IDENTIFICATION OF A CHLOROPLASTIC “RNA TRACTOR” WITHIN THE GENOME OF POTATO VIRUS X (PVX)

By

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A thesis submitted in conformity with the requirements for the Degree of Master of Science
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IDENTIFICATION OF A CHLOROPLASTIC “RNA TRACTOR” WITHIN THE GENOME OF *POTATO VIRUS X* (PVX)

Degree of Master of Science, 2009
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ABSTRACT

Previous studies have detected the presence of the capsid protein (CP) and CP RNA of PVX in chloroplasts of transgenic plants expressing the PVX CP and 8kD genes from nuclear transgenes. CP RNA was also found in the chloroplast of transgenic plants with mutations that eliminated either the CP or the 8kD protein. To further investigate the potential “RNA Tractor” activity of the PVX RNA, various constructs containing limited regions of the PVX 8kD and CP genes were produced and used to transform tobacco plants. RT-PCR analyses of chloroplastic RNA ascertained the presence of the RNA transcript within chloroplasts when as little as 125bp of the PVX sequence was used to transform plants. From this, it was concluded that the PVX proteins did not contribute to the movement of the viral RNA and that a region within the 125bp PVX sequence is acting as a chloroplastic “RNA Tractor”.

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5' cap    m7GpppGp
A. tumefaciens  Agrobacterium tumefaciens
BaMV      Bamboo Mosaic Virus
BAP       6-benzylaminopurine
bp        base pair
BSA       Bovine Serum Albumin
CaMV      Cauliflower Mosaic Virus
CP        capsid protein
ddH2O     double distilled water
DEPC      Diethylpyrocarbonate
dNTP      deoxynucleotide triphosphate
DNase     deoxyribonuclease
DTT       dithiothreitol
E. coli   Escherichia coli
EDTA      Ethylenediaminetetraacetic acid
EF-1      elongation factor 1
ER        endoplasmic reticulum
HEPES     (N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid)
Hsp       Heat shock proteins
IRBS      Internal Ribosome Binding Site
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRES</td>
<td>Internal Ribosome Entry Site</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pair</td>
</tr>
<tr>
<td>kD</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani (media)</td>
</tr>
<tr>
<td>LBA</td>
<td>LB media with 15 g/L agar</td>
</tr>
<tr>
<td>mA</td>
<td>milliamperes</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td><em>N. Xanthi</em></td>
<td><em>Nicotiana Xanthi</em></td>
</tr>
<tr>
<td>NAA</td>
<td>Naphthalene acetic acid</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>pBS</td>
<td>Blue-script plasmid</td>
</tr>
<tr>
<td>pC1300</td>
<td>Cambia plasmid strain 1300</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerate kinase</td>
</tr>
<tr>
<td>PPV</td>
<td><em>Plum pox virus</em></td>
</tr>
<tr>
<td>PSI</td>
<td>photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>photosystem II</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PVX</td>
<td>Potato Virus X</td>
</tr>
<tr>
<td>RMP</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNP</td>
<td>ribonucleoprotein</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEL</td>
<td>size exclusion limit</td>
</tr>
<tr>
<td>sGFP</td>
<td>synthetic GFP</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNAs</td>
</tr>
<tr>
<td>TE&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1: 10 dilution of 10mM Tris-HCl (pH 8), and 1mM EDTA</td>
</tr>
<tr>
<td>TGB</td>
<td>triple gene block</td>
</tr>
<tr>
<td>TIC</td>
<td>translocon of the inner envelope membrane of the chloroplast</td>
</tr>
<tr>
<td>TMV</td>
<td>Tobacco mosaic virus</td>
</tr>
<tr>
<td>Tnos</td>
<td>Nopalin synthase terminator</td>
</tr>
<tr>
<td>TOC</td>
<td>translocon of the outer envelope membrane of the chloroplast</td>
</tr>
<tr>
<td>ToMV</td>
<td>Tomato mosaic virus</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>TRV</td>
<td>Tobacco Rattle Virus</td>
</tr>
<tr>
<td>TYMV</td>
<td>Turnip yellow mosaic virus</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
</tbody>
</table>
1. General Properties of the Chloroplast

1.1. The structure and genome of the chloroplast

Chloroplasts are membrane enclosed organelles that are characteristic of photosynthetic plants and algae (Vothknecht and Soll, 2005). Chloroplasts in land plants typically measure 5-10 µm in diameter and are surrounded by both an outer and an inner membrane (Block et al., 2007; Figure 1). These organelles also possess a continuous third photosynthetic membrane that forms disc-like sub-organelles called thylakoids. The thylakoids are divided into two categories; those that are stacked into grana and the lamella that connect the individual granum to one another (Buchanan et al., 2000). Of all the organelles contained within a eukaryotic cell, chloroplasts and mitochondria are unique because they carry some of their own genetic information and are able to synthesize some of their own proteins (Buchanan et al., 2000). Chloroplasts contain double-stranded, circular chromosomes that range in size from 120 to 160 kB and typically contain four segments: a large region of single copy genes, a small region of single copy genes and 2 copies of inverted repeats (Buchanan et al., 2000). These genomes encode various components that are necessary for protein synthesis such as 4 ribosomal RNAs (rRNAs), 30 transfer RNAs (tRNAs), 21 ribosomal proteins and 4 RNA polymerase subunits. The chloroplast genome also encodes proteins that are involved in photosynthesis such as 28 thylakoid proteins and the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (Harris et al., 1994).
Figure 1: Diagrammatic representation of a chloroplast.
The chloroplast has three different membranes: the outer membrane that separates the inside of the organelle from the cytoplasm, the inner membrane that divides two aqueous environments (the intermembrane space and the stroma), and the thylakoid membrane (where photosynthesis takes place). The thylakoids are stacked into grana, which are connected to one another via lamella (adapted from Alberts et al., 2002).
1.2. The prokaryotic nature of the chloroplast

It has been proposed that chloroplasts originated from an endosymbiotic relationship that developed between a prokaryotic ancestor and the host cell that engulfed it (Buchanan et al., 2000). This endosymbiotic event is believed to have occurred once, thus all present day chloroplasts are thought to have evolved from one common predecessor (Palmer, 2000). Through the comparison of rRNA sequences and internal structures, many similarities have been established between plastids and the modern day *Synechococcus lividus* cyanobacteria, therefore it has been suggested that they both evolved from the same ancient cyanobacterium (Vothknecht and Soll, 2005).

Although chloroplasts possess their own genome and protein synthesis machinery, these organelles are unable to exist autonomously outside of the eukaryotic cell. This is likely due to a considerable relocation of genetic information from the chloroplastic genome to the host nucleus (Martin and Hermann, 1998). This suggests that the maintenance of the chloroplast is likely to require rigid coordination of both transcription and translation in the nucleus as well as in the chloroplast (Stengel et al., 2007).

Despite importing numerous peptides, the chloroplast also utilizes prokaryotic protein synthesis machinery to generate many of its own proteins. Although prokaryotic protein synthesis follows the same three steps required for eukaryotic translation (Initiation, Elongation and Termination), there are a few major differences. For example, while eukaryotic messenger RNAs (mRNAs) possess 5’ caps (m7GpppGp) and 3’ poly-A tails, prokaryotic RNA transcripts are missing both of these structures. Without the 5’ cap, a prokaryotic ribosome identifies the translational start site within an mRNA transcript by binding to a Shine-Dalgarno sequence (typically GGAGG in chloroplasts) upstream of the
initiator AUG (Buchanan et al. 2000; Alberts et al., 2002). The prokaryotic and eukaryotic systems also differ in the sizes of their ribosomal subunits, in the number of initiation factors involved in translation, and in the number of citrons contained within their mRNA transcripts (Alberts et al., 2002).

2. Chloroplastic Proteins

2.1. The prokaryotic translation of chloroplastic RNA transcripts

The chloroplast utilizes a prokaryotic system to synthesize proteins encoded in its own genome. Prokaryotic translation can be divided into 3 stages: Initiation, Elongation, and Termination.

Protein synthesis initiates when the 16S rRNA of the 30S small ribosomal subunit base pairs with the Shine-Dalgarno sequence upstream of the initiator AUG in the mRNA transcript. Meanwhile, Initiation Factor 2 (IF-2) binds to a tRNA aminoacylated with formylmethionine (tRNA\(^{fMet}\)) and facilitates the base pairing between this tRNA and the start codon of the mRNA (Alberts et al., 2002). Finally, the 50S large ribosomal subunit unites with the previously mentioned components to complete the initiation complex. The formation of this complex is promoted by two additional initiation factors. Initiation Factor 3 (IF-3) binds to the 30S subunit and prevents it from joining the 50S subunit when no mRNA transcript is present, and Initiation Factor 1 (IF-1) promotes the dissociation of the 70S ribosome (Kozak, 1999).
In the second phase of protein synthesis, the peptide chain is elongated through the addition of amino acids. First, a new amino-acyl tRNA molecule bound to an EF-Tu elongation factor enters the ribosome, and if the correct codon-anticodon pairing is made, a molecule of Guanosine Triphosphate (GTP) within the EF-Tu is hydrolyzed and the elongation factor dissociates from the tRNA (Alberts et al., 2002). The amino-acyl tRNA then moves into the A site of the ribosome and peptidyl transferase catalyzes the formation of a new peptide bond between the amino acids in the A and P sites. Next, another elongation factor, EF-G, enters the ribosome, which triggers the hydrolysis of an attached GTP molecule. This hydrolysis triggers a drastic change in the conformation of the ribosome that shifts the tRNAs located in the A and P sites to the P and E sites, respectively. The uncharged tRNA that is now located in the E site is expelled from the ribosome and the A site is now free to accept a new amino-acyl tRNA molecule (Alberts et al., 2002).

The final stage of protein synthesis is called termination and this occurs when one of the three termination codons enters the A site of the ribosome (Alberts et al., 2002). Since these codons are not recognized by any tRNA molecule, an additional amino acid is not added. Rather, these codons are recognized by release factors that cleave the polypeptide from the final tRNA and release the newly synthesized protein. Release Factor 1 (RF-1) recognizes the UAA and UAG codons, while Release Factor 2 (RF-2) recognizes the UAA and UGA codons. A third Release Factor (RF-3) promotes the release of RF-1 and RF-2 as the final step in the translation process (Marin-Navarro et al., 2007).
2.2. The chloroplastic import of nuclear encoded proteins

Although chloroplasts contain their own DNA and they synthesize many proteins, most chloroplastic proteins are encoded in the nuclear genome and are imported into the organelle after synthesis in the cytoplasm. This implies that plant cells have had to evolve a mechanism for specifically targeting cytosolic proteins to the organelle and another mechanism for transporting these proteins across both the inner and outer membranes of the chloroplast.

The most common method for targeting cytosolic proteins to the chloroplast involves the incorporation of a 40-50 amino acid transit peptide in the N-terminal region of the targeted protein (Soll and Schleiff, 2004). Transit peptides, also known as signal sequences, are both necessary and sufficient for chloroplast import. In other words, proteins that lack this transit peptide are not imported into the chloroplast and attaching this signal sequence onto the N-terminus of a foreign protein directs translocation into the organelle (Buchanan et al., 2000).

Although transit peptides are necessary for protein translocation into the chloroplast, these peptides do not possess a consensus motif in their primary amino acid sequence. However, there are some factors that seem to increase the likelihood that a peptide will be transported into the chloroplast, such as having an N-terminal region that is rich in basic amino acids and deficient in acidic residues (Gnanasambandam et al., 2007). The efficiency of chloroplastic import also appears to increase after the phosphorylation of chloroplast-targeted proteins by a cytosolic serine/threonine kinase; however this phosphorylation is not a necessary prerequisite for import to occur (Waegemann and Soil, 1991; Nakrieko et al.,...
The transit peptide of the chloroplast has been shown to differ from the signal sequences of other organelles in both amino acid composition and peptide secondary structure (Gnanasambandam et al., 2007). Chloroplast transit peptides have also been shown to be target specific and they prevent aberrant delivery to other organelles, such as the mitochondria (Rudhe et al., 2002).

Two functional steps have been proposed to describe the import of chloroplast-destined proteins. First, the transit peptide is recognized by receptors on the outer membrane of the chloroplast and the translocation process is initiated by the hydrolysis of a GTP molecule within the receptor (Stengel et al., 2007). Chaperone proteins then pull the peptide through the Toc (translocon of the outer envelope membrane of the chloroplast) and Tic (translocon of the inner envelope membrane of the chloroplast) complexes, which form an uninterrupted passageway (Schnell and Blobel, 1993). The Toc and Tic translocation complexes mediate at least 90% of the protein import into the organelle (Inaba and Schnell, 2008). The second step occurs once the polypeptide arrives in the stroma of the chloroplast, where chloroplastic proteases remove the transit peptide and chaperones assist in protein folding (Richter and Lamppa, 1998).
3. The relationship between plant viruses and the chloroplast

3.1. The impact of host proteins on the replication of plant viruses

It has been suggested that both viral and host proteins are involved in the replication of many RNA plant viruses. For example, the RNA polymerase of *Vesicular stomatitis virus* (VSV) has been shown to specifically associate with translation elongation factor -1 (EF-1); a host protein which is also believed to participate in the activity of the polymerase (Das *et al.*, 1998). EF-1 has also been implicated in the replication of *Tobacco mosaic virus* (TMV) and *Turnip yellow mosaic virus* (TYMV) (Zeenko *et al.*, 2002; Matsuda *et al.*, 2004). Several host heat shock proteins (Hsp) have also been associated with viral replicative efficiency. For example Hsp70 seems to enhance *Tomovirus* replicase activity and the inhibition of Hsp90 with geldanamycin and radicicol greatly reduces the replication of VSV (Serva and Nagy, 2006; Connor *et al.*, 2007). Additionally, a yeast 2 hybrid system demonstrated the interaction between the TMV replicase and a host AAA-ATPase (ATPase Associated with various Activities). When *Nicotiana benthamiana* plants that had the ATPase gene silenced were infected with TMV, there was a 2 fold reduction in virion accumulation; which suggested that the ATPase might assist in the replication of the virus (Abbink *et al.*, 2002).

3.2. Chloroplastic proteins and the accumulation of viruses within plants

Several studies have demonstrated that chloroplastic proteins influence the accumulation of plant viruses within plant cells. For example, *Plum pox virus* (PPV) infection has been shown to alter chloroplastic structure, resulting in dilated thylakoids, an increased number of plastoglobuli, a decreased starch concentration and increased hydrogen
peroxide levels (Diaz-Vivancos et al., 2008). Analyses of proteins extracted from PPV infected pea plants demonstrated that most of the changes produced by PPV infection were related to photosynthesis and carbohydrate metabolism, including decreasing the amounts of Rubisco, oxygen-evolving enhancer proteins, and photosystem II (PSII) stability factor proteins (Diaz-Vivancos et al., 2008). Thus the precipitation of PPV symptoms in pea plants might be the result of the virus interacting with chloroplastic proteins.

It has also been shown that the RNA helicase domain of the TMV replicase protein interacts with the 33K subunit of PSII (Abbink et al., 2002). Using a Tobacco rattle virus vector, this gene was silenced in Nicotiana benthamiana plants. When these plants were subsequently infected with TMV, a 10-fold increases in viral accumulation was observed. TMV levels also increased when PSII was inhibited using the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DMSU). Furthermore, when healthy Nicotiana benthamiana plants were infected with TMV, there was a subsequent decrease in the 33K mRNA within the cell. From these results, the authors suggested that TMV might be silencing the 33K gene in vivo in order to evade the host’s innate defence mechanisms (Abbink et al., 2002).

Most recently, ultraviolet cross-linking competition assays were used to identify an interaction between phosphoglycerate kinase (PGK), a chloroplastic protein, and the 3’ untranslated region (UTR) of the Bamboo Mosaic Virus (BaMV) RNA (Lin et al., 2007). When the PGK gene was silenced in Nicotiana benthamiana and subsequently challenged with a BaMV infection, the accumulation of BaMV coat protein was significantly reduced. It was thus suggested that the efficient accumulation of BaMV required the presence of the chloroplastic protein PGK (Lin et al., 2007).
3.3. The detection of Tobacco Mosaic Virus (TMV) within chloroplasts

TMV has been known to accumulate in the chloroplasts of infected plants since 1958, when Zaitlin and Boardman successfully isolated TMV virions from this organelle (Zaitlin and Boardman, 1958). However, it was later demonstrated that some of the isolated virions were only one third the length of the wild-type virus, and that not all of the virus-like particles actually contained TMV RNA (Shalla et al., 1975). It was also shown that the TMV coat protein was able to encapsidate some chloroplastic RNAs, and encapsidation was more likely to occur with chloroplastic transcripts than with nuclear transcripts (Siegel, 1971). It was then suggested that the TMV coat protein is able to encapsidate chloroplast RNA transcripts inside the chloroplast itself, and this leads to the formation of pseudovirions within the organelle (Rochon and Siegel, 1984). Thus it was established that both the TMV coat protein and virus-like rods are present within the chloroplasts of infected plants (Schoelz and Zaitlin, 1989).

