2) appeared to be untransformed, giving the same

hybridization pattern as the wild-type.

DNA isolated from leaves from $R_1$ progeny of GUS chloroplast transformants was
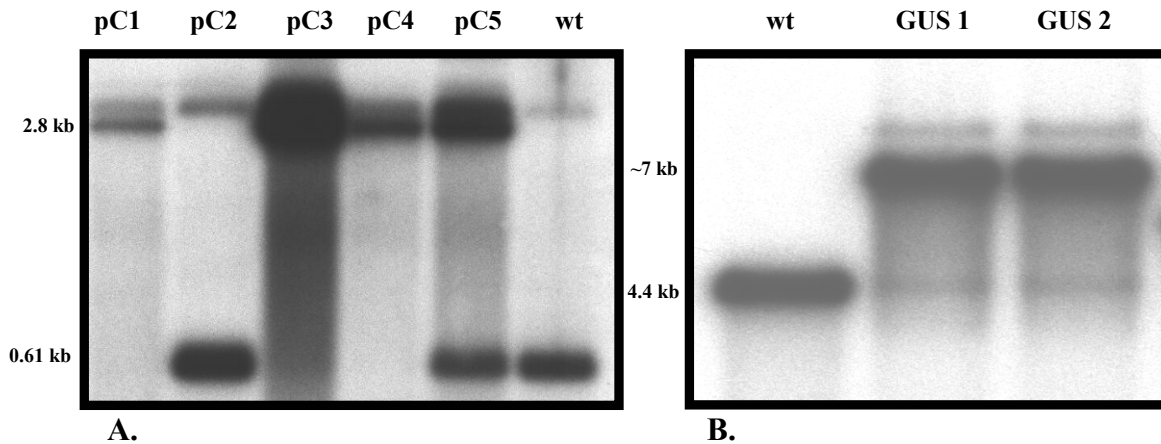
**Figure 3-5: Results of Southern analysis** of A) pCPO chloroplast transformants and B) GUS chloroplast transformants.

also analyzed by Souther blotting following digestion of genomic DNA with *Bgl*II., which is expected to cleave upstream of *rrn* operon and downstream from the insertion site within *trnA*. The same 0.61 kb probe (Figure 3-3B) was employed to confirm insertion and achievement of homoplasmy. The resulting hybridization pattern demonstrated that both GUS lines investigated were homoplasmic with bands at 7 kb for transformants as compared to 4.4 kb for untransformed wild-type plants (Figure 3-5B). An unexpected hybridization band was seen every time the Southern analysis was performed, regardless of the transformed line being investigated, possibly due to incomplete digestion.

## *Foreign Genes are Predominantly Transcribed by Plastid RNA Polymerase*

Northern analyses of CPO and pCPO lines were performed to confirm transcription of foreign gene in chloroplast transformants. The complex hybridization patterns observed in these lines were typical of those seen for transgenes integrated into chloroplast polycistronic transcription units such as the *rrn* operon (Quesada-Vargas,

2005).  Transcript analysis of CPO lines C2, C3 and C4 (Figure3-6A) indicated that the

predominant mRNA in leaf tissue was most likely the *aada:cpo* dicistron corresponding

to transcript c in Figure 3-7.  The nuclear transformant for *cpo* included in this analysis

harbored only the monocistron for *cpo* as expected.  The exposure time of 4 hours that

was allowed for this autoradiograph (Figure 3-6A) was insufficient for visual detection of

nuclear *cpo* transcripts but this transcript was detectable by phosphorimaging.  An

extended exposure time of approximately 16 hours enabled visual detection of the nuclear

transcript (not shown).

Quantification of transcript number indicated a 9- to 16-fold greater abundance of

*cpo* (*aadA:cpo)* mRNA in chloroplast transformants over the nuclear transformant

(Figure 3-6B).  Chloroplast transformant C1 was presumed to have escaped selection and

putatively carries the 16S mutation conferring spectinomycin resistance.



**A.**　　　　　　　　　　　　　　　　　　　　　　**B.**
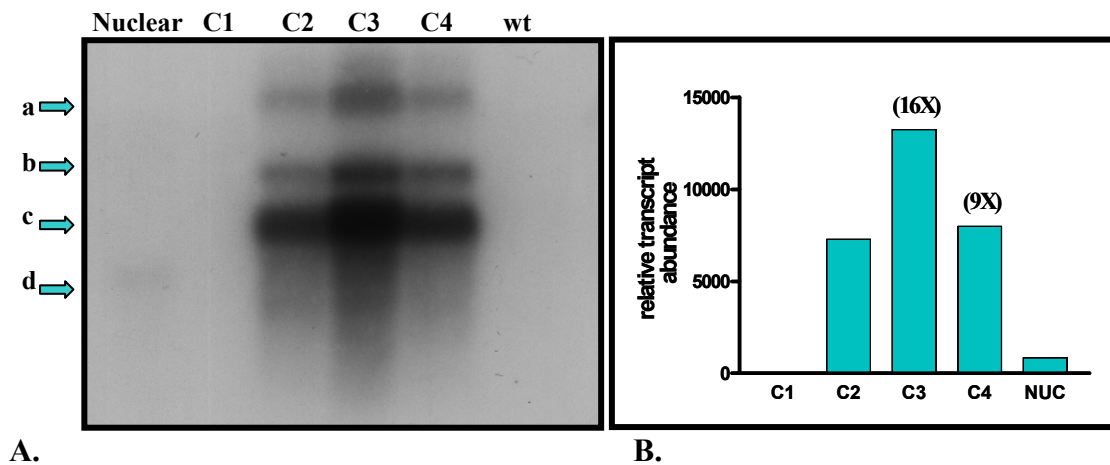
**Figure 3-6:  Results of northern analysis of CPO lines**.  A) Autoradiographic image of
transcripts hybridized to ^{32}P labeled, full length *cpo*.  B)  Quantitation of transcript
number by STORM phophorimager/ ImageQuant software.

Inclusion of *psbA* 5' UTR facilitated cleavage of the *aada:cpo* dicistron into

monocistronic units (Figures 3-7, 3-8A, transcripts c and d).  In analysis of transcript

abundance for pCPO lines the values for both the dicistron and the monocistron were

combined (pooled) for comparison to CPO lines. No significant difference (P> 0.05) was detected in relative transcript abundance between the pooled values for pCPO lines and the dicistron values for CPO lines evaluated (Figure 3-8B). Neither was the difference significant (P> 0.05) between relative abundance of the monocistron and the dicistron within pCPO lines (Figure 3-8C). Abundance of larger transcripts corresponding to a and b in Figure 3-7 was not considered.
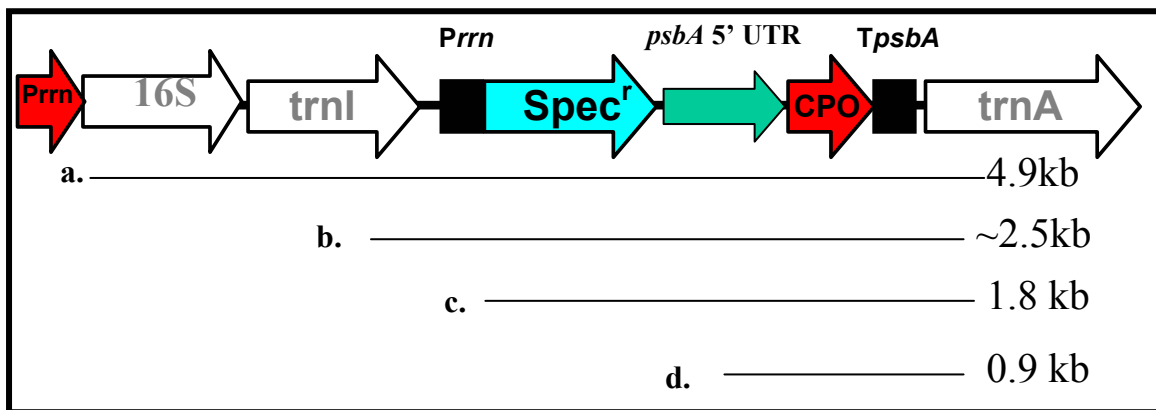