3.4. The role of viral coat proteins within the chloroplast

Previous work has confirmed that the TMV coat protein can be isolated from the chloroplasts of infected tobacco leaves and a positive correlation connecting the severity of chlorotic symptoms and the concentration of the coat protein within the organelle was established (Reinero and Beachy, 1986). A comparison of a mild strain of TMV with a strain of TMV that induces severe chlorosis revealed that far more coat protein accumulated in the chloroplasts of the chlorotic strain as compared to the mild strain (Reinero and Beachy, 1989; Banerjee et al., 1995). Also, the chlorotic strain was shown to inhibit PSII both in
vitro and in vivo, but the mild strain did not (Reinero and Beachy, 1989). Furthermore, plants that displayed severe chlorosis also had a significant reduction in the concentration of the entire PSII core complex and the 33kDa protein of the oxygen evolving complex (Lehto et al., 2003). The coat proteins of both TMV and Tomato mosaic virus (ToMV) have also been shown to associate directly with the thylakoid membrane and it has been proposed that the interaction of viral and chloroplastic proteins might contribute to the formation of chlorotic symptoms (Banerjee et al., 1995; Zhang et al., 2008).

4. mRNA Localization

4.1. The advantages of mRNA localization

The ability to localize mRNA confers several advantages upon a cell. First, a single mRNA molecule can act as a template for the generation of several protein molecules, thus it would be more efficient for the cell to move a single mRNA molecule as compared to translocating numerous proteins (Du et al., 2007). Also, mRNA localization can be used to spatially control the translation of proteins; which can restrict the synthesis of peptides to their specific subcellular locations and even facilitate the development of protein gradients (Martin and Ephrussi, 2009). In addition, mRNA targeting can promote the assembly of protein complexes by locally concentrating the RNA transcripts of all of the necessary subunits; thus increasing the likelihood that the resulting proteins will interact with one another (Palacios, 2007). Lastly, mRNA localization might facilitate the import of proteins targeted to specific organelles. In other words, if a protein is synthesized in close proximity
to its target organelle, it avoids many potential obstacles that might interfere with its arrival at its intended destination (Du et al., 2007).

4.2. Mechanisms of mRNA localization

The general purpose of mRNA localization is to target RNA transcripts to a particular subcellular region, so that when translated, the synthesized proteins also remain in the target vicinity (Hoyle and Ashe, 2008). Like many transit peptides, most mRNA localization signals are not believed to be integrated into the primary nucleic acid sequence. Rather, these localization signals, or “zip codes”, rely on the formation of secondary or tertiary structures in order to be recognized by cellular trans-acting factors (Hamilton and Davis, 2007). The most commonly used mechanism for translocating mRNA across the cell involves the recognition of specific cis-acting localization signals within the mRNA transcript by cellular trans-acting factors (Bullock, 2007). These factors then recruit motor proteins to form a large ribonucleoprotein transport complex; which moves along the cytoskeleton towards the intended destination. Once the complex arrives, the mRNA must be anchored within the region in order to prevent the diffusion of the targeted transcript (Bullock, 2007). mRNA localization can also occur via localized anchoring; where RNA transcripts move through the cell either by diffusion or by cytoplasmic streaming and are subsequently trapped by receptor proteins within the target destinations (Palacios, 2007). Alternatively, in localized protection, mRNA transcripts are degraded or destabilized in all areas of the cell, except for a specific target region (Palacios, 2007).
4.3. Aggregation of mRNA transcripts near chloroplasts

Although the localization of mRNA has been extensively studied in many animal and insect models, very little is known regarding the localization of mRNA within the plant kingdom. However, it has been previously shown that RNA-based gene silencing signals can spread systemically throughout the plant, and this is likely mediated by the movement of small interfering RNAs (siRNAs) travelling through the plant phloem (Du et al., 2007). Therefore, if it is possible for RNA signals to travel throughout the plant, it might also be possible for mRNAs to be targeted to subcellular organelles.

The targeting of mRNA transcripts to chloroplasts was investigated by selecting three nuclear encoded genes whose protein products are specifically found within the organelle (Marrison et al., 1996). The selected genes included the CHLH gene; which encodes a magnesium protoporphyrin chelatase subunit, the Por gene; which encodes a protochlorophyll oxidoreductase, and the Lhcb1*2 gene; which encodes a chlorophyll a/b binding protein. Through in situ hybridization, the investigators detected the aggregation of all three transcripts around the surface of the chloroplast. This led them to suggest that the RNA transcripts are somehow targeted to the chloroplast, and that the proximity to the organelle might facilitate the import of the locally synthesized peptides. It has also been proposed that the accumulation of these RNAs can be attributed to the localized protection of specific RNA transcripts associated with the chloroplast or the selective degradation of these same RNAs when they are located in any other region of the cell (Okita et al., 2002).
4.4. TMV RNA enters chloroplasts \textit{in vivo}

Previous studies have demonstrated the presence of TMV genomic length viral RNA within the chloroplasts of plants that had been both directly inoculated and systemically infected with the aforementioned virus (Schoelz and Zaitlin, 1989). However, it was still unclear as to whether the entire virion was being translocated into the chloroplast, or if it was only the unencapsidated viral RNA. To investigate this, a TMV mutant (Ts38) that can encapsidate its RNA and systemically infect plants at 20 °C but not at 35 °C, was used to infect \textit{Nicotiana tobacum} plants; which were grown at both 20 °C and 35 °C. The TMV RNA was detected in chloroplastic samples both when the virus was encapsidated at 20 °C, and when it was not (at 35 °C). This suggested that the unencapsidated TMV RNA is transported into chloroplasts. To confirm this, a C\textsubscript{c} strain of TMV, where all of the subgenomic RNAs are encapsidated, was used to infect plants. Since all subgenomics are present within the encapsidated virion, one would expect to see even the smallest subgenomics within the chloroplast if the entire virion is being translocated. However, only the genomic RNA was detected in these chloroplasts, thus it was concluded that only the unencapsidated viral RNA was transported into chloroplasts \textit{in vivo} (Schoelz and Zaitlin, 1989).
4.5. The accumulation of Avsunviroidae viroids within the chloroplast

Viroids are single-stranded, circular RNA plant pathogens that are approximately 247-401 nucleotides in length (Daros et al., 2006). Viroids are divided into two families, the Pospiviroidae and the Avsunviroidae. The four members of the Avsunviroidae family are the Avocado sunblotch viroid (ASBVd), the Peach latent mosaic viroid (PLMVd), the Chrysanthemum chlorotic mottle viroid (CChMVd), and the Eggplant latent viroid (ELVd) (Molina-Serrano et al., 2007). Viroids do not code for any proteins and they rely on interactions with their hosts for replication (Daros et al., 1994). It has also been shown that members of the Avsunviroidae family accumulate and replicate within the chloroplasts of infected plants (Lima et al., 1994; Bussiere et al., 1999), and that they may use the nuclear encoded polymerase (NEP) of the chloroplast for their replication (Navarro et al., 2000). Therefore, it has been proposed that these viroids enter the chloroplast using some endogenous RNA translocation pathway, however the mechanism of this RNA import has yet to be described (Ding, 2009). Furthermore, there is very little sequence conservation between the four members of the Avsunviroidae outside of their hammerhead structures, therefore secondary structure might play a more important role in the import of the RNA into the chloroplast (Flores et al., 2005).
1. Potato Virus X (PVX): Genome, Gene Products and Potential Internal Ribosome Binding Site (IRBS)

1.1. The PVX Genome

*Potato Virus X* (PVX) is the type member of the potexvirus genus and systemically infects many species of the *Solanaceae* family (Uhde-Holzem *et al.*, 2007). It is a rod-shaped, filamentous virus that possesses a single-stranded, 6435 nucleotide positive-sense RNA genome. This polycistronic RNA is capped at the 5’ end ($m^7$GpppGp), polyadenylated at the 3’ end, and contains five open reading frames (ORFs) as depicted in Figure 2 (Huisman *et al.*, 1988). The first ORF encodes a 166kD RNA replicase (RNA-dependent RNA polymerase). The second, third and fourth ORFs encode a 25kD protein, a 12kD protein, and an 8kD protein, respectively. These proteins are known as the “triple gene block” (TGB) and are thought to be involved in the cell-to-cell movement of the virus (Fedorkin *et al.*, 2001). The fifth ORF encodes a capsid protein (CP), which encapsidates the viral RNA into a 515 nm flexuous virion (Tollin and Wilson, 1988; Karpova *et al.*, 2006); and has also been implicated in viral intercellular movement (Morozov *et al.*, 1991).

The first ORF (the replicase gene), is expressed directly from the genomic RNA. In contrast, the expression of downstream cistrons occur through a series of capped subgenomic RNAs that are generated from the negative sense strand of the virus (Hefferon *et al.*, 1997).
Figure 2: Genomic Organization of PVX.
Potato Virus X (PVX) possesses a polycistronic RNA genome that contains five open reading frames: the RNA replicase (166kD), the triple gene block (25kD, 12kD, and 8kD) that has been implicated in the cell-to-cell movement of the viral RNA, and a capsid protein (CP); which is also believed to be involved in viral intercellular movement. The location of the proposed IRBS is also indicated.
1.2. The roles of the PVX TGB and CP in viral RNA movement

It has been previously shown that the PVX CP and TGB proteins play a role in the intercellular movement of the PVX RNA during viral infection (Fedorkin et al., 2001). Immunogold labelling of the 25kD protein has demonstrated that this protein is targeted to and accumulates within plasmodesmata (Erhardt et al., 1999; 2000). The 25kD protein is also known to modulate plasmodesmata gating, where the size exclusion limit (SEL) of the plasmodesmata is increased by the 25kD protein so that it can move between cells (Angell et al., 1996; Yang et al., 2000; Howard et al., 2004). The 25kD protein also acts as a suppressor of gene silencing during viral infection by blocking the systemic spread of plant defence silencing signals (Baulcombe et al., 2002; Voinnet et al., 2000; Lecellier and Voinnet, 2004). Furthermore, mutations that eliminate the protein’s ability to suppress silencing also inhibit the cell-to-cell movement of the virus; which implies that the prevention of gene silencing is crucial for promoting the plasmodesmata transport of the viral RNA (Bayne et al., 2005).

Although the 8kD and 12kD proteins have not been localized to the plasmodesmata, these proteins have been identified in the endoplasmic reticulum (ER) and it is believed that they are trafficked from this region to the periphery of the cell (Krishnamurthy et al., 2003, Mitra et al., 2003). The 12kD protein has also been found to affect the permeability of the plasmodesmata (Tamai and Meshi 2001), and amino acid substitutions in the central region of this protein inhibit viral cell-to-cell movement (Ju et al., 2007). While the 8kD protein does not appear to be necessary for the cell-to-cell movement of PVX, its presence greatly increases the efficiency of PVX intercellular movement (Lough et al., 2000; Solovyev et al., 2000; Tamai and Meshi, 2000; Lim et al., 2008). Also, both the 12kD and the 8kD proteins
possess hydrophobic domains; which are postulated to modulate the function of the 25kD protein during viral movement (Morozov et al., 1991; Yang et al., 2000).

The CP of PVX has been shown to accumulate within plasmodesmata in PVX-infected cells, however, the CP does not seem to control the gating of these structures (Angell et al., 1996; Oparka et al., 1996; Lough et al., 2000; Howard et al., 2004). Thus, it has been posited that the CP forms a ribonucleoprotein docking complex with the 25kD protein and the viral RNA; which then moves through the plasmodesmata during the systemic spread of the virus (Lough et al., 1998; Cruz et al., 1998, Lucas and Lee, 2004). This hypothesis has been supported in vitro by gel shift mobility assays that confirmed the interaction between the CP and the 25kD proteins (Lough et al., 2000). Furthermore, it has been shown that the CP belonging to PVY was able to complement cell-to-cell movement of PVX CP defective mutants but did not encapsidate the PVX genome. These finding suggest that the CP is important for viral intercellular movement and that the CP’s role in viral movement is distinct from its role in encapsidation (Fedorkin et al., 2000).

1.3. The potential PVX Internal Ribosome Binding Site (IRBS)

It is known that polycistronic positive-sense RNA viruses express their internal genes through the production of numerous capped, subgenomic RNAs. However, it has been previously shown that a construct corresponding to the C-terminal half of the 12kD gene, the entire 8kD gene, the entire CP gene, and the 3’ non-coding region of PVX RNA is expressed in transgenic plants under the control of a Cauliflower Mosaic Virus (CaMV) 35S promoter (Hefferon et al., 1997). Proteins isolated from these transgenic potato plants were subjected to Western blot analyses and it was found that both the CP and 8kD proteins were expressed
*in vivo* in these plants. Furthermore, Northern blot analysis of total RNA from plants transformed with the aforementioned construct indicated the presence of a single dicistronic mRNA from which both proteins were synthesized. When a stable hairpin structure, known to suppress the initiation of translation of downstream AUG codons, was placed upstream of the 8kD-CP dicistronic construct, the expression of the 8kD protein was inhibited but CP expression was unaffected. Although the phenomenon of internal initiation could be explained by alternative expression mechanisms such as leaky scanning, these mechanisms would have been blocked by the stable hairpin. Thus, the existence of an Internal Ribosome Binding Site (IRBS) was proposed (Hefferon *et al.*, 1997; **Figure 3**). It is believed that the IRBS is located upstream of the CP gene and that it directs the expression of the CP using a cap-independent translation initiation strategy (Hefferon *et al.*, 1997). Cap-independent translation has also been identified in other multicistronic viruses such as members of the picornaviridae (Tsukiyama-Kohara *et al.*, 1992; Belsham, 2000; Bieleski and Talbot, 2001; Serrano *et al.*, 2009). However, these internal ribosome entry sites (IRES) do not seem to share any sequence or structural similarities with the proposed PVX IRBS (Hefferon *et al.*, 1997).
8kD ⇔
5’ atggaagtaatacatctcaacgcaatcatacttgtgttgtaacaatcatagcagtca

Ttagtacctcttagtagaatgcagaaaccatctcagtaacatctcaagattactggagaatcaatcacagttggctt

Gcaagttagatgcagaaaccatcagagccattgcgtcataagttcgccttcggttaacggttaaagttt

CP ⇔
ccattgatactcgaagaagagtcagccaccagctagcacaacaca 3’

Figure 3: The location of a Shine-Dalgarno-like sequence upstream of the CP gene in PVX.

Hefferon et al. (2000) demonstrated the existence of a Shine-Dalgarno-like sequence in the region upstream of the PVX CP gene (highlighted in yellow), and this region was shown to function as internal initiation site for the translation of the PVX CP. The potential IRBS sequence is also believed to be located between the latter half of the 8kD gene and the beginning of the CP gene; which includes the Shine-Dalgano-like sequence (Hefferon et al., 2000). The start codons (ATG) for both the 8kD and CP genes are underlined.
1.4. PVX CP and CP RNA within transgenic potato chloroplasts

Previous research conducted in our laboratory revealed the presence of PVX CP within the chloroplasts of both PVX-infected potato plants and transgenic potato plants containing the 8kD and CP sequences (Hefferon et al., 2000). Northern blot analyses also confirmed the existence of PVX CP mRNA inside the chloroplasts of PVX-infected and transgenic plants (Hefferon et al., 2000).

In order to determine whether the CP was transported into chloroplasts from the cytoplasm or translated within the chloroplast itself, the 8kD and CP genes of the PVX genome were used to construct a binary vector plasmid, and transgenic potato plants were produced. Protoplasts isolated from the transgenic plants were incubated in the presence or absence of chloramphenicol, a prokaryotic ribosome inhibitor. Immunoprecipitation and dot blot analysis were used to detect the production of PVX CP within chloroplasts, while simultaneously observing the production of chloroplastic (P700) and cytoplasmic (actin) proteins. It was shown that the PVX CP was only detected in chloroplasts of cells grown in the absence of chloramphenicol. This was also true for P700, a chloroplast specific protein associated with photosystem I (PSI). On the other hand, actin levels were unaffected by chloramphenicol treatment, but were drastically reduced by incubating the protoplasts in cyclohexamide (a eukaryotic protein biosynthesis inhibitor). Thus, it was concluded that the CP was not synthesized in the cytoplasm and later transported into the chloroplast, but that the translation of the CP mRNA occurred within the chloroplast itself (Hefferon et al., 2000).

It was then proposed that the Shine-Dalgarno-like sequence located upstream of the CP might be acting as an internal translational initiation site for the expression of the CP (Figure 3). This Shine-Dalgarno-like sequence has been shown to be complementary to
chloroplast rRNA, and to possess sequence homology to the 16S rRNA of *E. coli* (Hefferon *et al.*, 2000). Furthermore, other viruses, including TMV and various carlaviruses, have also been shown to possess similar Shine-Dalgarno-like sequences upstream of their respective CP genes (Turner and Foster, 1997). Hefferon *et al.* (1997) thus suggested that the 8kD-CP sequence was transported into the chloroplast from the cytosol, and was then translated by chloroplastic ribosomes using a Shine-Dalgarno-like internal translation initiation site.

1.5. The roles of the PVX gene products in the translocation of PVX RNA

Previous studies have demonstrated that PVX RNA is translocated from the cytosol to the chloroplasts of transgenic potato plants (Hefferon *et al.*, 2000). However, it was unclear whether the PVX RNA was being transported into the chloroplast by one of its gene products (i.e. the 8kD protein or the CP) or whether the movement was due to the PVX sequence (i.e. the nucleotide sequence or RNA secondary structure) working in conjunction with a host factor(s).

To investigate this phenomenon, several constructs containing the PVX sequence were produced (Figure 4). The first construct contained full copies of both the 8kD and CP genes (Figure 4A). The second construct was designed to disable the 8kD protein through the insertion of a G in the 80th nucleotide position of the 8kD ORF. This insertion produced a frameshift mutation in the resulting protein (Figure 4B). The third construct had a full copy of the 8kD gene, but only the first thirteen nucleotides of the CP gene positioned in frame with the AUG of the sGFP gene, thus producing a CP-sGFP fusion protein (Figure 4C). These three constructs were used to transform *N. tabacum* cv. *Xanthi* plants.
Confocal microscopy was used to examine sGFP expression in leaf samples from nontransformed plants as well as from plants transformed with the empty pCambia plasmid (where expression of the sGFP gene is under the control of the CaMV 35S promoter), the pC:8K-CP construct, the pC:8K(insG^{80})-CP construct and the pC:8K construct (Cheng, 2007). sGFP expression was not observed in nontransformed plants, or in plants transformed with the pC:8K-CP and pC:8K(insG^{80})-CP constructs. In contrast, sGFP expression was observed in empty pCambia transgenic leaves, and to a lesser extent, in leaves harvested from pC:8K transgenic plants. This data provided further evidence for the existence of a functional IRBS upstream of the CP gene (Cheng, 2007).

Since the PVX CP and 8kD proteins have been previously implicated in the cell-to-cell movement of the virus (Fedorkin et al., 2001), it was hypothesized that these proteins may also be involved in the translocation of the PVX RNA into chloroplasts. To further explore the involvement of the PVX gene products in the translocation phenomenon, chloroplasts were isolated from plants transformed with the PVX constructs and reverse transcription polymerase chain reaction (RT-PCR) was used to detect the presence of the 8kD and CP RNAs within this organelle. For plants transformed with the pC:8K-CP and pC:8K(insG^{80})-CP constructs, the CP RNA was detected within chloroplasts. For plants transformed with the pC:8K construct, the 8kD RNA was present within chloroplasts, despite the absence of the CP protein. This data suggested that neither the 8kD nor the CP protein is essential for the translocation of the PVX RNA into chloroplasts (Cheng, 2007).
To investigate the translocation of PVX RNA into chloroplasts, regions of the PVX sequence were cloned into a modified version of the pCambia 1300 binary vector for the transformation of transgenic tobacco plants. A) pC:8K-CP: This construct contains both the 8kD and CP genes and was produced to confirm the previously-observed translocation of CP mRNA into chloroplasts B) pC:8K(insG^{80})-CP: the pC:8K-CP clone was digested with EcoNI and filled with a Klenow fragment; which caused the insertion of a G in the 80\textsuperscript{th} nucleotide position of the 8kD gene. This produced a frame shift mutation in the resulting protein. C) pC:8K: In this construct, the CP gene was truncated so that only the first 13 nucleotides remained. Also, the AUG of the CP gene is in frame with the AUG of the sGFP gene. 35S: Cauliflower Mosaic Virus 35S promoter. 8k: 8kD gene. CP: coat protein. sGFP: synthetic green fluorescent protein.

Figure 4: Schematic Representation of the PVX expression constructs in the pCambia 1300 plasmid.
2. Research Proposal

2.1. Research Objectives

The major goal of the proposed research is to determine whether the translocation of the PVX RNA into chloroplasts is dependent upon a particular region of the PVX nucleotide sequence. This mechanism will be investigated by two means:

a) Eliminating the involvement of the CP and 8kD proteins in the translocation phenomenon

First, it is necessary to confirm the roles of the CP and 8kD proteins in the translocation of PVX RNA. Since previous work in our lab has indicated that the elimination of either the CP or the 8kD protein did not affect the translocation of the RNA into chloroplasts, it is still theoretically possible that both proteins are able to transport the PVX RNA, and they can do so even when the other is absent. Thus, a construct must be created that contains mutations in both the 8kD gene and the CP gene so that neither protein is complete or functional. This construct will be used to produce transgenic tobacco plants and RT-PCR analyses of both total plant RNA and chloroplastic RNA will be performed to ascertain the presence or absence of PVX mRNA in chloroplasts.

b) Localizing the sequence of the proposed “RNA Tractor”

After the involvement of the CP and 8kD proteins in the translocation of PVX RNA has been clarified, further steps will be taken to investigate the possible existence of an RNA signal sequence within the PVX RNA. This will be accomplished through the production of additional constructs that contain a smaller portion of the PVX sequence in order to better localize the specific region that might be acting as the “RNA Tractor”. These constructs will
then be used to produce transgenic plants and the localization of the PVX RNA will be observed.

2.2. Hypothesis

We hypothesize that there is a certain RNA signal sequence, the “RNA tractor”, located between the 8kD gene and the CP gene that is recognized by either host proteins or viral proteins and is responsible for the movement of the PVX RNA into chloroplasts. Although the triple gene block and the CP have been previously implicated in the intercellular movement of the PVX RNA, previous research conducted in our lab has suggested that these proteins might not be involved in the translocation of the PVX virus into chloroplasts of transgenic plants. Alternatively, the PVX RNA itself, in conjunction with some factor(s) from the host, might be responsible for translocating both its own RNA and foreign RNA into the chloroplast.
MATERIALS AND METHODS

1. General Molecular Techniques

1.1. Plasmid DNA isolation from *E. coli* (Mini-Prep)

A glycerol stock of *Escherichia coli* transformed with a particular plasmid was used to inoculate 2 ml of LB broth (1% bacto tryptone, 0.5% bacto yeast extract and 1% sodium chloride, pH 7.5) supplemented with the appropriate selection antibiotic (ex. 60 µg/ml Ampicillin for pBS plasmids and 50 µg/ml Kanamycin for pCambia plasmids) and the cells were cultured overnight in a 37 °C shaker. The plasmid DNA was then extracted from 1.5 ml aliquots of cells using a modified version of the alkaline lysis method described in Maniatis *et al.* (1982).

The *E. coli* cells were harvested by centrifugation at 12 000 g for one minute. The supernatant was decanted and the pellets were resuspended in 100 µl of ice-cold Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8, and 2 mg/ml lysozyme). After a 5 minute incubation period at room temperature, 200 µl of freshly prepared Solution II (0.2 N sodium hydroxide and 1% sodium dodecyl sulphate) was added to each sample. The solution was thoroughly mixed by gently inverting the tubes 10 times, and was subsequently incubated on ice for 15 minutes. Next, 150 µl of Solution III (3 M sodium acetate, pH 4.8) was added to each tube and the suspension was inverted 10 times and kept on ice for one hour. Following this incubation, the tubes underwent centrifugation at 12 000 g for 5 minutes at 4 °C and the supernatant was transferred to a new tube.
To precipitate the plasmid DNA, 0.6 volumes of isopropanol was added to the supernatant and incubated at room temperature for 1 hour. Plasmid DNA was pelleted by centrifugation at 12,000 g for 1 minute at room temperature. The pellets were then washed once with 500 µl 70% ethanol and once with 500 µl 95% ethanol and left to air dry in the fume hood. Once all of the ethanol had evaporated, the pellets were dissolved in 30 µl of TE\(^{-1}\) buffer (1 mM Tris, pH 8, and 0.1 mM EDTA) and stored at -20 °C.

The extracted plasmid DNA was then analyzed using gel electrophoresis. A 0.5X TBE buffer (0.1 M Tris, 0.1 M Boric acid, and 2 mM EDTA, pH 8) was used for both the preparation of the agarose gel as well as the running buffer. One µl of each plasmid DNA sample was mixed with 4 µl of TE\(^{-1}\) and 1 µl of loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 50% glycerol), and subjected to electrophoresis through a 1.2% agarose gel at constant current of 30 mA, until the bromophenol blue had migrated half-way through the gel. The gel was then stained with ethidium bromide and photographed under ultraviolet light (300 nm) with a transilluminator.

1.2. Heat shock transformation of *E. coli* and *A. tumefaciens*

Bacterial strains were made competent and transformed using a calcium-chloride heat shock protocol outlined by Maniatis *et al.* (1982). Although the following protocol describes the transformation of *E. coli* strain DH5\(\alpha\) cells, *Agrobacterium tumefaciens* strain GV3103 were also transformed using this method, however, the cells were incubated at 28 °C rather than 37 °C, the incubation times were twice as long to accommodate for the difference in doubling time between the strains (i.e. the doubling time for *E. coli* is 20 minutes compared
to the 40 minutes required by *A. tumefaciens*), and the *A. tumefaciens* cells were cultured in the presence of 30 µg/ml Gentamycin.

A glycerol stock of DH5α was used to inoculate 3 ml of LB media, and the culture was grown overnight in a 37 ºC shaker. On the following day, 250 µl of this culture was used to inoculate 25 ml of fresh LB and the new culture was grown in the 37 ºC shaker until the optical density at 600 nm (OD<sub>600</sub>) was between 0.4-0.6. Once this OD was obtained, the cultured cells were incubated on ice for 20 minutes and were subsequently pelleted by centrifugation at 1200 g for 5 minutes at 4 ºC. The supernatant was decanted in the fume hood and the pellet was resuspended in 5 ml of ice-cold 50 mM calcium chloride. The cells were then incubated on ice for 20 minutes, followed by a second centrifugation at 1200 g for 5 minutes at 4 ºC. The supernatant was discarded in the fume hood and the pellet was resuspended in 667 µl of ice-cold 100 mM calcium chloride and kept on ice for at least 30 minutes. Next, the DNA sample (i.e. 1 µl of plasmid DNA or 10-15 µl of a ligation reaction) was mixed with a 200 µl aliquot of competent cells and incubated on ice for 30 minutes. The samples were then heat shocked at 42 ºC for 45 seconds and immediately cooled on ice for 10 minutes. Eight-hundred µl of LB media was added to each sample and the tubes were incubated at 37 ºC for 45 minutes. The cells were pelleted by centrifugation at 12 000 g for 30 seconds at room temperature and all but 200 µl of the supernatant was discarded. The remaining supernatant was used to resuspend the pellet and the mixture was plated on LBA (LB media with 15 g/L agar) with the appropriate selection antibiotic. The agar plates were inverted and incubated at 37 ºC overnight to allow the colonies to grow.

Single colonies were then picked and grown in 3 ml of LB supplemented with the appropriate selection antibiotic. Plasmid DNA was extracted from these cultures and
restriction digests were performed to ascertain the successful transformation of the bacteria with the correct plasmid.

1.3. Glycerol Stock Preparation

A single colony of bacteria harboring the appropriate plasmid was used to inoculate 3 ml of LB media and the culture was grown overnight in a 37 °C shaker. The following day, a 500 µl aliquot of this culture was placed in a sterile tube along with an equal volume of autoclaved glycerol. The samples were thoroughly mixed by vortexing and stored at -80 °C.

1.4. Phenol-chloroform DNA/RNA extraction

TE\(^{-1}\) was added to each sample of DNA/RNA so that the final volume was 100 µl. An equal volume of phenol (saturated with 0.1 M Tris, pH 8) was added to each sample and thoroughly vortexed. Next, the samples underwent centrifugation for 5 minutes at room temperature. The top phase was removed from each sample without disturbing the interface and placed in a new tube. Two volumes of cold chloroform were added to each sample and the mixture was thoroughly vortexed and underwent 5 minutes of centrifugation at room temperature. The chloroform was removed and this step was repeated. The DNA/RNA was then placed in a new tube and potassium acetate was added to a final concentration of 0.1 M along with 2.5 volumes of cold 95% ethanol. The samples were thoroughly mixed and incubated at -20 °C overnight. The next day, the DNA/RNA was pelleted by centrifugation at 12 000 g for 5 minutes at 4 °C. The resulting pellet was washed once with 500 µl 70% ethanol and once with 500 µl 95% ethanol and left to air dry in the fume hood. Once all of
the ethanol had evaporated, the pellets were dissolved in 30 µl of TE$^{-1}$ buffer or 30 µl sterile double distilled water treated with Diethyl Pyrocarbonate (DEPC) and stored at -20 °C.

1.5. Gene cloning and screening using plasmid DNA in *E. coli* DH5α

Plasmid DNA was digested using the recommended buffers and reaction conditions described by the restriction enzyme suppliers (i.e. NEB and Fermentas). Typically, DNA (both plasmid DNA and oligonucleotide inserts) was digested in 25 µl reactions containing 2.5 µl 10X buffer and 5 units of enzyme overnight at 37 °C. The DNA was then deproteinized by phenol-chloroform extraction and used for ligation reactions at a 10:1 molar ratio of insert to vector, respectively. Inserts were ligated into linearized plasmids overnight at 16°C in a total volume of 25 µl with 2.5 µl 10X ligation buffer (400 mM Tris-HCL, pH 7.8, 100 mM MgCl$_2$, 100 mM DTT, and 5 mM ATP), 0.1 pM of plasmid, 1 pM of insert and 5 units of T4 DNA ligase. On the following day, the reaction volume was raised to 50 µl, supplemented with 5 more units of ligase and incubated at room temperature for 4 hours. Following ligation, the number of plasmids lacking the desired insert was reduced by digestion with restriction enzymes and half of this reaction was used to transform DH5α competent cells. Once constructs were confirmed through the analysis of restriction digestion products and through sequencing, the modified plasmids were used to transform *Agrobacterium tumefaciens* strain GV3103 cells for the production of transgenic plants.
<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPMut Sense</td>
<td>CTAGTAGGCCCTACGTTAGTTA</td>
</tr>
<tr>
<td>CPMut Antisense</td>
<td>CTAGTAACTAACGTAAGGCCTA</td>
</tr>
<tr>
<td>L8K_F</td>
<td>AATATTGTTACCCAGGCCCTCATATCTCAACGCAATCAT</td>
</tr>
<tr>
<td>S8K_F</td>
<td>AATATTGTTACCCAGGCCCTGGAGAATCAATCACAGTG</td>
</tr>
<tr>
<td>8K_R</td>
<td>ACTACTGCTAGCGCGTGCTGACATCTTTTCGAGTATC</td>
</tr>
<tr>
<td>PCR27 Sense</td>
<td>TAGGCCTATTGATACTCGAAAGATGTCAGCACCAT</td>
</tr>
<tr>
<td>PCR27 Antisense</td>
<td>CTAGATGGTGCTGACATCTCTTCGAGTATCAATAGGCCT</td>
</tr>
<tr>
<td>sGFP_R</td>
<td>GAACCTCCAGGGTCAGCTTGC</td>
</tr>
</tbody>
</table>

Table 1. Sequences of oligonucleotides used for construct production and construct sequencing.
The CP mutant oligonucleotides (CPMut sense and CPMut antisense) were used to create an insert for the production of the pBS:8K(insG80)-CP(ins14-35S13X) clone. The 8kD forward (L8K_F) and reverse (8K_R) primers were used for the production of the pTR:224bp clone. The 8kD forward (S8K_F) and reverse (8K_R) primers were used for the production of the pTR:125bp clone. The PCR 27 oligonucleotides (PCR27 sense and PCR27 antisense) were used to produce the pTR:27bp clone. The sGFP reverse (sGFP_R) primer was used to confirm the sequences of all of the previously described clones.
1.6. PCR

Polymerase Chain Reaction (PCR) was used for the synthesis and amplification of double stranded DNA inserts used to produce various PVX constructs. PCR was also used to confirm the presence of transgenes in the chromosomal DNA of transgenic plants.