**Figure 3-7: Schematic representation of transcripts generated by chloroplast transformants.** Diagram indicates transcript sizes for pCPO lines (figure 3-8); CPO transcripts were found to be similar except that they were ~100 nucleotides shorter overall due to exclusion of *psbA* 5' UTR.
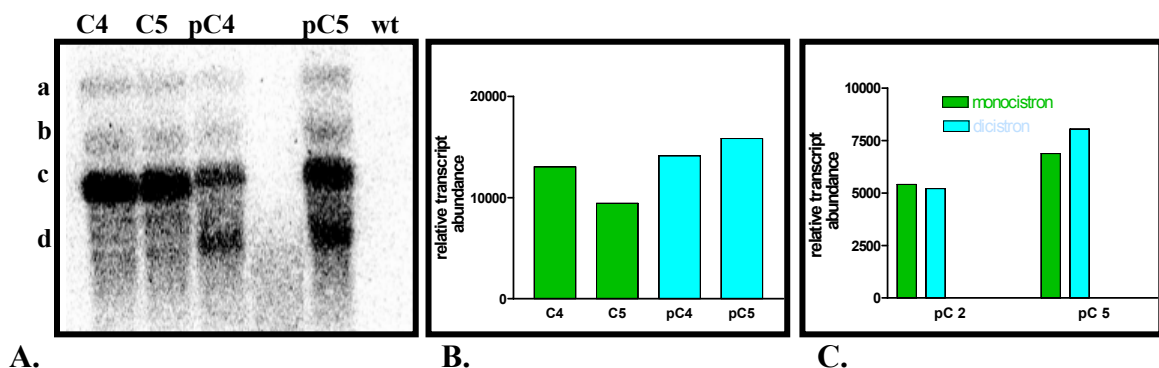


**Figure 3-8: Results of northern analysis of pCPO lines.** A) Phosphorimage of transcripts hybridized to ^{32}P labeled, full length *cpo*. B) Comparison of abundance between pooled (di- and monocistron) transcripts for pCPO lines and CPO lines (dicistron).

## *CPO Protein Accumulates in Chloroplast Transformants*

To determine if CPO protein accumulated in transplastomic plants western

blotting and quantification of chemiluminescent signals was performed. These analyses

confirmed expression of the enzyme in leaf tissue of chloroplast transformants. The

expected 31 kilo Dalton (kDa) protein product was detected in samples from chloroplast

transformants. The corresponding band was evident in the nuclear transformant and

detected in the purified enzyme control, but was absent in the wild-type sample (Figure

3-9A, lower). Equal loading of total soluble protein (TSP) was indicated by visual

inspection of Coomassie stained gels for initial experiments with CPO $R_1$ progeny

(Figure 3-9A, upper). Inclusion of samples of purified CPO enzyme of known

concentration in these experiments allowed quantification of the chemiluminescent

signal. Chloroplast transformants demonstrated a significantly higher ($P \leq 0.01$) level of

CPO expression than the nuclear transformant (Figure 3-9B) in three independent
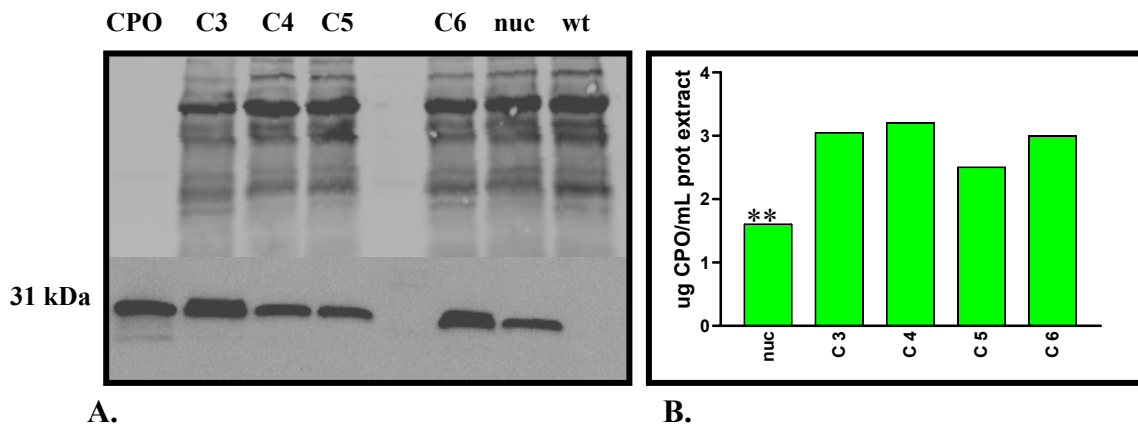


**Figure 3-9: Confirmation of CPO protein expression in chloroplast transformants**.
A) Coomassie stained gel indicates equal loading of TSP (above) and western blot
(below) of $R_1$ progeny confirms expression of CPO protein. B) Quantitation of CPO
abundance in chloroplast and nuclear transformants. Asterisks (**) indicates a
confidence level of 99%.

experiments.  However this level was lower than that predicted for expression in the chloroplast (DeCosa *et al.*, 2001; Tregoning *et al.*, 2003).

Replacement of the ribosome binding site (RBS) with *psbA* 5' UTR was expected to enhance translation of CPO in chloroplast transformants so these plants were also analyzed for protein content.  Sypro™ Ruby protein blot stain allowed determination of equal loading to be carried out on the same membrane as was treated with antibodies. Western blotting confirmed expression of CPO in both types of transplastomic plants, those with an RBS (C) and those in which the *psbA* 5' UTR (pC) was included (Figure 3-10A).  The expected 31 kDa protein was evident in both the nuclear transformant and the control purified enzyme, and was absent in the wild-type sample (Figure 3-10A). Quantification of chemiluminescent signals was unable to detect a significant difference ($P > 0.05$) in protein accumulation between RBS plants and *psbA* 5'UTR plants grown in the lab under fluorescent light (Figure 3-10B).



A.                                                                                      B.
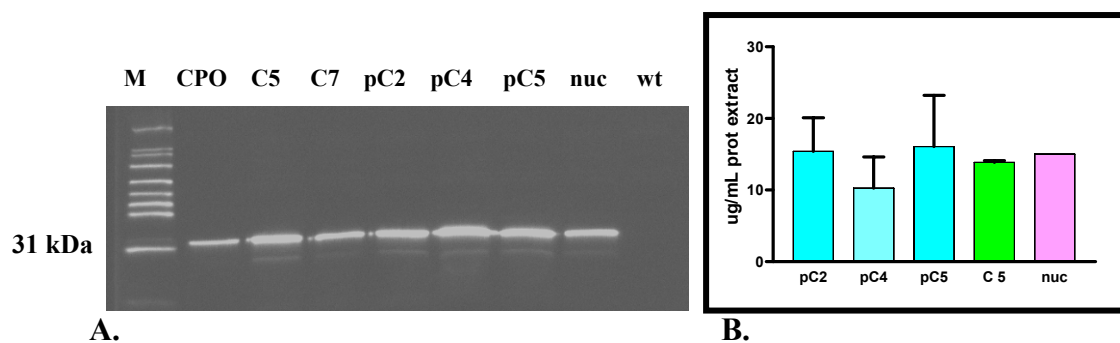
**Figure 3-10:  Comparison of CPO protein expression in chloroplast transformants with differing 5' regulatory sequences** A)  Western blot confirms expression of CPO protein.  B).  Quantification of CPO abundance in RBS and *psbA* 5' UTR chloroplast transformants and nuclear transformant