An aliquot of DNA template (either plasmid DNA, chromosomal DNA or cDNA) was mixed with 2.5 µl 10 X PCR buffer, 2.5 mM dNTPs, 2.5 mM magnesium chloride, 10 pmol each of sense and antisense primers (please see Table 2 for primer sequences), 2.5 units of Taq DNA Polymerase, and filled to 25 µl with sterile ddH₂O. A layer of mineral oil was placed on top of the reactions to prevent evaporation and the samples were placed in a thermal cycler for 30 cycles for amplification.
### Table 2. Sequences of primers used for RT-PCR analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L8K_F</td>
<td>AATATTGGTACCAGCCTCATATCTGGAATACGTCTGAGATAC</td>
</tr>
<tr>
<td>S8K_F</td>
<td>AATATTGGTACCAGCCTGGAGAATCAATCACAGTGTTG</td>
</tr>
<tr>
<td>8K_R</td>
<td>ACTGCTAGCCTGGTACGTAGCACATCTGAGTAC</td>
</tr>
<tr>
<td>CP_F</td>
<td>GGAACTGGATGTAAACTAACAAC</td>
</tr>
<tr>
<td>CP_R</td>
<td>AAAATACGTATGAAGACTGGGGGTAG</td>
</tr>
<tr>
<td>sGFP1_F</td>
<td>GCAAGCTGACCGGCTGAGTTG</td>
</tr>
<tr>
<td>sGFP2_R</td>
<td>GTGTTCTGCTGAGTGTGGT</td>
</tr>
<tr>
<td>sGFP3_R</td>
<td>GGTGCAGTTAAGTTG</td>
</tr>
</tbody>
</table>

The 8kD forward (L8K_F), 8kD reverse (8K_R), CP forward (CP_F) and CP reverse (CP_R) primers were used for the RT-PCR analysis of transgenic plants transformed with constructs pC:8K-CP, pC:8K(insG^{80})-CP, and pC:8K. The 8kD forward (S8K_F) and reverse (8K_R) primers were used for the RT-PCR analysis of transgenic plants transformed with the PCR clones pTR:224bp and pTR:125bp. The CP forward (CP_F) and reverse (CP_R) primers were used for the RT-PCR analysis of PVX total, chloroplastic and viral RNA. The sGFP reverse primer (GFP3_R) was used to produce cDNA from plants transformed with the empty pCambia plasmid, the pC:8K construct and non-transformed plants and this cDNA was amplified by PCR using sGFP forward (sGFP1_F) and sGFP reverse (sGFP2_R) primers.
2. Production of PVX Constructs

2.1. pBS (Blue-Script plasmid) clones

i. pBS: 8K(insG\textsuperscript{80})-CP(Δ\textsuperscript{18-146})

A previously constructed pBS clone (pBS:8K(insG\textsuperscript{80})-CP, Figure 4) was digested with \textit{NheI} at 37 °C overnight; releasing a 129bp fragment. The reaction was then deproteinated using a phenol chloroform extraction protocol and the resulting 5' overhang was filled using a Klenow fragment for 2 hours at 37 °C in a 50 µl reaction containing 5 µl of 10X Klenow buffer (500 mM Tris-HCl, pH 8.0, 50 mM MgCl\textsubscript{2}, and 10 mM DTT), 1 mM dNTP’s and 5 units of the Klenow enzyme. The Klenow-filled plasmid was then ligated overnight at 4°C, followed by a room temperature ligation for 4 hours the following day. Background plasmids were reduced by digesting the ligation reaction with \textit{NheI} and this reaction was used to transform \textit{E. coli} DH5α competent cells, which were plated on 60 µg/ml Ampicillin LBA plates.

A triple digest with \textit{KpnI}, \textit{SalI}, and \textit{NheI} was used to screen for potential clones. The correct clones released two fragments: one 850bp fragment that contains the 35S promoter and one 1250bp fragment that contains the mutated 8kD and CP genes. Although the \textit{NheI} site is located within the 1250bp fragment, this restriction site is destroyed by Klenow-filling and remains intact in correct clones. However, in the original pBS:8K(insG\textsuperscript{80})-CP construct, the \textit{NheI} site remains intact and the 1250bp fragment is cut into three pieces of approximately 129bp, 430bp and 700bp. The digests were subjected to electrophoresis through a 1.5% agarose gel and the correct clones were verified through sequencing.
ii. pBS:8K(insG<sup>80</sup>)-CP(ins<sup>14-35</sup>S13X)

The previously constructed pBS clone (pBS:8K(insG<sup>80</sup>)-CP, Figure 4) was digested with *NheI* at 37 °C overnight. An oligonucleotide insert was designed to fit into the plasmids *NheI* restriction site and to introduce a frameshift mutation into the CP gene. A *StuI* site was also added to the oligonucleotide insert to facilitate screening for the desired clones. The double stranded insert was produced by annealing the sense and antisense strands (CPMut Sense and CPMut Antisense respectively, Table 1) by first boiling them for 5 minutes and then allowing them to cool to room temperature. One pmol of the double stranded insert was then ligated into 0.1 pmol of the linearized pBS plasmid overnight at 16 °C with T4 DNA ligase. The ligation reaction was then digested with *NheI* to reduce background colonies this reaction was used to transform *E. coli* DH5α competent cells; which were plated on 60 µg/ml Ampicillin LBA plates.

A digest with *StuI* was used to screen for the correct plasmids, with clones linearizing while empty plasmids remained uncut. The clones were further confirmed via sequencing.

2.2. pCambia 1300 clones

i. pTR:224bp

A region of the pBS:8K(insG<sup>80</sup>)-CP construct was amplified using PCR in order to produce a clone that contained mutations in both the 8kD and CP genes. A *KpnI* site was incorporated into the forward primer (L8K_F, Table 1) and a *NheI* site was incorporated into the reverse primer (8K_R, Table 1), so that the PCR products could be digested with *KpnI* and *NheI* to produce sticky ends. Concurrently, empty pCambia 1300 plasmids were digested
with KpnI and XbaI. The linearized plasmids and the digested amplicons were deproteinated and ligated together overnight at 16 °C with T4 DNA ligase. The sticky ends of the two KpnI sites (one located in the digested amplicon and one located in the linearized plasmid) ligated together, while the NheI site of the amplicon was ligated to the XbaI site of the linearized plasmid. Background plasmids were reduced by NheI digestion for 2 hours at 37 °C and this reaction was used to transform competent DH5α cells; which were plated on 50 µg/ml Kanamycin plates.

Potential clones were digested with NcoI and the resulting fragments were analyzed on a 1.5% agarose gel. Correct clones released a 776bp fragment, whereas empty pCambia plasmids released a 557bp fragment. pTR:224bp clones were further confirmed with sequencing and were used to transform tobacco plants.

ii. pTR:125bp

In order to investigate whether a limited portion of the PVX nucleotide sequence could function as an “RNA tractor”, a 125bp region of the PVX nucleotide sequence (including the last 102 nucleotides of the 8kD gene and the first 13 nucleotides of the CP gene) was amplified by PCR. A KpnI site was incorporated into the forward primer (L8K_F, Table 1) and a NheI site was incorporated into the reverse primer (8K_R, Table 1), so that the PCR products could be digested with KpnI and NheI to produce sticky ends.

Concurrently, empty pCambia 1300 plasmids were digested with KpnI and XbaI. The linearized plasmids and the digested amplicons were deproteinated and ligated together overnight at 16 °C with T4 DNA ligase. Background plasmids were reduced by NheI
digestion for 2 hours at 37 °C and this reaction was used to transform competent DH5α cells; which were plated on 50 µg/ml Kanamycin plates.

Potential clones were digested with NcoI and the resulting fragments were analyzed on a 1.5% agarose gel. Correct clones released a 677bp fragment, whereas empty pCambia plasmids released a 557bp fragment. pTR:125bp clones were further confirmed with sequencing and were used to transform tobacco plants.

**iii. pTR:27bp**

To further limit the PVX sequence involved in the translocation of PVX RNA into tobacco chloroplasts, a construct was created that contained only 5bp of the 8kD gene and 13bp of the CP gene. To achieve this, a double stranded oligonucleotide containing a KpnI site at the 5’ end and an XbaI site at the 3’ end was designed. The double stranded insert was produced by annealing the sense and antisense strands (PCR27 Sense and PCR27 Antisense, respectively, Table 1) by first boiling them for 5 minutes and then allowing them to cool to room temperature. Concurrently, empty pCambia plasmids were linearized using KpnI and XbaI. The double stranded oligonucleotide insert was then ligated into the linearized plasmid with T4 DNA ligase at 16 °C overnight. Background plasmids were reduced by KpnI digestion for 2 hours at 37 °C and this reaction was used to transform competent DH5α cells; which were plated on 50 µg/ml Kanamycin plates. Potential clones were digested with NcoI and the resulting fragments were analyzed on a 1.5% agarose gel. Correct clones released a 578bp fragment, whereas empty pCambia plasmids released a 557bp fragment. pTR:27bp clones were further confirmed with sequencing and were used to transform tobacco plants.
3. Production of Transgenic \textit{N. tabacum cv. Xanthi} and Expression Analyses

3.1. Agrobacterium-mediated stable transformation of \textit{N. tabacum cv. Xanthi}

A glycerol stock of agrobacteria strain GV3103 harboring the desired PVX construct in a modified version of the binary vector pCambia 1300, was used to inoculate 3 ml of LB in the presence of 30 µg/ml Gentamycin and 50 µg/ml Kanamycin. The culture was incubated overnight in a 28 °C shaker. On the following day, 300 µl of this culture was used to subculture 30 ml of LB with 30 µg/ml Gentamycin and 50 µg/ml Kanamycin; which was left to grow in a 28 °C shaker overnight once more.

On the day of transformation, 10-15 medium-sized \textit{N. tabacum cv. Xanthi} leaves were sterilized in 70% ethanol and rinsed with distilled water. The leaves were then placed in 1% bleach for 20 minutes. After the incubation period was over, the leaves were thoroughly rinsed with sterile ddH$_2$O to remove any residual bleach. In the fume hood, small circles were cut from the leaves to create bacterial-entry sites. The leaf discs were then incubated in the 30 ml agrobacteria cultures for 5 minutes. Once infected, the leaf discs were blotted dry on sterile filter paper and transferred to Petri plates of MS41 (4.4 g/L MS salts, 3% sucrose, 2 g MES, 1 mg/L 6-benzyl-aminopurine (BAP), 0.4 mg/L naphthalene acetic acid (NAA), and 9 g/L agar, pH 5.8). The Petri plates were incubated for 3 days under constant light at 24 °C. The leaf circles were then transferred to Petri plates with MS41a selection media (MS41, 400 µg/ml Carbenicillin and 20 µg/ml Hygromycin) to stimulate the formation of transgenic calli.

Approximately 3-4 weeks after the appearance of transgenic calli, primary shoots began to develop on the calli. These shoots were then transferred to sterile magenta boxes.
containing MS42a rooting media (2.2 g/L MS salts, 1.5% sucrose, 2 g MES, 0.9% agar, pH 5.8, 400 µg/ml Carbenicillin, and 20 µg/ml Hygromycin). The resulting plants were propagated by selecting a few leaves from the rooted plants and placing them on fresh MS41a media to induce the formation of new shoots. After propagation, the rooted plants were transferred to soil to grow in the green house. In order to ease the transition from growing in media in magenta boxes to growing in soil in the green house environment, humidity was supplemented for the first week by covering the plants with plastic wrap.

The successful transformation of transgenic plant lines were confirmed using both PCR analysis of plant chromosomal DNA and reverse transcription polymerase chain reaction (RT-PCR) analysis of total plant RNA. Various primers were used for these analyses and they are shown in Table 2. In the transgenic lines that were transformed with the empty pC1300 construct and the pC:8K construct, sGFP expression was also used to confirm the success of transformation.

3.2. Chromosomal DNA isolation from plant tissue

One plant leaf was sterilized with 70% ethanol and rinsed with distilled water. A small area of the plant leaf (5 cm²) was homogenized with a sterile bleach-treated mortar and pestle on ice. 500 µl of Extraction Buffer (250 mM Tris-HCl, pH 7.5, 250 mM sodium chloride, 25 mM EDTA, and 0.5% SDS) was added to the homogenate and the solution was transferred to a sterile 1.5 ml tube. The solution was thoroughly vortexed for 5 seconds and was subjected to centrifugation at 12 000 g for 1 minute at room temperature. The resulting supernatant was transferred to a new tube and was mixed with an equal amount of
isopropanol by vortexing. The solution was incubated at room temperature for 2 minutes and then pelleted by centrifugation at 12 000 g for 5 minutes at room temperature. The resulting pellet was washed once with 500 µl 70% ethanol and once with 500 µl 95% ethanol and left to air dry in the fume hood. The pellet was then dissolved in 30 µl of TE buffer (1 mM Tris, pH 8, and 0.1 mM EDTA) and stored at -20 °C. One µl of the extracted chromosomal DNA was used as a template for amplification by PCR in order to confirm the appropriate transformation of transgenic tobacco plants.

3.3. Total RNA extraction from plant tissue

_N. tabacum cv. Xanthi_ leaves were sterilized with 70% ethanol and rinsed with distilled water. The leaves were then ground into a fine powder with a sterile bleach-treated mortar and pestle in the presence of liquid nitrogen. The powder was then immediately transferred to a sterile DEPC treated 1.5 ml tube containing 250 µl phenol and 250 µl chloroform and vigorously vortexed. 500 µl of TES buffer (10 mM Tris-EDTA, and 0.1 M sodium chloride) was then added to the tube and the entire mixture was centrifuged at room temperature for 1 minute at 12 000 g. The supernatant was transferred to a new tube and washed twice with 1 ml chloroform each time. The supernatant was then transferred to a new tube and supplemented with 0.1M sodium acetate and 2.5 volumes ice-cold 95% ethanol and incubated overnight at -20 °C. On the following day, the RNA was pelleted by centrifugation at 4 °C for 20 minutes. The resulting pellet was washed once with 500 µl 70% ethanol and once with 500 µl 95% ethanol and left to air dry in the fume hood. Once all of the ethanol had evaporated, the pellet was dissolved in 20 µl of 10 mM Tris, pH 7.2, and stored at -20 °C.
3.4. Infection of *N. tabacum cv. Xanthi* with PVX and subsequent virus isolation

Inoculums of frozen tobacco leaves previously infected with the PVX virus were ground to a smooth paste with a mortar and pestle in the presence of 50 mM sodium phosphate buffer, pH 7.2. One medium-sized frozen PVX-infected leaf was used for every three plants to be infected. Once the plants reached the 4-leaf stage, carborundum powder was lightly sprinkled onto the cuticle of two leaves of each plant to be infected. With a gloved hand, the inoculum paste was gently applied to each leaf. After 20 minutes, the infected plants were washed to remove any residual inoculums and were kept under greenhouse conditions (23-26 °C and a 16 hour photoperiod). Fifteen days after the initial infection, the plants were ready to be harvested for virus isolation.

35 g of infected *N. tabacum cv. Xanthi* leaves were homogenized using a pre-chilled blender in 250 ml of grinding buffer (0.1 M Tris-borate, pH 7.5, and 0.25% B-mercaptoethanol). The homogenate was filtered through 4 layers of sterile cheesecloth on ice. Iso-butanol was added to the filtered homogenate to a final concentration of 6%. The mixture was constantly stirred on ice for 30 minutes and subjected to centrifugation at 12 000 g for 20 minutes at 4 °C. The supernatant was collected in a flask and the virus was precipitated on ice for 30 minutes with 8% PEG8000 and 2% sodium chloride. The mixture was then subjected to centrifugation at 12 000 g for 20 minutes at 4 °C. The pellets were left to dissolve overnight in a 4 °C refrigerator in a total of 7 ml of 0.1 M Tris-borate buffer, pH 7.2. The next morning, the dissolved pellets were subjected to centrifugation at 12 000 g for 5 minutes at 4 °C; which sedimented any leftover cell debris while the virus remained in the supernatant. The viral supernatant was collected in a sterile tube and crystals of sodium azide were added to the sample to prevent bacterial contamination. The purity of the viral
extraction was determined by taking optical density readings at OD\textsubscript{260} and OD\textsubscript{280} (the OD\textsubscript{260} /OD\textsubscript{280} ratio = 1.48). The virion concentration was determined by multiplying the OD\textsubscript{260} reading by the dilution factor used, assuming an extinction coefficient of 3 for 1mg/ml (purified virion preparations gave a yield of 4.6 mg/ml, or, 32.2 mg per 35 g of leaf tissue).

3.5. Isolation of Virion RNA

An aliquot of purified virions (250 µg) was incubated for 10 minutes at 37 °C in the presence of 0.1% SDS in order to dissociate the coat proteins. An equal volume of phenol was then added and the solution was thoroughly vortexed and subjected to centrifugation at 12 000 g for 5 minutes at 4 °C. The supernatant was transferred to a new tube and washed twice with 2 volumes cold chloroform. After the final chloroform wash, the aqueous top phase was transferred to a new tube and the viral RNA was precipitated with 100mM sodium acetate and 2.5 volumes 95% ethanol at -20 °C for 2 hours. The RNA was then pelleted by centrifugation at 12 000 g for 20 minutes at 4 °C. The resulting pellets were washed once with 500 µl 70% ethanol and once with 500 µl 95% ethanol and left to air dry in the fume hood. Once all of the ethanol had evaporated, the RNA pellet was dissolved in 15 µl of 10 mM Tris, pH 7.2, and stored at -20 °C. The isolated viral RNA was utilized in a reconstruction control for chloroplastic RNA isolation as well as a positive control for reverse transcription polymerase chain reaction (RT-PCR) analyses.
3.6. Chloroplast isolation from leaves and subsequent RNA extraction

Chloroplasts were isolated from transgenic, non-transformed and PVX-infected plants using the method described by Hefferon et al. (2000), illustrated in Figure 5.

Fifteen grams of *N. tabacum cv. Xanthi* leaves were harvested and sterilized by first rinsing with 70% ethanol followed by a thorough rinse with distilled water. The leaves were then homogenized in 15 ml of cold grinding buffer (50 mM HEPES-KOH, pH 7.3, 330 mM sorbitol, 0.1% BSA, 1 mM magnesium chloride, 2 mM Na$_2$EDTA, and 1 mM DTT) and the homogenate was filtered through a fine sieve. The solution was pelleted at 500 g for 1 minute at 4 °C to remove cellular debris and unbroken cells. The supernatant was then transferred to a new tube and centrifuged at 1200 g for 8 minutes at 4 °C for in order to pellet the chloroplasts. The chloroplast pellet was then resuspended in 1 ml of grinding buffer and overlaid on a 50% Ficoll gradient. The tubes were subjected to centrifugation at 13 000 g for 15 minutes and the ring of intact chloroplasts was transferred to a new tube containing 5 ml of washing buffer (50 mM HEPES-KOH, pH 8.0, and 330 mM sorbitol). The suspension underwent centrifugation once again at 1200 g for 8 minutes at 4 °C and all but 0.5 ml of the supernatant was discarded. RNase A (20 µg/ml) was then added to the solution and incubated for 30 minutes on ice in order to eliminate any externally associated cytoplasmic RNAs. The RNase treatment was followed by the addition of Proteinase K (400 µg/ml), which was also incubated on ice for 30 minutes in order to remove any residual proteins; including the RNase enzyme. The chloroplasts were then washed three times with washing buffer. The integrity of the purified chloroplasts was ascertained by observation under the confocal microscope. Chloroplastic RNA was extracted from purified chloroplasts using a phenol-chloroform extraction protocol (similar to page 42) and this RNA was analyzed by RT-PCR.
Filtered homogenate

Low speed centrifugation

Pellet contains cell debris

Low speed centrifugation of supernatant

Pellet contains chloroplasts

Pellet placed on Ficoll gradient

“ring” of intact chloroplasts

“ring” washed 3X with buffer

Pellet of purified chloroplasts

**Figure 5: Diagrammatic representation of the chloroplast isolation protocol.**

Tobacco leaves were homogenized and filtered through a fine sieve to remove any residual intact tissue. The homogenate was then subjected to centrifugation at low speed (500 g) to remove cell debris. The supernatant underwent centrifugation at a higher speed (1200 g) to sediment the chloroplasts. The pellet of chloroplasts was then subjected to ultracentrifugation (13 000 g) on 50% Ficoll; which separates the intact chloroplasts (located in the “ring”) from the broken chloroplasts (located in the smear above the “ring”). The “ring” of intact chloroplasts was then collected and washed 3 times with buffer to remove any residual Ficoll.
3.7. The treatment of RNA samples with DNase I

Prior to RT-PCR analysis, all RNA samples were treated with DNase I to remove any contaminant DNA from the RNA sample. The following reagents were added to a DEPC-treated RNase-free tube containing the RNA sample: 2.5 µl 10X reaction buffer (100mM Tris-HCl, pH 7.5, 25mM MgCl₂, and 1mM CaCl₂), 1 unit of the DNase I enzyme, 40 units of recombinant RNase inhibitor, and DEPC-treated ddH₂O to a final volume of 25 µl. The reaction was incubated at 37 °C for 30 minutes and was subsequently deproteinated by phenol-chloroform extraction prior to being used for RT-PCR analysis.

3.8. Reverse transcription (RT)-PCR

Samples of both total RNA and chloroplastic RNA were isolated from transformed, non-transformed and PVX-infected tobacco plants. This RNA was then reverse transcribed into cDNA, which was subsequently used as a template for PCR amplification.

1 µg of extracted RNA was mixed with 5 pmol of the desired antisense primer and sterile DEPC treated ddH₂O was added to a final volume of 30 µl. The mixture was boiled for 5 minutes and then cooled until the water bath reached 65 °C. The following reagents provided by Invitrogen were then added to each sample: 2.5 µl of 2.5mM dNTPs, 10 µl of 5X First Strand buffer, 5 µl of 0.1M DTT, 40 units of recombinant RNase inhibitor, and 200 units of Superscript II Reverse Transcriptase. The samples were then incubated at 40 °C for two hours. The resulting cDNA (1 µl) would serve as a template for PCR amplification.
RESULTS

1. Expression and Analyses of PVX Constructs

1.1. Sequence verification of previously constructed clones

In order to elucidate the roles of the PVX CP and 8kD proteins in the translocation of the viral RNA into tobacco chloroplasts, constructs were produced that had mutations in either the CP gene or the 8kD gene. As depicted in Figure 4, PVX sequences were cloned into modified pCambia 1300 binary vectors. Each construct was under the control of a CaMV 35S promoter to facilitate gene transcription, contained a gene coding for the sGFP protein, and was followed by a Tnos transcription terminator (Nopaline synthase terminator). All clones that were previously constructed by past graduate students were confirmed by digesting the plasmids with restriction enzymes and analyzing the resulting fragments on 1.5% agarose gels (Figure 6). The nucleotide sequences of these clones were further verified by sequencing using sequence specific primers (Figures 7 and 8). The confirmed plasmids were then used to transform *N. tabacum cv. Xanthi* plants.
Figure 6: A 1.5% agarose gel demonstrating the digestion of previously constructed PVX clones with NcoI.

Previously constructed clones were confirmed via enzymatic digestion with NcoI. **Lane B** contains the negative control (uncut pCambia plasmid). **Lane C** contains the positive control, (empty pCambia plasmid digested with NcoI, which releases a 557bp fragment). **Lane D** contains the pC:8K-CP construct digested with NcoI; which releases a 1.7kb fragment. **Lane E** contains the pC:8K(insG<sup>80</sup>)-CP construct digested with NcoI; which also releases a 1.7kb fragment. **Lane F** contains the pC:8K construct digested with NcoI; which releases a 900bp fragment. **Lane A** contains a 100bp DNA ladder.
| A) 5’ | ATG GAA GTA AAT ACA TAT CTC AAC GCA ATC ATA CTT GTG CTT GTG GTA ACA ATC ATA GCA GTC ATT AGT ACT TCC TTA GTG AGG ACT GAA CCT TGT GTC ATC AAG ATT ACT GGA GAA TCA ATC ACA GTG TTG GCT TGC AAG TTA GAT GCA GAA ACC ATC AGA GCC ATT GCC GAT CTC AAG CCA CTC TCC GTT GAA CGG TTA AGT TTC CAT TGA 3’ |
| B) 5’ | ATG GAA GTA AAT ACA TAT CTC AAC GCA ATC ATA CTT GTG CTT GTG GTA ACA ATC ATA GCA GTC ATT AGT ACT TCC TTA C[CG] GAG GAC TGA ACC TTG TGT CAT CAA GAT TAC TGG AGA ATC AAT CAC AGT GTT GGC TTG CAA GAT AGA TGC AGA AAC CAT CAG AGC CAT TGC CGA TCT CAA GCC ACT CTC CGT TGA ACG GTT AAG TTT CCA TTG A 3’ |

**Figure 7: A comparison of the sequences of the 8kD gene from clones pC:8K-CP and pC:8K(insG<sup>80</sup>)-CP.**

A) The nucleotide sequence of the 8kD gene from clone pC:8K-CP

B) The nucleotide sequence of the mutated 8kD gene from clone pC:8K(insG<sup>80</sup>)-CP; which was produced by digesting the pC:8K-CP clone with EcoNI, filling in the 5’ overhang with a Klenow fragment, and ligating the plasmid back together. The resulting G insertion (highlighted in yellow) produced a frameshift mutation in the 8kD protein. The start (ATG) and stop (TGA) codons are underlined and the EcoNI restriction site is highlighted in purple.
Figure 8: The pC:8K nucleotide sequence demonstrating the CP truncation and CP-sGFP fusion.

The entire 8kD sequence was inserted into a modified pC1300 vector (Figure 16). The first 13 nucleotides of the CP gene are also present within this construct, and the ATG of the CP gene is in frame with the ATG of the sGFP ORF, producing a CP-sGFP fusion protein (indicated by the amino acids M, S, A, P, A, R, G, S, M). The start (ATG) codon of each gene is underlined and the position of the CP truncation is indicated by a slash.
1.2. Construction of pBS clones

To clarify the roles of the PVX CP and 8kD proteins in the translocation of PVX RNA into chloroplasts, pBS constructs that had mutations in both the 8kD and CP genes were produced. All mutations were introduced into a previously constructed clone, pBS:8K(insG$^{80}$)-CP (Figure 9), that already had an extra G inserted into the 80$^{th}$ nucleotide position of the 8kD gene, placing the 8kD protein out of frame. Thus, all of the pBS clones contained a CaMV 35S promoter at the 5’ end for gene expression, a GFP gene, and a Tnos transcription terminator at the 3’ end. Potential clones were digested with restriction enzymes and the resulting fragments were analyzed on agarose gels. The nucleotide sequences of potential clones were further verified by sequencing using sequence specific primers.
Figure 9: Schematic representation of the previously constructed pBS:8K(insG^80)-CP clone.
The pBS:8K(insG^80)-CP construct has the insertion of an extra G in the 80^th nucleotide position of the 8kD gene (represented by the “8k”); producing a frameshift mutation in the resulting 8kD protein. Two strategies were used to mutate the CP gene in this clone: A) The pBS:8K(insG^80)-CP plasmid was digested with *NheI* (releasing a 129bp fragment), blunt ended with a Klenow fragment, and the two blunt ends were ligated together. Upon analysis of the resulting nucleotide sequence, it was determined that this method failed to mutate the CP. B) The pBS:8K(insG^80)-CP plasmid was also digested with *NheI*, and a double stranded oligonucleotide insert was ligated into the digestion site. This method produced a frameshift mutation in the CP.
i) \( pBS:8K(insG^{80})-CP(\Delta^{18-146}) \)

To create a construct with both the 8kD and CP genes mutated, the \( pBS:8K(insG^{80})-CP(\Delta^{18-146}) \) construct was digested with \( NheI \), filled with a Klenow fragment and the blunt ends of the vector were ligated together again. A \( SalI \), \( KpnI \) and \( NheI \) triple digest was used to screen for potential \( pBS:8K(insG^{80})-CP(\Delta^{18-146}) \) clones (Figure 10). Since the two \( NheI \) sites (located within the CP gene) would be destroyed by the Klenow-filling, potential clones would release an 850bp fragment (containing the 35S promoter) and an intact 1250bp fragment (containing the PVX sequence). In contrast, the original \( pBS:8K(insG^{80})-CP \) construct would retain the \( NheI \) restriction sites, and the triple digest of these constructs would cut the 1250bp PVX fragment into three pieces: a 129bp fragment, a 430bp fragment, and a 700bp fragment. Potential clones were sequence-verified and the resulting nucleotide sequence (Figure 11) was translated into the corresponding amino acid sequence (Figure 12). Although the digestion of \( pBS:8K(insG^{80})-CP \) with \( NheI \) and the subsequent Klenow filling led to the deletion of nucleotides 18-146 in the PVX CP gene, the CP failed to be put out of frame.
Figure 10: A 1.5% agarose gel demonstrating the triple digestion of pBS:8K(insG^{80})-CP(Δ^{18-146}) with SalI, KpnI, and Nhel.

To generate the pBS:8K(insG^{80})-CP(Δ^{18-146}) clone, the pBS:8K(insG^{80})-CP construct was digested with Nhel (releasing a 129bp fragment), filled with a Klenow fragment and the blunt ends of the vector were ligated together. A SalI, KpnI and Nhel triple digest was used to screen for potential pBS:8K(insG^{80})-CP(Δ^{18-146}) clones. Lanes A, B and D contain potential clones. The 1250bp fragment containing the mutated 8kD and CP ORFs remained uncut because the Nhel site was destroyed when the digestion site was filled with the Klenow fragment. In contrast, the Nhel site remained intact in the pBS:8K(insG^{80})-CP construct, thus when this plasmid was digested with the three enzymes, the 1250bp fragment was cut into three fragments of approximately 129bp (not shown), 430bp, and 700bp. An additional fragment containing the 35S CaMV promoter (approximately 850bp) can also be seen on this gel. Lane C contains a 100bp DNA ladder.
Figure 11: pBS:8K(insG\textsuperscript{80})-CP(Δ\textsuperscript{18-146}) nucleotide sequence demonstrating the 8kD and CP mutation sites.