To further investigate the ability of *psbA* 5' UTR to enhance foreign protein expression in chloroplast transformants, selected *psbA* 5' UTR plants (pC2 and pC4) as well as an RBS plant (C5) were moved to the greenhouse for maximum light exposure

while another member of pC4 line was retained in the lab. All individuals included in this survey had aged and were sampled at ~1m in height, leaves ~30 cm x15 cm, but prior to emergence of inflorescence. Western blots confirmed expression of the CPO enzyme in all transformants, chloroplast and nuclear, as evidenced by 31 kDa band corresponding to that of the purified enzyme and absence of this band in the wild-type sample (Figure 3-11A). Quantification of the chemiluminescent signal indicated a significant (3 to 4 fold, P$\leq$ 0.01 and P$\leq$ 0.001 respectively) increase in CPO accumulation in leaf tissue of the *psbA* 5' UTR chloroplast transformants exposed to full sunlight over both the RBS plants in the same environment and the *psbA* 5' UTR plant maintained in the lab (Figure 3-11B).



**A.**                                                                          **B.**
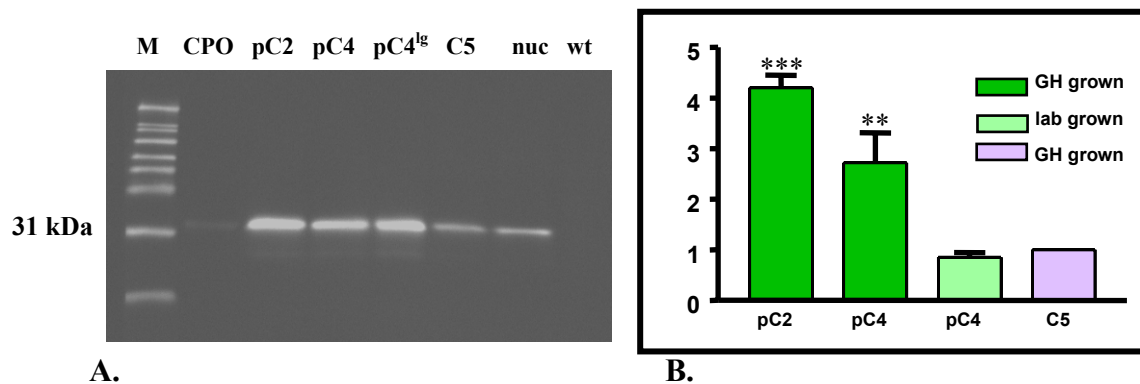
**Figure 3-11:  Comparison of CPO protein expression in lab grown versus greenhouse grown plants.** A)  Western blot confirms expression of CPO protein, superscript lg indicates lab grown  B)  Quantification of CPO abundance in RBS and *psbA* 5' UTR chloroplast transformants exposed to full sunlight versus lab grown. Values on Y axis are ratios of µgCPO/mL protein extract standardized to RBS plant. Asterisks (**, ***) indicate confidence level of 99%and 99.9% respectively.

## *Enzyme Assay Confirms CPO Activity in Chloroplast Transformants*

To determine if the CPO protein produced by transplastomic plants was enzymatically active spectrophotometric assays were carried out (Figure 3-12).  Leaf extracts of three primary chloroplast transformants catalyzed the conversion of the substrate monochlorodimedon (MCD) to monochloromonobromodimedon (MCMBD) by

the addition of a bromide ion in the presence of acetate and hydrogen peroxide resulting

in the reduction of $A_{290}$ values over time. These three lines, C2, C3 and C4 were positive

for CPO expression by northern (Figure 3-6) and western (Figure 3-9) analyses.
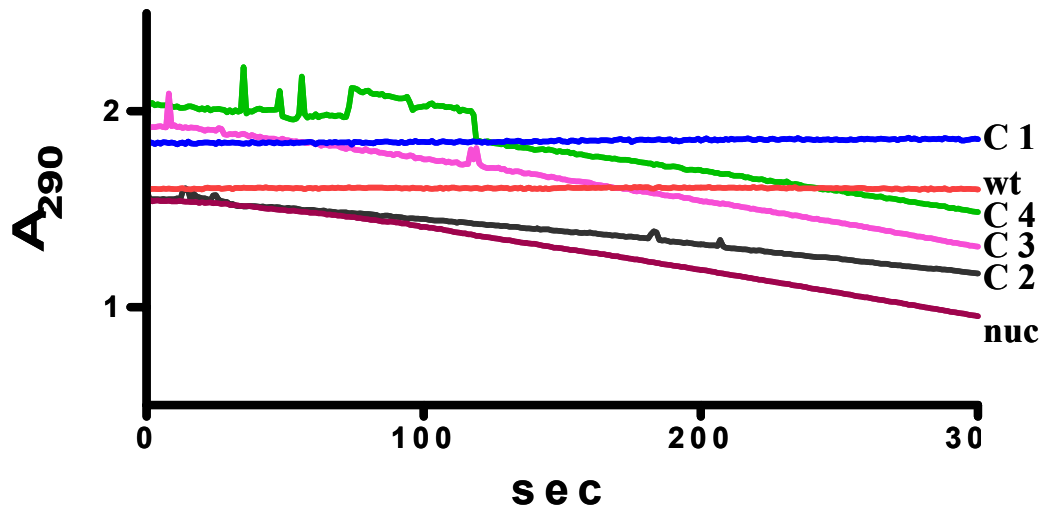


**Figure 3-12: Spectrophotometric analysis of CPO enzyme activity in chloroplast and nuclear transformants.** Extracts of primary ribosome binding site chloroplast transformants (C) were able to catalyze the conversion of the substrate MCD to MCMBD resulting in the reduction of $A_{290}$ values over time. C1, assumed to have escaped antibiotic selection due to a resistance mutation, did not demonstrate any activity in this assay.

### *Cross Pollination Experiments Confirm Maternal Inheritance*

Cross pollination experiments were carried out with CPO transformants and

non transformed wild-type plants to confirm maternal inheritance of the *aadA* transgene.

For each type of cross, ten capsules were collected and 250 seeds from each of the four

types (1000 seeds total) were germinated on antibiotic media. When the maternal parent

was the chloroplast transformant, progeny are uniformly green when germinated on

spectinomycin media whether the paternal parent was wild-type or transformed. When

the paternal parent was transformed but maternal parent is of the wild-type, progeny were uniformly bleached on spectinomycin media (Figure 3-13) as expected if the resistance is inherited through the maternal cytoplasm.
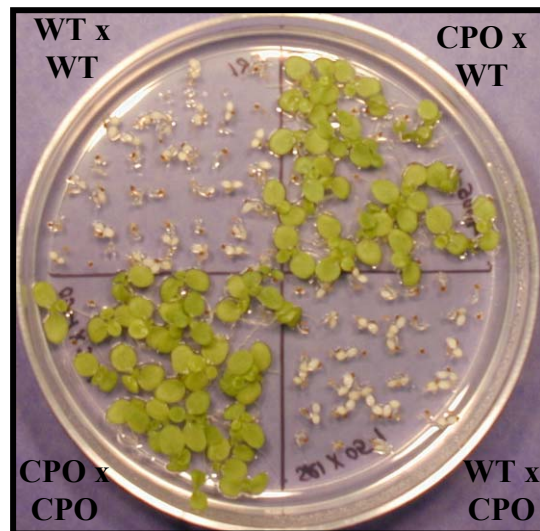


**Figure 3-13: Confirmation of maternal inheritance.** Progeny demonstrate susceptible phenotype when maternal parent is wild-type, resistant phenotype when maternal parent carries *aadA* transgene.

## *β-glucuronidase is Expressed in GUS Chloroplast Transformants*

To determine if plants transformed with the GUS gene were expression the active protein fluorometric and histochemical assays were performed. Translation of the GUS protein in chloroplast transformants was driven by a RBS (GGAGG) upstream of the translation initiation codon. Fluorometric analysis indicated an approximately 2 to 3 fold enhancement of GUS activity in leaf tissue of chloroplast transformants over the nuclear transformant (Figure 3-14B). Histochemical staining of leaf disks taken from chloroplast transformants confirmed expression of the GUS protein in chloroplast transformants (Figure 3-14A) as indicated by the appearance of blue color encircling the disks.
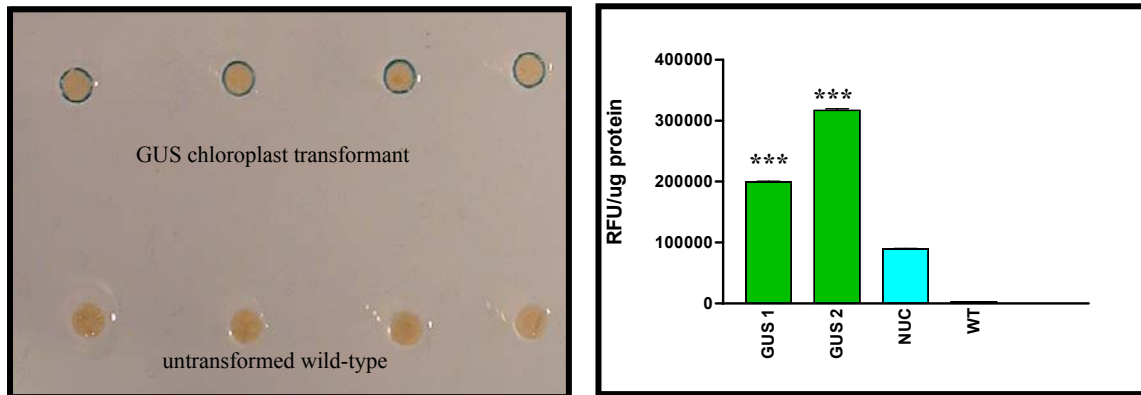
**A.**                                           **B.**

**Figure 3-14: GUS expression in chloroplast transformants.** A) Histochemical staining for GUS expression in leaf disks; blue ring is indicative GUS expression. B) Fluorometric analysis indicated significantly more GUS activity in the chloroplast transformants than in a nuclear transformant. Asterisks (***) denote confidence level of 99.9 %.

## *Evaluation of CPO Lines for In Planta Resistance to Pseudomonas syringae*

Chloroplast transformants for CPO and pCPO were assayed for resistance to

*Pseudomonas syringae* pv *tabaci* (ATCC 17914) (Pst) along with a nuclear transformant

and an untransformed, wild-type control. In two independent experiments Spearman's

and Pearson's correlation analysis indicated that the relationship between the number of

colony forming units and time was not significant (P≤ 0.05). CPO chloroplast

transformants were not effective in limiting Pst population establishment *in planta*

(Figure 3-15).
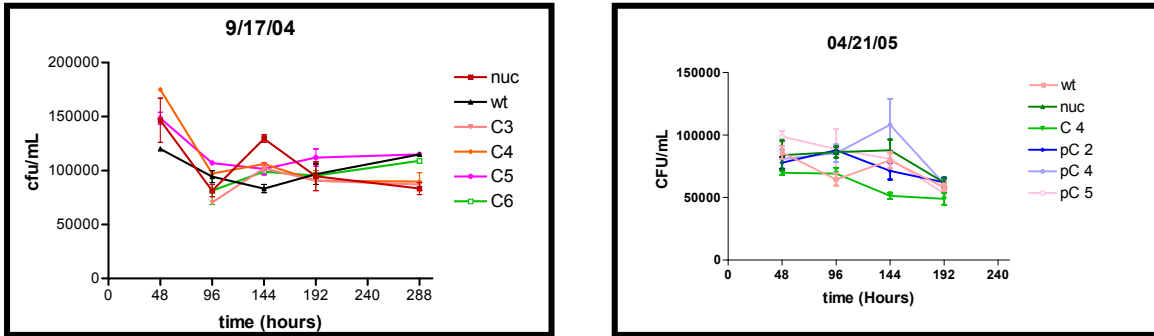
**Figure 3-15: Graphic results for Pst *in planta* population assays**. Transplastomic plants expressing CPO enzyme were not effective in reducing Pst populations over time. Abbreviation: colony forming unit (CFU)

## *In Vitro Evaluation of CPO Transformants for Antifungal Activity*

To determine if chloroplast transformants were able to inhibit colony formation by selected fungi, pregerminated conidia were incubated with crude leaf extracts from tobacco plants transformed with the *cpo* gene. CPO (C), pCPO (pC, lab grown) chloroplast transformants and the nuclear transformant (nuc), significantly reduced colony formation by pregerminated conidia of the cotton fungal pathogen *Verticillium dahliae* compared to non-transformed wild-type extracts ($P \le 0.001$). The level of control exhibited by representatives of lines C5, C6 and pC differed significantly from the CPO nuclear transformant ($P \le 0.001$), whereas nuc, pC2 and pC5 were not significantly different from each other ($P > 0.05$) (figure 3-16).
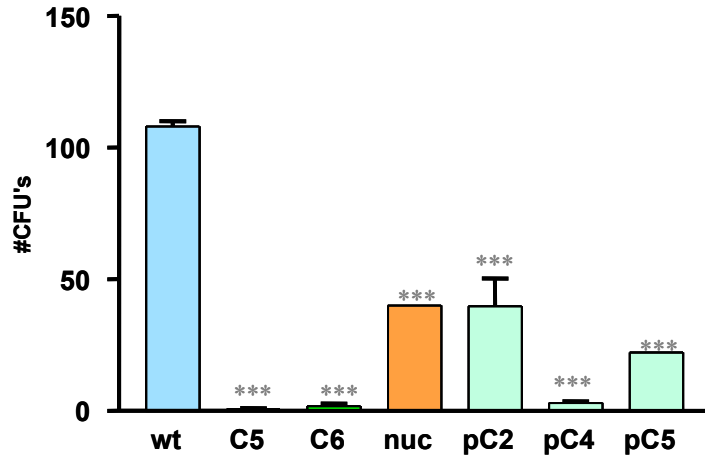
**Figure 3-16: Inhibition of pregerminated conidia of *V. dahliae*** by leaf extracts of tobacco plants transformed with the *cpo* gene. Asterisks (***) indicate a confidence level of 99.9 %, n=3. Abbreviation: colony forming unit (CFU).

Crude leaf extracts from CPO tobacco chloroplast transformants significantly reduced colony formation by pregerminated conidia of the cotton fungal pathogen *Fusarium verticillioides* compared to non transformed wild-type plants (P≤ 0.001) (Figure 3-17). Chloroplast transformants with the *psbA* 5' UTR incorporated were not included in *F. verticillioides* assay.
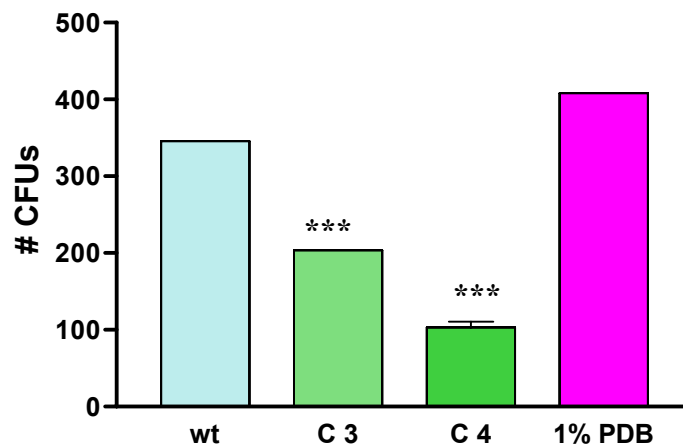


**Figure 3-17: Inhibition of pregerminated conidia of *F. verticillioides*** by leaf extracts of tobacco CPO chloroplast transformants. Asterisks (***) indicate a confidence level of 99.9 %, n=3. Abbreviation: colony forming unit (CFU).