The pBS:8K(insG\textsuperscript{80})-CP(Δ\textsuperscript{18-146}) clones were further verified by sequencing. The above nucleotide sequence illustrates the original G insertion in the 80\textsuperscript{th} position of the 8kD ORF (highlighted in pink). Also, the deletion of nucleotides 18-146 is indicated by a slash. The start (ATG) codon of each gene is underlined.
A) 5’ MetSAPAS/TTQATGSTTSTTTKTAGATPATASGLFTTPDGDFSTARAIVA/SNAVATNEDSLKIEAIWKDMKVTDTMAQAAWDLVRHCADVGSSAQTEMIDTGPYSNGISRARLAAAIKEVCTLRLQFCMKYYAPVWNWMILTNNSPPANWQAQGFKEHKFAAFDFFNGVTNPAAIMPKEGLIRPPSEAEMNAAQTAAVAFTVKITKXRAQSNDFASTDAAVTRGRITGGTAAELLXLSHHITTSSTOP3’

B) 5’ MetSAPAS/SNAVATNEDSLKIEAIWKDMKVPTDTMAQAAWDLVRHCADVGSSAQTEMIDTGPYSNGISRARLAIAIKEVCTLRLQFCMKYYAPVWNWMILTNNSPPANWQAQGFKEHKFAAFDFFNGVTNPAAIMPKEGLIRPPSEAEMNAAQMSAPASRPYVSY3’

Figure 12: Protein translation of the pBS:8K(insG80)-CP(Δ18-146) nucleotide sequence, as compared to the original CP amino acid sequence.
The pBS:8K(insG80)-CP(Δ18-146) nucleotide sequence was translated into a protein sequence using the ExPASy translation tool. A) The amino acid sequence of the original CP is depicted here. The amino acids that were deleted in the pBS:8K(insG80)-CP(Δ18-146) clone are highlighted in yellow. B) The amino acid sequence derived from the pBS:8K(insG80)-CP(Δ18-146) clone is depicted here, with the deleted amino acids represented by a slash.
ii) pBS:8K(insG\textsuperscript{80})-CP(ins\textsuperscript{14-35}S13X)

In a second attempt to create a construct with both the 8kD and CP genes mutated, the pBS:8K(insG\textsuperscript{80})-CP construct was again digested with \textit{N}heI and a double stranded oligonucleotide (CPMut Sense and CPMut Antisense, \textbf{Table 1}) was ligated into the digestion site. A \textit{Stu}I digest was used to screen for potential pBS:8K(insG\textsuperscript{80})-CP(ins\textsuperscript{14-35}S13X) clones (\textbf{Figure 13}). Since a \textit{Stu}I site was incorporated into the double stranded oligonucleotide, clones that had the insert ligated into them were linearized by a \textit{Stu}I restriction digest. The original pBS:8K(insG\textsuperscript{80})-CP construct, however, does not possess a \textit{Stu}I site and remained uncut after this restriction digest. Potential clones were then sequence-verified and the resulting nucleotide sequence (\textbf{Figure 14}) was translated into the corresponding amino acid sequence. As seen in \textbf{Figure 15}, the insertion of the double stranded oligonucleotide introduced a premature stop codon in the 13\textsuperscript{th} amino acid position of the CP.
Figure 13: A 1.5% agarose gel of potential pBS:8K(insG^{80})-CP(ins^{14-35}S13X) clones digested with StuI.

To generate the pBS:8K(insG^{80})-CP(ins^{14-35}S13X) clone, the pBS:8K(insG^{80})-CP construct was digested with NheI and an double stranded oligonucleotide insert was ligated into the digestion site. This insertion introduced a premature stop codon into the CP ORF. A StuI digest was used to screen for potential pBS:8K(insG^{80})-CP(ins^{14-35}S13X) clones. Lanes A, C, D, E, F, G, H, I, K, L, M, N, O, and Q contain the pBS:8K(insG^{80})-CP construct cut with StuI. Since this construct lacks a StuI site, the vector remains uncut. Lanes B, J, and P contain potential clones, which possess a StuI site due to the inserted double stranded oligonucleotide and are linearized by this digest.
Figure 14: pBS:8K(insG\textsuperscript{80})-CP(ins\textsuperscript{14-35}S13X) nucleotide sequence demonstrating the 8kD and CP mutation sites. The pBS:8K(insG\textsuperscript{80})-CP(ins\textsuperscript{14-35}S13X) clones were further verified by sequencing. The above nucleotide sequence illustrates the original G insertion in the 80\textsuperscript{th} position of the 8kD ORF (highlighted in pink). Also, the sequence of the oligonucleotide insert is highlighted in red and the \textit{StuI} restriction site located within this insert is capitalized. The start (ATG) codon of each gene is underlined and the premature stop (TAG) codon of the CP is indicated in bold.
A) 5’  Met S A P A S T Q A T G S T T S T T K T A G A T P A T A S G L F T I P D
GDFFSTARAVASNAV AT NEDLSKIEAIWKDMKVPTD
TMAQA AWDLVRHCADVGSSAQTEMIDTPYSN GIS R
ARLAAAIKEVCRLQFCCMKYAPVWNVWLMTNNSPPA
NWQAQGFKPEHKFAAAFFFGNVTNPAAIMPKEGLIRP
PSEAEMNAAQTAAFVKITKXRAQSDFASLDAAVTRG
RITGTTTAELLXLSHHITTST Stop 3’

B) 5’  Met S A P A S R P Y V S Y Stop  3’

Figure 15: Protein translation of the pBS:8K(insG\textsuperscript{80})-CP(ins\textsuperscript{14-35}S13X) nucleotide sequence, as compared to the original CP amino acid sequence. The pBS:8K(insG\textsuperscript{80})-CP(ins\textsuperscript{14-35}S13X) nucleotide sequence was translated into its corresponding amino acid sequence using the ExPASy translation tool. A) The amino acid sequence of the original CP. B) The amino acid sequence derived from the pBS:8K(insG\textsuperscript{80})-CP(ins\textsuperscript{14-35}S13X) clone, illustrating the introduction of a premature stop codon in the 13\textsuperscript{th} amino acid position of the protein.
1.3. Construction of pCambia 1300 pTractor (pTR) clones

In order to investigate the role of the PVX nucleotide sequence in the translocation of its own RNA into tobacco chloroplasts, three constructs containing different portions of the 8kD and CP genes were produced (Figure 17). PCR was used to amplify regions of the pBS:8K(insG$^{80}$)-CP clone to produce double stranded DNA inserts. Each construct was cloned into a modified version of the pCambia 1300 binary vector (Figure 16) using KpnI and XbaI restriction sites. Thus, all clones contain a 35S CaMV promoter for the initiation of transcription, a gene for sGFP, and a Tnos transcription terminator. Potential clones were identified by digesting plasmids with NcoI and the nucleotide sequences of these clones were further confirmed by sequencing using a sGFP reverse (sGFP_R, Table 1) primer. The confirmed plasmids were then used to transform N. tabacum cv. Xanthi plants.

i) pTR:224bp

In order to produce a binary plasmid that contained both the 8kD and CP genes mutated, a region of the PVX sequence was amplified by PCR using L8K_F and 8K_R primers (Table 1). The resulting PCR amplicons were digested with KpnI and NhelI and were ligated into empty pCambia plasmids that had been digested with KpnI and XbaI. Potential clones were digested with NcoI and released a fragment of approximately 776bp, whereas empty pCambia plasmids released a 557bp fragment (Figure 18). The desired clones contained 224bp of the PVX sequence, including the last 201 nucleotides of the 8kD gene and first 13 nucleotides of the CP gene; which was out of frame with the ATG of the sGFP gene. Correct clones were confirmed with sequencing (Figure 19) and were used to transform N. tabacum cv. Xanthi plants.
Figure 16: Schematic representation of the modified pCambia 1300 plasmid.
The original pCambia 1300 plasmid was modified by Aatir Butt (2006); who removed the original promoter and replaced it with a cauliflower mosaic virus (CaMV) 35S promoter that had unique KpnI and XbaI sites downstream from it. All of the pCambia 1300 pTR constructs were cloned into this modified binary vector in order to transform tobacco plants. sGFP: synthetic green fluorescent protein. Tnos: nopaline synthase terminator.
Figure 17: Schematic representation of the pTR clones in modified pC1300 plasmids. PCR inserts were cloned into the modified version of pCambia 1300 binary vector using KpnI and XbaI, and the constructs were used to transform tobacco plants. A) pTR:224bp: containing 224bp of the PVX sequence, including 201 nucleotides from the 8kD gene and 13 nucleotides from the CP gene. B) pTR:125bp: containing 125bp of the PVX sequence, including 102 nucleotides from the 8kD gene and 13 nucleotides from the CP gene. C) pTR:27bp: containing 27bp of the PVX sequence, including 5 nucleotides from the 8kD gene and 13 nucleotides from the CP gene. 35S: CaMV 35S promoter. sGFP: synthetic green fluorescent protein. Tnos: Nopaline synthase terminator.
Figure 18: A 1.5% agarose gel demonstrating the digestion of pTR:224bp with NcoI. PCR products containing a portion of the PVX 8kD and CP genes were cloned into a modified version of the pCambia 1300 vector using KpnI and XbaI restriction sites. Potential clones were expected to release a 776bp fragment (Lanes D, E, and F), while empty pCambia plasmids released a 557bp fragment (Lane C). Uncut pCambia plasmid can be seen in Lane B. Lane A contains a 100bp DNA ladder.
Figure 19: The pTR:224bp nucleotide sequence illustrating the annealing locations of the L8K_F and 8K_R PCR primers.

The nucleotide sequence of the pTR:224bp clone was further verified by sequencing. The above nucleotide sequence illustrates the original G insertion in the 80th position of the 8kD ORF (highlighted in yellow) as well as the locations of the forward (L8K_F, Table 1) and reverse (8K_R, Table 1) primers used to amplify the PCR product insert. The start (ATG) codon of each gene is underlined and the inactivated XbaI site is highlighted in red. Also, the intact KpnI site used to clone in the PCR fragment is highlighted in blue.
ii) pTR:125bp

In order to examine the possible existence of a RNA signal sequence within the PVX RNA, limited regions of the 8kD and CP genes were amplified by PCR using S8K_F and 8K_R primers (Table 1), digested with KpnI and Nhel, and ligated into empty pCambia 1300 plasmids that had been linearized with KpnI and XbaI. When digested with NcoI, potential clones released a 677bp, whereas empty pCambia plasmids released a 557bp fragment (Figure 20). The desired clones contained 125bp of the PVX sequence, including the last 102 nucleotides of the 8kD gene and the first 13 nucleotides of the CP gene; which was out of frame with the ATG of the sGFP gene. Correct clones were confirmed with sequencing (Figure 21) and were used to transform N. tabacum cv. Xanthi plants.

iii) pTR:27bp

The region of the PVX RNA that could be acting as the “RNA tractor” was further investigated by producing a clone that contains only small portions of the 8kD and CP genes. A double stranded oligonucleotide containing 27bp of the PVX sequence, including the last 5 nucleotides of the 8kD gene and the first 13 nucleotides of the CP gene, was cloned into empty pCambia 1300 plasmids. Potential clones were digested with NcoI and released a 578bp fragment, whereas empty pCambia plasmids released a 557bp fragment (Figure 22). Correct clones were sequence-verified (Figure 23) and used to transform N. tabacum cv. Xanthi plants.
Figure 20: A 1.5% agarose gel demonstrating the digestion of pTR:125bp with *NcoI*. PCR products containing limited regions of the PVX 8kD and CP genes were cloned into a modified version of the pCambia 1300 vector using *KpnI* and *XbaI* restriction sites. Potential clones were expected to release a 677bp fragment (**Lanes B, C, and D**), while empty pCambia plasmids released a 557bp fragment (**Lane A**). **Lane E** contains a 100bp DNA ladder.
Figure 21: The pTR:125bp nucleotide sequence illustrating the annealing locations of the S8K_F and 8K_R PCR primers.
The nucleotide sequence of the pTR:125bp clone was further verified by sequencing. The above nucleotide sequence illustrates the locations of the forward (S8K_F, Table 1) and reverse (8K_R, Table 1) primers used to amplify the PCR product insert. The start (ATG) codon of each gene is underlined and the inactivated \textit{Xba}I site is highlighted in red. Also, the intact \textit{Kpn}I site used to clone in the PCR fragment is highlighted in blue.
Figure 22: A 1.5% agarose gel demonstrating the digestion of pTR:27bp with NcoI. A double stranded oligonucleotide insert containing 27 nucleotides of the PVX sequence was cloned into a modified version of the pCambia 1300 vector using KpnI and XbaI restriction sites. Potential clones were expected to release a 578bp fragment (Lanes B, C, and D), while empty pCambia plasmids released a 557bp fragment (Lane A). Lane E contains a 100bp DNA ladder.
Figure 23: The pTR:27bp nucleotide sequence demonstrating the ligation of the synthesized double stranded oligonucleotide insert.
The nucleotide sequence of the pTR:27bp clone was further verified by sequencing. The nucleotide sequence of the double stranded oligonucleotide insert is indicated with upper case letters. The start (ATG) codon of each gene is underlined and the intact \textit{KpnI} and \textit{XbaI} restriction sites used to clone in the double stranded oligonucleotide are highlighted in blue and red, respectively.
2. Production of Transgenic *N. tabacum* cv. *Xanthi* and Expression analyses

2.1. Agrobacterium-mediated stable transformation of *N. tabacum* cv. *Xanthi* plants

Four week old *N. tabacum* cv. *Xanthi* leaf discs were transformed using *A. tumefaciens* strain GV3103 harbouring the following constructs: empty pCambia, pC:8K-CP, pC:8K(insG$_{80}$)-CP, pC:8K, pTR:224bp, and pTR:125bp. A total of 73 lines of transgenic plants were produced. Successful transformation of the tobacco plants was confirmed in at least 2 lines of plants for every construct by PCR analyses of chromosomal DNA and RT-PCR of total RNA.

2.2. Confirmation of transformation by PCR

The successful transformation of transgenic tobacco plants was confirmed by using PCR to detect the presence of the 8kD and CP genes in chromosomal DNA isolated from these plants.

Three different sets of primers were utilized to confirm the transgenic plants. For plants transformed with constructs pC:8K-CP, pC:8K(insG$_{80}$)-CP, and pC:8K, chromosomal DNA was analyzed using primers for both the 8kD gene (L8K_F and 8K_R, Table 1) and the CP gene (CP_F and CP_R, Table 2). When primers for the 8kD gene were used, the expected 255bp PCR product was detected in each of the three types of transformed plants. However, when primers were used for the CP gene, the expected 350bp PCR products were only detected in plants transformed with constructs pC:8K-CP and pC:8K(insG$_{80}$)-CP, but not pC:8K (Figure 24).
Plants transformed with constructs pTR:224bp and pTR:125bp were confirmed using S8K_F and 8K_R (Table 1) primers for the 8kD gene. The expected 156bp PCR product was detected in both sets of plants (Figure 25). Bands were not detected when template DNA was isolated from nontransformed plants, or from plants transformed with the empty pCambia 1300 plasmid.

### 2.3. Confirmation of transformation by RT-PCR

Transgenic tobacco plants were also confirmed by analyzing the RT-PCR products derived from total plant RNA. Prior to RT-PCR analyses, all RNA samples were treated with DNase I in order to eliminate any contaminating chromosomal DNA within the sample. When these DNase I treated RNA samples were used directly as templates for PCR, no PCR products were observed. Thus, it was concluded that the DNase I treatment was effective (Figure 26).

Total RNA was isolated from plants transformed with pC:8K-CP, pC:8K(insG^80)-CP, pC:8K, pTR:224bp, and pTR:125bp constructs. RNA from plants transformed with constructs pC:8K-CP, pC:8K(insG^80)-CP, and pC:8K was detected using L8K_F and 8K_R primers (Table 1), and the expected 255bp fragment was observed for these samples (Figure 27). RNA from plants transformed with constructs pTR:224bp and pTR:125bp was detected using S8K_F and 8K_R primers (Table 1), and the expected 156bp band was also observed for these samples (Figure 28). Total RNA from untransformed plants was also subjected to RT-PCR, however no bands were seen for this sample. Plasmid DNA was used as a positive control for the PCR reaction, while PVX RNA was used as a positive control for the RT reaction.
Chromosomal DNA was isolated from plants transformed with the pC:8K-CP, pC:8K(insG<sup>80</sup>)-CP, and pC:8K constructs and PCR was conducted to ascertain the presence of both the 8kD and CP genes within the plant genomes. Primers specific to the 8kD gene (L8K_F and L8K_R, 255bp fragment, Table 1) and to the CP gene (CP_F and CP_R, 350bp fragment Table 2) were used to produce the PCR amplicons. When primers for the 8kD gene were used, the expected 255bp PCR product was detected in lanes from all three transgenic plants: pC:8K-CP (Lane G), pC:8K(insG<sup>80</sup>)-CP (Lane I), and pC:8K (Lane K). However, when primers were used for the CP gene, the 350bp product was only detected in lanes containing samples from plants transformed with pC:8K-CP (Lane H), and pC:8K(insG<sup>80</sup>)-CP (Lane J), and not pC:8K (Lane L). PCR products were also detected when plasmid DNA was used as a positive control (Lane A). However, the PCR products were not detected in samples from nontransformed plants using either the 8kD primers (Lane B) or the CP primers (Lane C). Also, the PCR products were not detected in samples from plants transformed with the empty pCambia 1300 plasmid using either the 8kD primers (Lane D) or the CP primers (Lane E). Lane F contains a 100bp DNA ladder.
Figure 25: PCR products of chromosomal DNA isolated from plants transformed with constructs pTR:224bp and pTR:125bp.