Crude leaf extracts from CPO tobacco chloroplast transformants significantly reduced colony formation by pregerminated conidia of the mycotoxin-producing saprophyte *Aspergillus flavus* compared to non transformed wild-type extracts (Figure 3-18)  In experiments where transformants carried RBS (Figure 3-18A), representatives of two lines, C4 and C5 had the greatest inhibitory effect (P≤ 0.01), while C5 had a significant effect (P≤ 0.05).

In assays where chloroplast transformants carried the *psbA* 5' UTR (Figure 3-18B) plants grown in full sunlight exhibited the greater inhibitory effect but leaf extracts from all plants included in this assay significantly reduced the establishment of *A. flavus* colonies arising from pregerminated conidia compared to those that had not been exposed to plant extracts (P≤ 0.001).  The greenhouse grown RBS plant was more effective than the lab grown *psbA* plant (P≤ 0.001).  There was no significant difference between one *psbA* individual (*psbA*-4) grown in the greenhouse and the RBS plant (P> 0.05).  Among transformants carrying the *psbA* 5' UTR (*psbA*), greenhouse grown plants were more effective in reducing colony establishment than a lab grown plant (P≤ 0.001).  The difference in colony reduction between the RBS plant and the *psbA*-2 plant grown in the greenhouse was highly significant (P≤ 0.001).

**A.**                                                                              **B.**
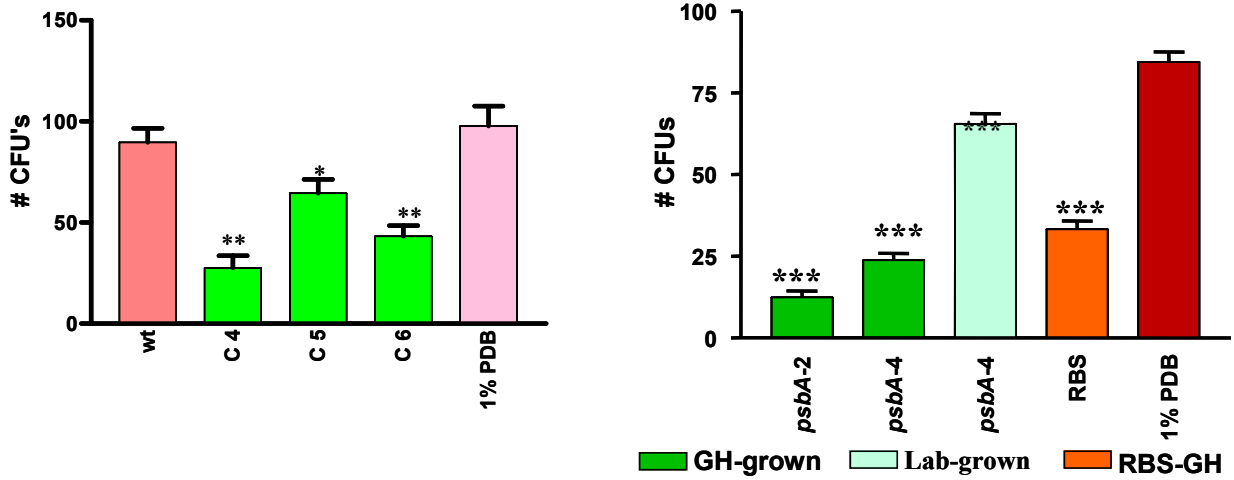
**Figure 3-18: Inhibition of pregerminated conidia of *A. flavus* by leaf extracts of tobacco CPO chloroplast transformants.** A) Performance of RBS plants. B) Performance of *psbA* 5'UTR plants as affected by light intensity. On this graph *psbA*-2 & 4 correspond to pC2 & pC4; RBS corresponds to C4. Asterisks (\*, \*\*, \*\*\*) are indicative of confidence levels, 95%, 99%, and 99.9% respectively; n=3. Abbreviation: colony forming unit (CFU).

# Discussion

Chloroplast biotechnology in higher plants provides the opportunity to engineer species of agricultural interest for improved agronomic traits, such as microbial resistance, while reducing the environmental risk of transgene escape. Plastids possess he potential to accumulate foreign proteins to high levels making them an attractive platform for transgene expression. The lower levels of expression often associated with nuclear transformation can lead to enhanced resistance of pests to the very proteins introduced as a control measure. Additionally, at lower concentrations, introduced antimicrobial compounds may be insufficient to effectively control less susceptible species such as *Aspergillus flavus.*

Our original objective was to achieve a higher level of accumulation of the active CPO enzyme in tobacco chloroplasts greater than that previously demonstrated in CPO nuclear transformants. The *cpo* gene was successfully integrated into the tobacco chloroplast genome in a site-specific manner and that the CPO lines under investigation were homoplasmic for the transgene, as demonstrated by Southern analyses (Figures 3-4 & 3-5). Chloroplast transformants did produce more (~1.5-fold) of the enzyme than the nuclear transformant used for comparison (Figure 3-9) by western analysis. While this difference was significant (P≤ 0.01) we were not satisfied with the magnitude of the difference. Studies have reported significant improvements (50- to 250-fold) in foreign protein accumulation in chloroplast transformants (Kang *et al.*, 2003; Viitanen *et al.*, 2004) compared to nuclear. Such dramatic differences could be attributed to sequestration of the protein within the chloroplast in cases where the foreign protein may

be toxic to the host cell. Additionally, accumulation of the protein may be limited in nuclear transformants due to degradation by host cytoplasmic proteases.

We considered some possible limiting factors which could affect the level of CPO expression observed in our transformants. Northern analysis clearly demonstrated that transcript abundance alone was not a limiting factor (Figure 3-6). Multiple transcripts containing the *aadA:cpo* message are produced in high abundance. The dicistron corresponding to transcript (c) in Figure 3-7 was the most prevalent and was found to be present in 9 to16 times higher copy number than the *cpo* transcript in the nuclear line.

We also considered codon usage as a potential limitation to optimal translation efficiency in plastid transformants. Chloroplast genes tend towards A or U in the third position of the codon with GC content overall falling well below 50 % for all plastid genes (Table 4-1) (Shimada and Sugiura, 1991). The *cpo* constructs employed in these experiments utilized the native *Pseudomonas* sequence. This sequence has a GC content of 64 % overall, tending toward G or C

| Tobacco chloroplast genes | % GC content |
|---|---|
| Genetic system genes | 27.9 |
| Photosynthesis genes | 30.9 |
| NADH dehydrogenase genes | 27.0 |

**Table 4-1:** GC content of chloroplast genes.

in the third position. Translation efficiency may be improved through the synthetic optimization of the native *cpo* sequence GC content for chloroplast specific expression.

The original constructs for *cpo* as well as the GUS construct contained a Shine-Delgarno (SD) type ribosome binding site (RBS) upstream of the translational start codon

for these genes.  Interestingly fluorometric analysis of GUS chloroplast transformants indicated a greater enhancement of expression in leaf tissue over nuclear transformants for GUS (Figure 3-14) than was observed in the CPO lines.  Several reports have indicated that inclusion of the tobacco native *psbA* 5'UTR could facilitate high levels of foreign protein accumulation in chloroplast transformants (Table 2-2).  For human serum albumen (HSA) in particular, expression was dramatically improved through the inclusion of this regulatory element.  In chloroplast transformants where a RBS was employed, HSA accumulated to 0.02% of total soluble protein (TSP) in leaf tissue whereas inclusion of the *psbA* 5' UTR allowed HSA to reach 11.1 % of TSP (Fernandez-San Millan *et al.*, 2003).  In an effort to improve CPO expression in chloroplast transformants, we replaced the RBS with tobacco native *psbA* 5' UTR.

Western analysis of the new CPO lines carrying the *psbA* 5'UTR (pCPO) showed that CPO protein was produced in the transformants, however quantification of the chemiluminescent signal did not indicate a significant improvement over the original CPO lines (Figure 3-10).  As translation of *psbA* is known to be highly regulated by light, selected plants from pCPO lines were transferred from the growth bench in the lab to the greenhouse to determine if exposure to full sunlight would enhance accumulation of CPO in transformed chloroplasts.  A 3- to 4-fold increase in CPO accumulation was observed in pCPO plants in the greenhouse over pCPO grown in the lab or a RBS plant grown in the greenhouse (Figure 3-11).  This result was again below our expectation, and suggests that other, as yet unidentified events, possibly block CPO synthesis and/or accumulation in transformed chloroplasts.

One possibility is that the *psbA* 5' UTR does not function optimally when placed between *aadA* and *cpo* as constructed in these experiments. Northern analysis clearly demonstrates that *cpo* is transcribed as a member of a polycistronic unit as would be expected for transgenes incorporated into the plastid ribosomal operon (*rrn*). The initial precursor transcript for the operon of 7200 nucleotides in length is extensively processed posttrancriptionally (Marchfelder and Binder, 2004). There are 3 transcripts (see Figure 3-7) detectable on the original CPO radiograph (Figure 3-6A). Transcript (a) corresponds to the 4.9 kb message which, in the light, is transcribed predominantly by the plastid encoded RNA polymerase (PEP) from its native promoter at the beginning of the operon. Transcript (b) is presumed to be a cleavage product of (a) and possibly a larger transcript. Transcript (c), by far the most abundant in this example, corresponds to the 1.7 kb dicistron representing *aadA:cpo*. Though it is possible that some portion of this mRNA pool (c) results from the endonucleolytic cleavage of a larger transcript, it is more likely that this mRNA accumulates to a markedly higher level due to transcription by PEP via the engineered $\sigma^{70}$ type promoter introduced upstream of the transgenic sequence.

Northern analysis of pCPO transformants demonstrated an altered hybridization pattern (Figure 3-8A) than was seen for CPO lines with a RBS. Incorporation of the *psbA* 5' UTR appears to have introduced an endonucleolytic cleavage site which allows the separation of at least a portion of the dicistronic mRNA into monocistronic units. The band designated (d) corresponds to the ~0.9 kb transcript for *psbA* 5' UTR:*cpo* . This mRNA could only have resulted from the cleavage of a larger polycistron as no independent promoter element for transcription of monocistronic *cpo* was introduced in the new construct. Quantification of the pooled dicistron and monocistron in pCPO lines

showed there was no significant difference between the total abundance of the pooled

transcripts and the dicistron abundance for CPO lines.  Neither was there a difference in

transcript number between di-and monocistron abundance in pCPO lines.

　　　　To dismiss the results of northern analysis as "not significantly different" based

on transcript abundance alone would be an error.  At this juncture we must consider *at*

*least* two possibilities:  that the *psbA* 5' UTR cannot function in its intended capacity

when confined between two coding regions of an operon and/or, that some fraction of the

monocistronic cleavage product resulting from the inclusion of *psbA* 5'UTR within a
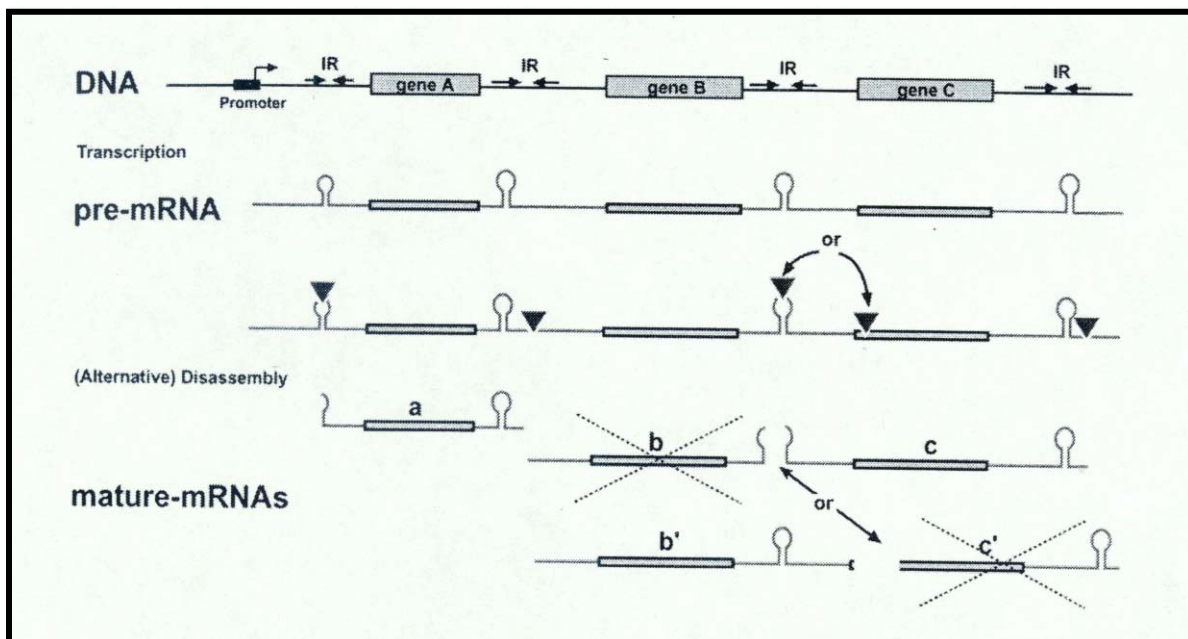


**Figure 4-1:  Schematic model of transcript processing**.  Large precursor messages
are processed after, or possibly during, transcription.  Endonucleolytic cleavage is
indicated by triangles.  Transcripts a, b' and c represent stable, translatable messages.
Transcript b lacks the 3'stem loop and is therefore unstable; c' lacks 5' elements and is
untranslatable. (from Marchfelder, 2004)

polycistron is not a translatable message (or it is translatable but at some reduced level).

The model presented in Figure 4-1 serves to demonstrate the potential of endonucleolytic

cleavage to generate untranslatable transcripts from a polycistronic precursor RNA.

Tobacco chloroplast transformants for the *Cry*2Aa2 operon (DeCosa *et al.*, 2001) have been examined by northern analysis and polyribosome (polysome) fractionation assays (Quesada-Vargas, 2005, in press).  Transcription of this 4 gene operon (*aadA-orf*1-*orf*2-*cry*2Aa2) is predominantly driven by the engineered P1 promoter ($\sigma^{70}$) immediately upstream of *aadA* in the pLD-CtV vector and there are no embedded promoters within the transgene sequence.  Computational analysis predicted a stem loop structure in the native bacterial sequence for c*ry*2A.  Approximately 50% of the total mRNA was comprised of the monocistron for the *cry*2A gene.  While some of these smaller transcripts were found to be associated with polysomes, they were observed mainly in the non-polysomal fraciton (Quesada-Vargas, 2005, in press).  These results may indicate that the polycistron is preferentially translated or, alternatively, that the monocistronic cleavage product is somehow impaired, perhaps by degredative activity of endogenous exonucleases (Drager *et al.*, 1999; Marchfelder and Binder, 2005).