Chromosomal DNA was isolated from plants transformed with the pTR:224bp and pTR:125bp constructs and PCR was conducted to ascertain the presence of the 8kD gene within the plant genomes using primers specific to this gene (S8K_F and 8K_R, Table 1). The expected 156bp PCR product was detected in both the pTR:224bp (Lane E) and pTR:125bp (Lane F) samples. PCR products were also detected when plasmid DNA was used as a positive control for PCR (Lane A). However, the 156bp product was not detected in samples from nontransformed plants (Lane B) or from plants transformed with the empty pCambia 1300 plasmid (Lane C). Lane D contains a 100bp DNA ladder.
Figure 26: A 2% agarose gel demonstrating the PCR and RT-PCR products of DNase I treated RNA.

All RNA samples were treated with DNase I prior to being used as templates for cDNA synthesis. When DNase-treated total (Lane E) and chloroplastic (Lane C) RNA samples derived from pC:8K-CP plants were used directly as templates for PCR, the expected 255bp fragment was not observed. These bands were observed when the total (Lane F) and chloroplastic (Lane D) RNA samples were first transcribed into cDNA and subsequently amplified by PCR. The band was also observed when plasmid DNA was used as a template for PCR (Lane A). Lane B contains a 100bp DNA ladder.
Figure 27: RT-PCR products of total RNA isolated from plants transformed with constructs pC:8K-CP, pC:8K(insG^{80})-CP, and pC:8K.

Total RNA isolated from plants transformed with constructs pC:8K-CP, pC:8K(insG^{80})-CP, and pC:8K was reverse transcribed into cDNA and was amplified by PCR, using primers specific to the 8kD gene sequence (L8K_F and 8K_R, Table 1). The resulting PCR products were analyzed on a 2% agarose gel and the expected 255bp PCR fragment was detected in lanes containing pC:8K-CP (Lane F), pC:8K(insG^{80})-CP (Lane G), and pC:8K (Lane H) RT-PCR products. PCR products were also detected when plasmid DNA was used as a positive control for PCR (Lane A) and when PVX RNA was used as a positive control for RT-PCR (Lane B). The 255bp product was not detected in samples from nontransformed plants (Lane C) or from plants transformed with the empty pCambia 1300 plasmid (Lane D); which were used as negative controls. Lane E contains a 100bp DNA ladder.
Figure 28: RT-PCR products of total RNA isolated from plants transformed with constructs pTR:224bp and pTR:125bp.

Total RNA isolated from plants transformed with constructs pTR:224bp and pTR:125bp was reverse transcribed into cDNA and was amplified by PCR, using primers specific to the 8kD gene sequence (S8K_F and 8K_R, Table 1). The resulting PCR products were analyzed on a 2% agarose gel and the expected 156bp PCR fragment was detected in lanes containing pTR:224bp (Lane F) and pTR:125bp (Lane G) RT-PCR products. PCR products were also detected when plasmid DNA was used as a positive control for PCR (Lane A) and when PVX RNA was used as a positive control for RT-PCR (Lane B). The 156bp product was not detected in samples from nontransformed plants (Lane C) or from plants transformed with the empty pCambia 1300 plasmid (Lane D). Lane E contains a 100bp DNA ladder.
2.4. Comparison of sGFP expression in both nontransformed and transgenic tobacco leaves

sGFP expression levels from both nontransformed and transgenic tobacco leaves were observed under a confocal microscope using two different filters (Figures 29 and 30). Images captured using a long pass filter that blocks all wavelengths below 585 nm (allowing chlorophyll fluorescence but blocking sGFP fluorescence) showed that all of the leaf samples were intact and healthy. Images captured using a GFP green filter that blocks all wavelengths that do not fall within the 505-530 nm range (allowing sGFP fluorescence but blocking chlorophyll fluorescence), showed areas of sGFP expression within plants cells. While all samples clearly demonstrated chlorophyll fluorescence, sGFP expression was only seen in the positive control (pCambia 1300 transgenic leaves, where sGFP expression is under the control of a CaMV 35S promoter) and to a lesser extent in pC:8K leaves; where sGFP expression is believed to be controlled by an IRBS located upstream of the CP gene (Figure 29). sGFP expression was not observed in samples of nontransformed leaves or in plants transformed with constructs pTR:224bp and pTR:125 bp; which have the AUG of the CP out of frame with the AUG of the sGFP (Figure 30).

2.5. Confocal imaging of isolated chloroplasts

Chloroplasts were isolated from nontransformed tobacco leaves and were observed under a confocal microscope using a long pass filter (blocking any light below 585nm). Images from isolated chloroplasts were compared to confocal images from intact untransformed leaves under the same conditions (Figure 31). Isolated chloroplasts were determined to be healthy and intact and were used for further analyses.
Figure 29: Confocal images demonstrating sGFP expression in both empty pCambia and p:8K transgenic leaves.

Tobacco leaves from nontransformed plants (panels A and B) as well as from plants transformed with empty pCambia 1300 constructs (panels C and D), and pC:8K constructs (panels E and F) were visualized using a confocal microscope under two different filters at 10X magnification. The images on the left were captured using a long pass filter that blocks all wavelengths below 585 nm, allowing the visualization of chlorophyll fluorescence but blocking sGFP fluorescence. The images on the right were captured using a GFP green filter that blocks all wavelengths that do not fall within the 505-530 nm range, allowing the visualization of sGFP fluorescence but blocking chlorophyll fluorescence. While all samples clearly demonstrated chlorophyll fluorescence (panels A, C and E), sGFP was seen in the positive control (pCambia 1300 transgenic leaves seen in panel D, where sGFP expression is under the control of a CaMV 35S promoter) and to a lesser extent in pC:8K leaves (panel F), where sGFP expression is believed to be controlled by an IRBS located upstream of the CP gene. No sGFP expression was seen in the nontransformed leaves (panel B).
Figure 30: Confocal images demonstrating the lack of sGFP expression in both pTR:224bp and pTR:125bp transgenic leaves.
Tobacco leaves transformed with the constructs pTR:224bp (panels A and B) and pTR:125bp (panels C and D) were visualized using a confocal microscope under two different filters at 10X magnification. The images on the left were captured using a long pass filter that blocks all wavelengths below 585 nm, allowing the visualization of chlorophyll fluorescence but blocking sGFP fluorescence. The images on the right were captured using a GFP green filter that blocks all wavelengths that do not fall within the 505-530 nm range, allowing the visualization of sGFP fluorescence but blocking chlorophyll fluorescence. While both samples clearly demonstrated chlorophyll fluorescence (panels A and C), sGFP expression was not observed in either sample (panels B and D).
Figure 31: Confocal images of intact chloroplasts before and after isolation from *N. tabacum cv. Xanthi* leaves.
Chloroplasts from both intact nontransformed leaves (panel A) and a sample of purified chloroplasts (panel B) were observed using a long pass filter (blocking any light below 585nm) under the confocal microscope at 40X magnification. Isolated chloroplasts were determined to be intact and were used for further analyses.
2.6. Analyses of Total and Chloroplastic RNA of PVX-infected tobacco plants

Total and chloroplastic RNA was isolated from leaves of plants infected with PVX. The RNA was reverse transcribed using a CP_R primer (Table 2) and the cDNA was amplified by PCR using CP_F and CP_R primers (Table 2). Total and chloroplastic RNAs were also isolated from nontransformed plants and the RT-PCR products derived from these samples were used as negative controls. PVX RNA and plasmid DNA were used as positive controls for the RT and PCR reactions, respectively. Additionally, a reconstruction control was produced by incubating chloroplasts isolated from nontransformed plants with 1μg of PVX RNA on ice for 15 minutes and subsequently treating those chloroplasts with RNase A and Proteinase K prior to chloroplastic RNA extraction.

The RT-PCR products were analyzed on a 2% agarose gel (Figure 32) and the expected 350bp fragment was detected in lanes containing samples of both total and chloroplastic RNA from PVX-infected plants. Bands were also seen in lanes with the plasmid DNA and PVX RNA positive controls. RT-PCR products were not detected in lanes containing total and chloroplastic RNA from untransformed plants, nor were they detected in the lane containing the reconstruction control.
Figure 32: A 2% agarose gel demonstrating the presence of PVX RNA in both total and chloroplastic RNA samples from PVX-infected tobacco leaves.

Total and chloroplastic RNA was isolated from plants infected with the PVX virus. This RNA was subjected to RT-PCR analysis using primers corresponding to the CP gene (CP_F and CP_R, Table 2). The resulting RT-PCR products were analyzed on a 2% agarose gel and the expected 350bp PCR fragment was detected in lanes containing reactions that derived their RNA templates from both total RNA (Lane G) and chloroplastic RNA (Lane H) isolated from PVX infected plants. The PCR product was also detected when plasmid DNA was used as a positive control for PCR (Lane A) and when PVX RNA was used as a positive control for RT-PCR (Lane B). The 350bp product was not detected in samples using either total (Lane C) or chloroplastic (Lane D) RNA from uninfected plants as a template. Also, the PCR product was not detected in the reconstruction control (Lane E), where chloroplasts isolated from nontransformed plants were incubated in PVX RNA, and subsequently treated with RNase A and Proteinase K prior to chloroplastic RNA extraction. Lane F contains a 100bp DNA ladder.
2.7. The determination of sGFP RNA localization within tobacco cells

To eliminate the possibility that any cytoplasmic RNA can diffuse into the chloroplast and be detected by RT-PCR, total and chloroplastic RNA was isolated from nontransformed plants, as well as from plants transformed with empty pCambia 1300 plasmids and pC:8K constructs. This RNA was subjected to RT-PCR analysis using primers corresponding to the sGFP gene (sGFP3_R for the production of the cDNA and sGFP1_F and sGFP2_R for PCR amplification, Table 2 and Figure 33). The resulting RT-PCR products were analyzed on a 2% agarose gel (Figure 34) and the expected 438bp PCR fragment was detected in lanes containing RT-PCR products from both total and chloroplastic RNA isolated from pC:8K transgenic plants. PCR products were also detected when plasmid DNA was used as a positive control. The 438bp product was not detected in samples of total or chloroplastic RNA from nontransformed plants. However, RT-PCR detected the presence of the sGFP RNA in total RNA samples from empty pC:1300 transgenic plants (where the sGFP gene is under the control of a CaMV 35S promoter), but not in chloroplastic RNA samples from these same plants. Confocal microscopy was also used to localize the sGFP protein within leaves harvested from plants transformed with empty pCambia 1300 plasmids (Figure 35). Chloroplasts within the tissue sample were visualized using a long pass filter that blocks all wavelengths below 585 nm (showing chlorophyll fluorescence) and a GFP green filter; which blocks all wavelengths that do not fall within the 505-530 nm range (showing sGFP fluorescence). A composite image was then created to elucidate the regions where chlorophyll fluorescence and sGFP expression overlap; which demonstrated a lack of sGFP expression in the chloroplasts. sGFP expression was also not seen in samples of isolated chloroplasts (Figure 36).
Figure 33: The sGFP nucleotide sequence illustrating the annealing locations of the sGFP1_F, sGFP2_R, and sGFP3_R primers.
The annealing locations of sGFP1_F (highlighted in red), sGFP2_R (highlighted in yellow) and sGFP3_R (highlighted in blue) are illustrated in the above nucleotide sequence. The start codon (ATG) of the sGFP gene is underlined.
Figure 34: A 2% agarose gel demonstrating the localization of sGFP RNA in both nontransformed plants and plants transformed with empty pCambia plasmid and pC:8K constructs.

Total and chloroplastic RNA was isolated from nontransformed plants as well as from plants transformed with empty pCambia 1300 constructs and pC:8K constructs. This RNA was subjected to RT-PCR analysis using primers corresponding to the sGFP gene (sGFP3_R for the production of the cDNA and sGFP1_F and sGFP2_R for PCR amplification, Table 2). The resulting RT-PCR products were analyzed on a 2% agarose gel and the expected 438bp PCR fragment was detected in lanes containing RT-PCR products from both total RNA (Lane F) and chloroplastic RNA (Lane G) isolated from pC:8K transgenic plants. PCR products were also detected when plasmid DNA was used as a positive control (Lane A). The 438bp product was not detected in samples using either total (Lane B) or chloroplastic (Lane C) RNA from nontransformed plants as a template. However, RT-PCR detected the presence of the sGFP RNA in total RNA samples from empty pC:1300 transgenic plants (Lane D), but not in chloroplastic RNA samples (Lane E) from these same plants. Thus, it can be determined that the sGFP RNA is not translocated into the chloroplasts of empty pC:1300 transgenic plants. Lane H contains a 100bp DNA ladder.
Figure 35: Confocal images demonstrating the localization of sGFP expression in *N. tabacum* cv. *Xanthi* leaves transformed with the empty pCambia 1300 construct. Tobacco leaves transformed with the empty pCambia 1300 construct were visualized using a confocal microscope (DIC image of leaf tissue illustrated in panel B). The images were captured using a long pass filter (as seen in panel A) that blocks all wavelengths below 585 nm (which shows chlorophyll fluorescence) and a GFP green filter (as seen in panel C) which shows sGFP fluorescence. A composite image of the three previously described pictures is illustrated in panel D, and any overlap of sGFP and chlorophyll fluorescence is indicated in yellow. Since the regions of red fluorescence (chlorophyll within chloroplasts) do not significantly overlap with the regions of green fluorescence (sGFP), it was determined that there is no significant sGFP fluorescence within chloroplasts.
Figure 36: Confocal images of isolated chloroplasts visualized under both red and green filters at 100X magnification.

Chloroplasts isolated from nontransformed plants (panels A and B) and plants transformed with empty pCambia 1300 constructs (panels C and D) were visualized using a confocal microscope under two different filters. The images on the left were captured using a long pass filter that blocks all wavelengths below 585 nm, allowing the visualization of chlorophyll fluorescence. The images on the right were captured using a GFP green filter that blocks all wavelengths that do not fall within the 505-530 nm range, allowing the visualization of sGFP fluorescence. While both samples clearly demonstrated chlorophyll fluorescence (panels A and C), sGFP expression was not observed in either sample (panels B and D).
2.8. RT-PCR analyses of chloroplastic RNA

RT-PCR was used to detect the presence of PVX 8kD RNA within the chloroplasts of transgenic plants. Chloroplasts were isolated from nontransformed plants, as well as from plants transformed with constructs pC:8K-CP, pC:8K(insG^{80})-CP, pC:8K, pTR:224bp, and pTR:125bp. Chloroplastic RNA was then extracted from the isolated chloroplasts and the presence of the 8kD RNA was detected using L8K_F and 8K_R primers (Table 1) for plants transformed with the pC:8K-CP, pC:8K(insG^{80})-CP, and pC:8K constructs. A 255bp RT-PCR product was observed in chloroplastic RNA samples from pC:8K-CP, pC:8K(insG^{80})-CP, pC:8K transgenic plants (Figure 37). For plants transformed with the pTR:224bp and pTR:125bp constructs, the 8kD RNA was detected using S8K_F and 8K_R (Table 1) primers. A 156bp RT-PCR product was observed in chloroplastic RNA samples from both pTR:224bp transgenic plants and pTR:125bp transgenic plants (Figure 38). PVX RNA and plasmid DNA were used as positive controls for the RT and PCR reactions, respectively. The expected RT-PCR product was not detected in chloroplastic RNA samples from nontransformed plants or from plants transformed with the empty pCambia 1300 plasmid. Also, the RT-PCR product was not detected in the reconstruction control; where chloroplasts isolated from nontransformed leaves were incubated in 1µg of PVX RNA on ice for 15 minutes and subsequently treated with RNase A and Proteinase K prior to chloroplastic RNA extraction.
Figure 37: A 2% agarose gel demonstrating the detection of the PVX 8kD RNA in the chloroplasts of pC:8K-CP, pC:8K(insG<sup>80</sup>)-CP, and pC:8K transgenic plants. Chloroplastic RNA from plants transformed with constructs pC:8K-CP, pC:8K(insG<sup>80</sup>)-CP, and pC:8K were reverse transcribed into cDNAs; which were then amplified by PCR, using primers specific to the 8kD gene sequence (L8K_F and 8K_R, Table 1). The resulting PCR products were analyzed on a 2% agarose gel and the expected 255bp PCR fragment was detected in lanes containing pC:8K-CP (Lane G), pC:8K(insG<sup>80</sup>)-CP (Lane H), and pC:8K (Lane I) RT-PCR products. PCR products were also detected when plasmid DNA was used as a positive control for PCR (Lane A) and when PVX RNA was used as a positive control for RT-PCR (Lane B). The 255bp product was not detected in samples from nontransformed plants (Lane C) or from plants transformed with the empty pCambia 1300 plasmid (Lane D); which were used as negative controls. Also, the PCR product was not detected in the reconstruction control (Lane E), where chloroplasts isolated from nontransformed plants were incubated with purified PVX RNA, and subsequently treated with RNase A and Proteinase K prior to chloroplastic RNA extraction. Lane F contains a 100bp DNA ladder.
Figure 38: A 2% agarose gel demonstrating the detection of the PVX 8kD RNA in the chloroplasts of pTR:224bp and pTR:125bp transgenic plants.