Among 70 protein-coding genes and 9 conserved ORFs present in the tobacco chloroplast genome, five (*ndhF, psbA, psbM, psbN,* and *rbcL*) are transcribed monocistronically (Sugita and Sugiura,1996).  A methyl jasmonate-induced change in the length of the 5' UTR was reported to impair translation of the barley *rbcL* transcript (Reinbothe *et al.*, 1993), suggesting that specific 5' processing is essential for certain chloroplast mRNAs to be functional.  Deletion of the stem loop structure of the *psbA* 5'UTR has been shown to reduce transcript abundance by half resulting in a fourfold depression in translation efficiency (Alexander *et al.*, 1998).  These results have been supported by a more recent study in which a series of deletion and single base mutants for the *psbA* 5' stem loop were developed (Zou *et al.*, 2003).  Translation efficiency, as

determined by GUS activity in relation to mRNA level, was depressed by 1.8- to 6-fold

in mutant lines over the lines with wild-type *psbA* 5' UTR.  Clearly inappropriate

cleavage within the *psbA* 5' UTR has the ability to impair translation efficiency.
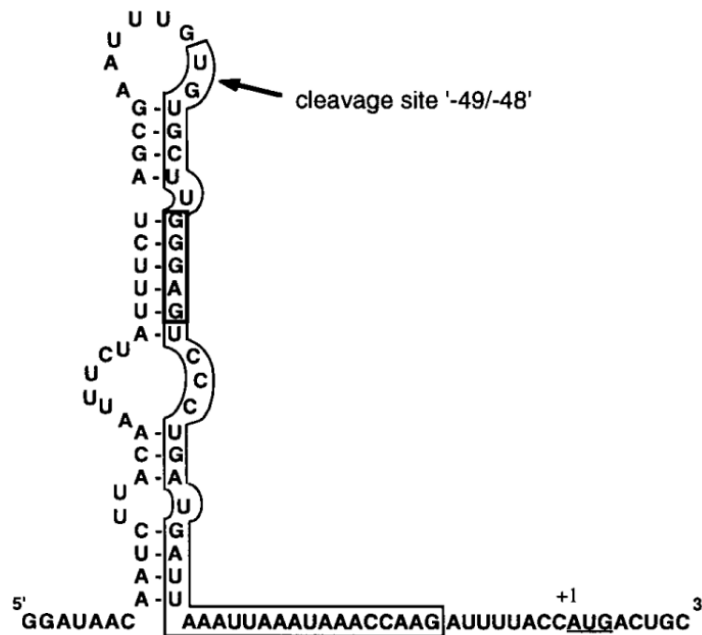


**Figure 4-2:  Representation of secondary structure for *psbA* 5'UTR.**  The protein binding element is highlighted in thin frame, SD sequence in dark frame. Endonucleolytic cleavage site is indicated by arrow (From Alexander, 1998).

Several nuclear encoded protein factors have been identified in *C. reinhardtii*

(Yohn *et al.*, 1998b), spinach (Alexander *et al.*, 1998) and *Arabidopsis* (Shen *et al.*, 2001)

that are required for the formation of an RNA binding complex that initiates *psbA*

translation.  The central protein binding element in spinach *psbA* 5' UTR is provided

Figure 4.2.  After cleavage at the indicated site, the protein complex can no longer form

on the mRNA and activation of translation is abolished; formation of the complex prior to

cleavage at this site protects the mRNA and translation can proceed.  Experimental

evidence (Danon and Mayfield, 1991; Yohn *et al.*, 1998a) suggests that these factors are

highly specific for *psbA* 5' UTR sequence elements. It seems very unlikely that these factors would be able to operate in concert with 5' UTR elements while they are embedded in a polycistronic unit and cleavage to the monocistron prior to complex binding renders the transcript biologically inactive.

Where *psbA* 5' UTR has been shown to be highly effective in promoting foreign protein accumulation it was incorporated into transgene constructs as the leader sequence for a monocistronic transcription unit (Staub and Maliga, 1994; Staub and Maliga, 1995). Alternatively, where the *psbA* leader sequence was incorporated between two transgenes, the full length leader, including the promoter element was utilized. In two examples where the latter was the case, the gene of interest was fused to the full length *psbA* leader (including promoter) and ligated into the same pLD-CtV vector used for the *cpo* transformation experiments described herein. Transplastomic tobacco plants expressing *Bacillus anthracis* protective antigen (PA) demonstrated foreign protein accumulation to 2.7 % of TSP in leaves under 16/8 h illumination and 18.1 % TSP after 3-5 days of continuous illumination in growth chambers (Watson *et al.*, 2004). Employing the same regulatory elements for the expression of the *Escherichia coli ubi*C gene reportedly facilitated accumulation of chorismate pyruvate-lyase (CPL) up to 35 % of TSP in mature leaves (Viitanen *et al.*, 2004). Unfortunately neither of these reports included northern analysis, or for that matter, RNA analyses of any kind. The cleavage event we observed in pCPO lines would be likely to occur in these experiments as well. If this event is involved in the lower than expected levels of expression then we must ask how the high levels of expression were achieved in the aforementioned examples. Inclusion of the *psbA* promoter in these constructs may have allowed transcription by the PEP of the

65

monocistronic message for the transgene in addition to the polycistron transcribed from the native *rrn* promoter and the dicistron from the engineered PEP promoter on the transformation vector. While the polycistronic message for these genes would be susceptible to the same proposed fate as pCPO mRNA, the monocistron would be able to associate with the RNA binding proteins specific to *psbA* in a manner appropriate to support translation of protein product.

A further consideration that should be addressed is the potential for problems associated with the localization of an enzyme such as chloroperoxidase in the plastid. If CPO is active in the chloroplast this could affect active oxygen species (AOS) concentration (see Equation 1). It has been established that AOS, such as hydrogen peroxide and the superoxide radical, play a role in redox signalling in yeast (Vivancos *et al.*, 2005) and plants (Dat *et al.*, 2000; Vranova *et al.*, 2002). The activity of CPO within the plastid could possibly interfere with the redox status of the organelle. Ironically, given that *psbA* is redox regulated, in lines that include this UTR CPO may be partially
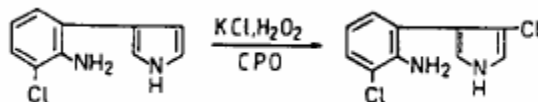


**Figure 4-3:** Chlorination of 4-(2-amino-3-chlorophenyl)pyrrole by CPO (From Wiesner, 1988)

responsible for its down-regulation. Also of interest is the ability of CPO to chlorinate pyrrole moieties (Wiesner *et al.*, 1988) (Figure 4-3). Pyrrole moieties are critical in biosynthesis of porphin (Figure 4-4B) and eventually protoporphyrin IX, a key intermediate in chlorophyll and heme synthesis (Goodwin *et al.*, 1983). A closer

**Figure 4-4: Representation of pyrrole and porphin**. A) Pyrrole molecule. B) Structure of porphin, a tetrapyrrole.

examination of the effect of CPO expression on plastid function in chloroplast transformants may be necessary, despite the apparent overall fitness of CPO chloroplast transformants whole plants (Figure 3-1C).