Chloroplastic RNA from plants transformed with constructs pTR:224bp and pTR:125bp were reverse transcribed into cDNAs; which were then amplified by PCR, using primers specific to the 8kD gene sequence (S8K_F and 8K_R, Table 1). The resulting PCR products were analyzed on a 2% agarose gel and the expected 156bp PCR fragment was detected in lanes containing pTR:224bp (Lane G) and pTR:125bp (Lane H) samples. PCR products were also detected when plasmid DNA was used as a positive control for PCR (Lane A) and when PVX RNA was used as a positive control for RT-PCR (Lane B). The 156bp product was not detected in samples from nontransformed plants (Lane C) or from plants transformed with the empty pCambia 1300 plasmid (Lane D); which were used as negative controls. Also, the PCR product was not detected in the reconstruction control (Lane E), where chloroplasts isolated from nontransformed plants were incubated with purified PVX RNA, and subsequently treated with RNase A and Proteinase K prior to chloroplastic RNA extraction. Lane F contains a 100bp DNA ladder.
DISCUSSION

1. Production of PVX Constructs and Expression Analyses

Previous studies have shown that the CP and CP RNA of PVX accumulate within chloroplasts of plants transformed with the 8kD-CP dicistronic construct. These studies also identified a potential IRBS upstream of the CP gene and it was hypothesized that this region acted as an internal translation initiation site for the expression of the CP (Hefferon et al., 1997; 2000).

Although these results established the presence of PVX RNA within chloroplasts, very little was known about the mechanism by which this RNA entered the organelle. At first, it was believed that the viral proteins (i.e. the CP and 8kD proteins) were involved in this translocation phenomenon, since they have been previously implicated in the intercellular movement of the viral RNA (Fedorkin et al., 2001). To further investigate the roles of the CP and 8kD proteins, two constructs were created. The first clone had the insertion of a G in the 80th nucleotide position of the 8kD gene, and although the 8kD protein was disabled, the CP RNA was still detected in the chloroplasts of plants transformed with this construct. Furthermore, the 8kD RNA was detected inside the chloroplasts of plants transformed with constructs where the CP gene was truncated. These results suggested that neither protein alone was responsible for moving the PVX RNA into the organelle. However, it was still possible that both proteins possess an “RNA Tractor” and that both are capable of translocation, even when one of the viral proteins is absent. In other words, it is possible for the CP to move the RNA when the 8kD protein is absent and vice versa.
To elucidate the roles of the viral proteins, a construct that had both proteins disabled was produced. This was first attempted in a previously constructed pBS clone, that already contained a G insertion in the 80th nucleotide position of the 8kD gene. At first, the plasmid was digested with NheI and filled with a Klenow fragment in an attempt to place the CP out of frame. This strategy was unsuccessful, thus a double stranded oligonucleotide was inserted into the NheI site of the plasmid and this introduced a premature stop codon into the CP. Although this construct successfully disabled both proteins, it needed to be transferred from the pBS plasmid into a binary vector in order to transform tobacco plants.

Rather than subcloning this fragment into a new vector, three additional constructs were produced and cloned directly into a modified version of the pCambia 1300 plasmid. One construct was designed to eliminate both viral proteins (pTR:224bp) and the other two constructs were designed to limit the region of PVX sequence integrated into transformed plants (pTR:125bp and pTR:27bp). The pCambia plasmid was chosen because it has been shown to have a high copy number in E. coli., and it possesses multiple restriction sites for ease of cloning. This plasmid also confers Kanamycin resistance to transformed bacterial colonies and Hygromycin B resistance for the selection of transgenic plants. Additionally, this plasmid contains the gene for synthetic GFP S35T (sGFP) which is known to produce a higher level of fluorescence and has an increased stability in plants due to its high G-C content (Chiu et al., 1996; Niwa, 2003). All seven constructs (pC:8K-CP, pC:8K(insG80)-CP, pC:8K, pTR:224bp, pTR:125bp, pTR:27, and empty pCambia plasmid) were confirmed with restriction digests and further verified with DNA sequencing.
2. Production of Transgenic *N. tabacum cv. Xanthi* and Expression Analyses

The previously described constructs were used to transform *N. tabacum cv. Xanthi* plants. Transgenic plants were identified by Hygromycin B resistance, and were further verified by confirming the presence of desired transgenes within chromosomal DNA with PCR. RT-PCR was also used to detect the 8kD RNA transcript in samples of total plant RNA. Some lines of transgenic tobacco also had their transformation confirmed by examining their leaves under a confocal microscope. The leaves were examined under two filters: a long pass filter; which blocks all wavelengths below 585 nm, and a GFP green filter that blocks all wavelengths that do not fall within the 505-530 nm range. It was found that plants transformed with the empty pCambia plasmid demonstrated high levels of fluorescence under the GFP green filter. This was expected since the transcription of this gene is under the control of a CaMV 35S promoter, and it is the first and only cistron on the RNA transcript. In contrast, leaves harvested from nontransformed plants showed no fluorescence at all. Interestingly, plants transformed with the pC:8K construct (where the ATG of the truncated CP gene is in frame with the ATG of the sGFP gene) also fluoresced. However, the level of fluorescence was far weaker than levels seen in the empty pCambia transgenic plants, as was shown by a previous student in our lab (Cheng, 2007). Since the 8kD gene is located upstream of the CP-sGFP ORF, expression of the sGFP protein is not expected. The observation of sGFP expression within leaves harvested from pC:8K transgenic plants provides further evidence towards the existence of a potential IRBS located upstream of the CP gene, as was hypothesized by Hefferon *et al.* (1997). The IRBS also explains the weaker fluorescence in these plants, since internal translation initiation sites are
known to be less efficient when compared to the initiation of translation by 5’ cap structures (Cherry et al., 2005).

It was also important to ascertain the presence of PVX RNA within chloroplasts of transformed plants, as was previously shown by Hefferon et al. (2000). To ensure that only chloroplastic RNA would be detected, all isolated chloroplasts were treated with RNase A and Proteinase K to remove any externally-associated cytoplasmic RNAs and proteins. Also, a reconstruction control was produced where chloroplasts isolated from healthy, nontransformed plants were incubated in the presence of PVX RNA and then subsequently treated with RNase A and Proteinase K to remove all possible RNAs and proteins attached to the exterior of the organelle. To confirm previous work that established the presence of PVX RNA within chloroplasts, nontransformed, healthy *N. tabacum* cv. Xanthi plants were infected with the PVX virus and primers were used to detect the presence of the CP RNA. RT-PCR verified the existence of this RNA in samples of both total and chloroplastic RNA obtained from infected plants, although the RNA was not detected in the total and chloroplastic RNA of healthy plants. It was also found that plants transformed with the dicistronic 8K-CP construct also had the 8kD RNA within both total and chloroplastic RNA samples, whereas nontransformed plants did not. These findings demonstrate that the PVX RNA transcript is in fact transported into chloroplasts; which was consistent with previously determined results (Hefferon et al., 2000; Cheng, 2007).

However, it was still unclear as to whether this movement was dependent on the PVX sequence, or if it was the result of the PVX RNA transcripts randomly diffusing into the organelle and being detected by the high sensitivity of RT-PCR. In order to ascertain a causative relationship between the PVX sequence and RNA movement, the total and
chloroplastic RNAs of plants transformed with the empty pCambia plasmid were examined. In these plants, sGFP is expressed by a CaMV 35S promoter, and numerous sGFP RNA transcripts are expected to be present within the cytoplasm. However, since sGFP is not believed to localize within chloroplasts on its own (Tanaka et al., 2005), it was hypothesized that the sGFP RNA would remain in the cytoplasm. RT-PCR was used to identify where the sGFP RNA accumulated in the cell and it was detected in samples of total RNA, but not within samples of chloroplastic RNA. The sGFP RNA was also absent from total and chloroplastic RNA of nontransformed plants. The RNA was however found in samples of both total and chloroplastic RNA derived from plants transformed with the pC:8K construct, where the sGFP gene is preceded by the PVX 8kD gene. This demonstrates that RNA transcripts present within the cytoplasm of transformed tobacco cells do not randomly diffuse into the chloroplasts of these same cells. These results also imply that the PVX sequence must be incorporated into the transforming construct in order to observe RNA translocation. Furthermore, these results illustrate that the PVX sequence is able to translocate a foreign RNA sequence (i.e. sGFP) into the chloroplast as well. Corroborating evidence was provided by examining sGFP expression within leaf tissues of plants transformed with the empty pCambia plasmid under a confocal microscope. Upon examination of the obtained images, it was concluded that the protein did not seem to localize within chloroplasts in either intact leaf tissue or within chloroplasts isolated from these same plants (Figures 35 and 36). These results are expected because fluorescent proteins that lack the chloroplastic target peptide are not believed to enter the organelle (Xiang et al., 2006). Rather, these untargeted fluorescent proteins are found in the cytoplasm and in the nucleus, whose nuclear pores are large enough to permit the entrance of these small proteins by diffusion (Berg and Beachy, 2008).
In order to elucidate the mechanism by which the PVX RNA was moving into chloroplasts of transgenic plants, it was first necessary to eliminate the possible involvement of the viral proteins in this translocation phenomenon. Since these proteins have been implicated in the cell-to-cell movement of the viral RNA, it was believed that they might also be involved in intracellular movements. Previous work has shown that eliminating either the CP or the 8kD protein did not inhibit the movement of PVX RNA transcript into the chloroplast. However, it was still possible that both proteins are capable of translocating the PVX RNA, so a construct was created that had both viral proteins simultaneously disabled. The translation of the sGFP protein was also prevented by placing the ATG of the sGFP gene out of frame with the ATG of the CP gene. Chloroplastic RNA was isolated from plants transformed with the pC:8K-CP, pC:8K(insG^{80})-CP, pC:8K and pTR:224bp constructs and RT-PCR was used to detect the presence of the 8kD RNA within these samples. As was previously shown, the 8kD RNA was detected in samples of both total and chloroplastic RNA of plants transformed with the 8kD-CP dicistronic construct. In contrast, the RNA was not detected in samples of either total or chloroplastic RNA of nontransformed or empty pCambia transgenic plants. However, the 8kD RNA was found inside the chloroplasts of transgenic plants that had either the CP (pC:8K) or the 8kD (pC:8K(insG^{80})-CP) protein disabled. This confirmed previous results demonstrating that neither protein was independently responsible for the PVX RNA movement. However, in order to definitively state that neither protein had any involvement in the movement of the PVX RNA into chloroplasts, it was necessary to examine the chloroplastic contents of plants transformed with the pTR:224bp construct; where both viral proteins are simultaneously disabled. The PVX transcript was detected in both total and chloroplastic RNA samples derived from these
plants. Since RNA translocation persisted despite the absence of both viral proteins, it was concluded that the viral proteins were not responsible for the movement of the PVX RNA into chloroplasts.

These results provided further evidence for the hypothesis that there was some element contained within the PVX RNA sequence that was directing its own translocation into chloroplasts of transformed plants. Experiments were then conducted to investigate whether a more limited portion of the PVX sequence retained this “RNA tractor” activity. Thus, a construct containing only 125bp of the PVX sequence was used to transform tobacco plants. Although the 125bp PVX sequence was not detected in the total and chloroplastic samples of nontransformed plants, it was identified in samples of both total and chloroplastic RNA derived from pTR:125bp transgenic plants, demonstrating that the proposed “RNA Tractor” is located within the 125bp region of the PVX sequence. However, if one assumes that there is a functional IRBS upstream of the CP, it is possible that the 125bp sequence produces a 5 amino acid peptide (containing the amino acids MSAPG) derived from the remaining nucleotides of the CP gene, and it is possible that this peptide might have some involvement in the translocation phenomenon. Although this situation is not likely, considering most chloroplastic transit peptide sequences are 40-50 amino acids long (Soll and Schleiff, 2004), it would be beneficial to produce an additional construct where the ATG of the CP is somehow altered so that no peptide is produced.

The results presented both in this thesis and in previous reports suggest that the movement of PVX RNA into chloroplasts is dependent upon a limited region of the PVX RNA transcript. However, from this work, it is unclear whether the chloroplastic translocation is dependent upon the primary sequence of the RNA transcript, the secondary
structure of this transcript, the truncated CP peptide, or if it is a combination of all of these factors. Thus, further work is necessary to clarify which of these factors is acting as the “RNA Tractor”. Furthermore, it is likely that this RNA transcript is interacting with some element of the host cell in order for translocation to occur. Thus, further work is necessary to elucidate any interacting factors that might be facilitating the targeting of the PVX RNA into chloroplasts.

3. Summary and Conclusions

The possible existence of an “RNA Tractor” within the PVX RNA genome was investigated through the production of many constructs that contained various portions of the PVX 8kD and CP genes (Figures 4 and 17). These constructs were used to transform N. tabacum cv. Xanthi plants and the transgenic plants were confirmed using PCR to detect transgenes within chromosomal DNA and RT-PCR to detect RNA transcripts within total plant RNA (Figures 24, 25, 27, and 28). RT-PCR was then used to detect the presence of the 8kD and CP RNA within the chloroplasts of transformed plants. The CP RNA was detected in both total and chloroplastic RNA of PVX-infected plants, as was previously observed by Hefferon et al. (2000; Figure 32). However, it was necessary to ascertain that the RNA movement was dependent on the PVX sequence and not the result of passive diffusion of the RNA from the cytoplasm. To accomplish this, sGFP primers were used to detect the presence of sGFP RNA within chloroplasts of plants transformed with empty pCambia plasmid, and no such RNA was detected (Figure 34). Thus, it was concluded that the PVX sequence must be present in order to observe chloroplastic RNA translocation.
The 8kD RNA was also detected within chloroplasts of plants transformed with constructs where both the CP and 8kD proteins were disabled either independently or in tandem (Figures 37 and 38). This suggests that neither protein is responsible for translocation of PVX RNA into the chloroplast. Furthermore, it was found that 125bp of the PVX sequence was sufficient for the RNA translocation phenomenon to be observed (Figure 38). According to these results, it was concluded that the viral proteins do not play a role in the movement of the PVX RNA into chloroplasts and that this RNA translocation is dependent on the 125bp PVX sequence.

4. Future Work

According to the results described in this thesis, a 125bp region of the PVX sequence is able to translocate its own RNA from the cytosol to the chloroplasts of transgenic tobacco plants. Additional work should be done to further limit this sequence in an attempt to discover the smallest possible region that retains “RNA Tractor” activity. This could be accomplished by examining the chloroplastic RNA of plants transformed with the pTR:27bp clone to see if translocation persists with this very limited sequence. It would also be interesting to investigate whether this translocation phenomenon occurs in related viruses with similar nucleotide sequences.

It has also been established that neither the CP nor the 8kD protein is responsible for the movement of PVX RNA into chloroplasts. It is therefore possible that some host factors are interacting with a region of the PVX RNA and facilitating its transport into the chloroplast. Further studies should be conducted to try and identify any potential host RNA-
binding proteins by using methods such as RNA affinity chromatography or gel-retardation assays to detect RNA-protein interactions, followed by mass spectrometry to analyze the sequence of the purified proteins (Hovhannisyan and Carstens, 2009).

Studies should also be conducted to investigate if the PVX RNA is being translocated to other organelles, such as the plant mitochondria. Since mitochondria are similar to chloroplasts in that they both have prokaryotic origins and carry their own prokaryotic-like ribosomes, we postulate that the “RNA tractor” might also transport PVX mRNA into the mitochondria (Gray, 1991). Many mitochondrial diseases are the consequence of mutations or deletions in either the mitochondrial genome and/or the nuclear genome. Once the exact mechanism of RNA transport into the mitochondria is understood, we can attempt to utilize the “RNA tractor” to deliver the necessary RNA into the mitochondria.
REFERENCES