In terms of our ultimate goal, the expression of CPO in transplastomic plants to confer microbial resistance, we have demonstrated considerable success. In experiments conducted *in vitro*, crude leaf extracts from both RBS CPO lines (CPO) and those with *psbA* 5' UTR (pCPO) have significantly reduced colony formation by pregerminated conidia of several microbial species: *V. dahliae* (Figure 3-16) and *F. verticillioides* (Figure 3-17), (important plant pathogens) and *A. flavus* (Figure 3-18), (a mycotoxin producing saprophyte. A visual inspection of the data from pCPO lines for protein accumulation as compared to inhibition of *A. flavus* suggests a correlation between CPO

abundance and colony reduction in *in vitro* bioassay (Figure 4-5). The samples for this

series of experiments were prepared from the same leaf tissue for protein analysis and
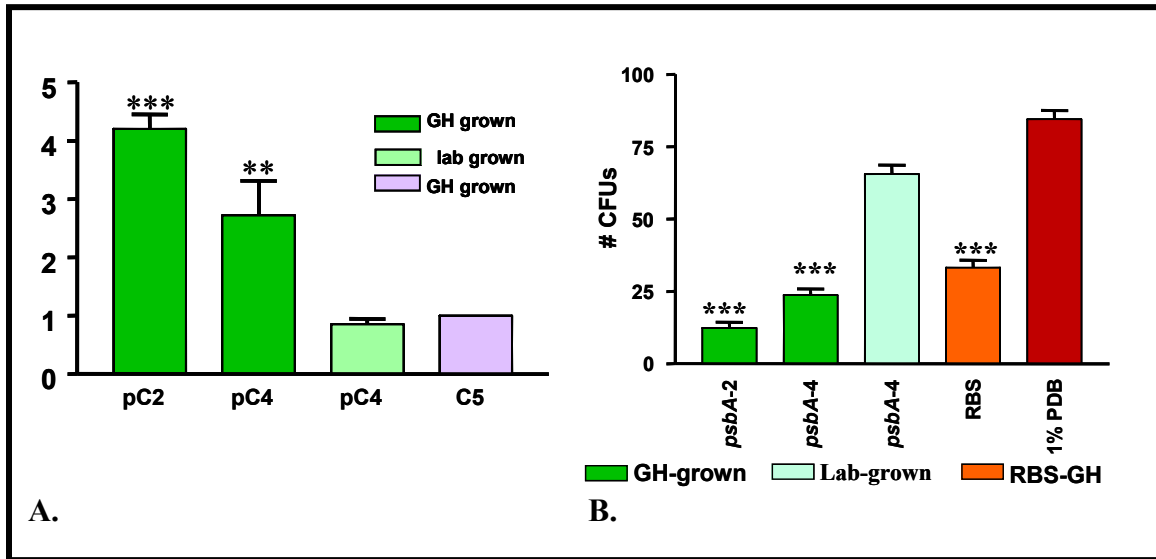


**Figure 4-5: Performance of CPO and pCPO lines in *A. flavus* bioassay compared to CPO protein abundance.** A) CPO abundance in chloroplast transformants; Y axis values are μg CPO/mL protein extract normalized to RBS (C5) plant. B) Efficacy of leaf extracts from CPO and pCPO lines in inhibition of colony formation by *A. flavus* spores. Note on X axis labels: pC2 and pC4 and C5 in (A) are equivalent to RBS, *psbA* 2 and *psbA* 4 in (B)respectively. Asterisks (**,***) indicate level of confidence, 99% and 99.9% respectively. Abbreviation: colony forming units, CFUs.

bioassay. The potential of CPO expression in transplastomic plants warrants further

investigation.

Work will continue in the lab to explore the efficacy of CPO transformants

against plant pathogens *in planta.* Development of a CPO construct optimized for plant

chloroplast codon usage is underway. Also future work should include an analysis of the

possible toxicicity and allergenicity of plants expressing CPO for microbial defense as

food and feed safety is the primary concern of this research.

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# Appendix

## *Recipes*

### *Protein extraction buffer:*

50 mM Tris pH 6.8
  5 mM MgCl
  1 mM EDTA
  1 mM EGTA
  5 mM β-mercaptoethanol
Protease inhibitor cocktail (Sigma #9599) per manufacturer's recommendation.

### *Western transfer buffer:*        *Laemmli sample buffer (1X)*

| Western transfer buffer | Laemmli sample buffer (1X) |
|---|---|
| 0.2 M glycine | 50 mM Tris pH 6.8 |
| 25.0 mM Trizma base | 100 mM DTT (dithiothreitol) |
| 20 % MeOH | 2 % SDS (sodium dodecyl sulfate) |
| | 10 % glycerol |
| | 2 % β-mercaptoethanol |

### *CPO Enzyme assay cocktail:*

| COMPONENT | VOLUME FOR ONE SAMPLE |
|---|---|
| 1.0 M sodium acetate buffer, pH 5.2 | 2.5 mL |
| 0.3 M $NaN_3$ | 100 μL |
| 3.0 M NaBr | 100 μL |
| 0.24 M $H_2O_2$ | 100 μL |
| 1.5 mM monochlorodimedon (MCD) | 100 μL |
| | 2.9 mL |

Note: MCD must be stored at -20 $^o$C in sealed container with desiccant. Remove MCD from freezer ~1 hour prior to opening.

*β-glucuronidase (GUS) assay components:*

GUS fluorometric extraction buffer:
50 mM NaPO$_4$ buffer (pH 7)
10 mM β-mercaptoethanol (70 µL in 100 mL)
10 mM Na$_2$EDTA (372 mg in 100 mL)
0.1 % sarkosyl (100 mg in 100 mL)
0.1 % Triton X-100 (100 µL in 100 mL)

GUS fluorometric assay buffer:
1 mM MUG (4-methylumbelliferyl- β-D-glucuronide; MW 352.3)
        Dissolve 35.2 mg of MUG in 80 mL GUS extraction buffer
        and 20 mL MeOH

4-MU standard:
Prepare 100 mL of 1 mM 4-MU by dissolving 19.82 mg of 4-MU   in 100 mL sterile
milli-Q water.  Place 10 µL of 1 mM stock in 10 mL of water to give final concentration
of 1 µM.  Aliquot 1 mL into microfuge tubes and store in -20 $^o$ C protected from light.


GUS histochemical assay buffer:          GUS staining solution:
0.1 M NaHPO$_4$                                    9 mL assay buffer
5 mM potassium ferricyanide           2 mL X-gluc (16.7 mg ml$^{-1}$ in DMSO)
5 mM potassium ferrocyanide          2 mL methanol (MeOH)
0.06 % Triton X-100

# Vita

Tracey Ann Ruhlman was born in Halifax, Nova Scotia, Canada in 1968. She attended Delagado Community College in New Orleans, Louisiana where she received a full merit scholarship and completed the Associate Degree program with honors in May 2001. She attended the University of New Orleans on the Nouvelle Orleans Transfer Scholarship and earned her Bachelor of Science Degree in Biological Sciences in May 2003. She entered the Master of Science Program at the University of New Orleans in June 2003 supported by the Governor's Biotechnology Initiative Scholarship. Her thesis research was carried out at the Southern Regional Research Center, United States Department of Agriculture, in New Orleans, Lousiana., under the direction of Drs. Kanniah Rajasekaran and Jeffery Cary. She has presented her thesis work at several professional meetings including the Society for In Vitro Biology World Congress 2004, the Southern Section of the American Society of Plant Biologists 2004, the American Society for Microbiology General Meeting 2004 and the USDA ARS Aflatoxin Elimination Workshop 2004. She also attended the first International Symposium on Chloroplast Bioengineering 2005 as an invited speaker. Tracey received the Sara E. Bryan Memorial Graduate Student Award from the University of New Orleans in May 2005 and the 2005 John S. Song Foundation Award from the Society for In Vitro Biology. In August 2005 Tracey received her Master of Science Degree from the University of New Orleans.